Developing new predictors of *Helicobacter pylori* associated disease and its progression

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Abstract

Since discovery *H. pylori* has been one of the most intensively researched bacteria. Although the majority of those infected remain asymptomatic it can lead to serious diseases which carry significant morbidity and mortality. The most serious complication of *H. pylori* infection is gastric cancer and one of the most effective ways to reduce the associated mortality is to detect pre-malignant disease, as this develops in a step wise manner.

Using advanced endoscopy is one way to detect pre-malignant conditions but due to the variety in endoscopic techniques and mucosal classifications the diagnosis is often dependent on histology. This work aimed to develop simple, accurate classification systems to detect *H. pylori* associated disease. The sensitivity and specificity of magnification Narrow Band Imaging for detecting *H. pylori* gastritis was 69% and 67%, intestinal metaplasia 87% and 97% and dysplasia 92% and 98% respectively.

H. pylori is also associated with iron deficiency anaemia but the mechanisms remain unclear. In children it has been proposed that *H. pylori* disrupts iron regulatory mechanisms via the peptide hepcidin but this has not been extensively researched in adults. Serum hepcidin was significantly lower *in H. pylori* infected anaemic individuals and anaemic individuals without evidence of infection when compared to controls (9-fold, $p=0.009$ and 5-fold, $p<0.0001$ respectively). These results are opposite to data from children, possibly explained by the presence of gastric atrophy. The cellular localisation of ferroportin was different in the *H. pylori* infected group which could be due to local cytokine production. Gaining a better understanding of this mechanism could aid the development of more targeted investigation and treatment.

However, with regards to allergic and autoimmune conditions, there is growing evidence to suggest *H. pylori* is inversely associated. It is believed that any benefit associated with *H. pylori* is confined to childhood when the immune system is developing. A significant reduction was seen in $IL10^{+}$ Tregs ($p=0.0029$) after successful eradication suggesting the removal of *H. pylori*

may have systemic consequences on the immune system that are still not fully understood.

This work has highlighted the use of endoscopic techniques to identify individuals at risk of disease. It has also described the effects of eradication on the immune system which potentially could have implications for individuals with allergic conditions with regards to eradication therapy.

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List of abbreviations

AFI	Autofluorescence
ASA	American society of anaesthesiologist classification
BabA	Blood group antigen-binding adhesion
B cells	B lymphocytes
BFA	Brefeldin A
CagA	Cytotoxin associated gene A
cagPAI	Cytotoxin associated gene pathogenicity island
CCD	Charge-Coupled Device
cDNA	Complementary DNA
CI	Confidence intervals
CLE	Confocal Laser Endomicroscopy
CLO test	Campylobacter like organism test/rapid urease test
$C0_2$	Carbon dioxide
CTLA-4	Cytotoxic T lymphocyte antigen – 4
CV	Collecting venules
DEIA	DNA enzyme immunoassay
DGP	Deamidated antigliadin antibodies
DNA	Deoxyribonucleic acid
DupA	Duodenal ulcer promoting A
EGFR	Epidermal growth factor receptors

EGGIM	Endoscopic classification for grading gastric intestinal metaplasia
ELISA	The enzyme linked immunosorbent assay
EMA	Endomysial antibodies
EPIIYA	Glu-Pro-Ile-Tyr-Ala
FACS	Fluorescent and cell sorting
FBS	Fetal bovine serum
FISH	Fluorescent in situ hybridization
FOXP3	Forkhead box P3
G-17	Gastrin-17
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGT	Gamma-glutamyl transpeptidase
GI	Gastrointestinal
GIST	Gastrointestinal stromal tumour
GORD	Gastro-oesophageal reflux disease
Hb	Haemoglobin
hBD2	Human β defensin 2
HD	High Definition
H&E	Haematoxalin and eosin
H. pylori	*Helicobacter pylori*
IDA	Iron deficiency anaemia
IELs	intraepithelial lymphocytes

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin Ig
ITP	Idiopathic thrombocytopenia purpura
K	Kappa scores
Li-PA	Line probe assay
LR	Likelihood Ratio
LT	Leukotrienes
MALT	Mucosa Associated Lymphoid Tissue Lymphoma
MAP	Mitogen activated proteins
MCH	Mean corpuscular haemoglobin
MCV	Mean corpuscular volume
ME	2- mercaptoethanol
MHC	Major Histocompatibility complex
mRNA	Messenger ribonucleic acid
NBI	Narrow Band Imaging
NBI-Z	Magnification narrow band imaging
NF	Nuclear factor
NH_4	Ammonium
NK	Natural killer cells
NS	Non significant
NSAID	Non-steroidal anti-inflammatory drugs

NTC	No template control
NUD	Non-ulcer dyspepsia
OipA	Outer membrane protein A
OLGA	Operative link for gastritis assessment
OR	Odd ratios
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-PHFA	PCR dependent preferential homoduplex formation assay
PGI	Pepsinogen I
PGII	Pepsinogen II
PGI/II ratio	Pepsinogen I and Pepsinogen II ratio
PPI	Proton pump inhibitor
RCT	Random controlled trials
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
PRRs	Pattern recognition receptors
RT-qPCR	Real time quantitative polymerase chain reaction
SECN	sub epithelial capillary network
SFKs	Src family of kinases

SHP-2	Src homology 2-containing protein tyrosine phosphatase-2
s1/m1	Signal and mid region 1
SOD	Superoxide dismutase
SSCP	Single strand confirmation polymorphism
T cells	T lymphocytes
Th cells	T helper cells
TLR	Toll like receptor
TNF	Tumour necrosis factor
Tregs	Regulatory T lymphocytes
T4SS	Type IV secretion system
TTG	Anti-tissue transglutaminase antibodies
UBT	Urea Breath Test
VA	Villous atrophy
VacA	Vacuolating cytotoxin A
VAS	Visual Analogue Scale
WLE	White Light Endoscopy
WLE-Z	Magnification White Light Endoscopy
ZO-1	Zona occludens 1 protein

Chapter 1: General Introduction

1.1 Introduction

In 1983 two scientists successfully isolated a spiral bacterium they called *Campylobacter pylori* and later re-named *Helicobacter pylori* (*H. pylori*) and were later awarded the Nobel Prize for Medicine or Physiology in 2005 (Marshall and Warren, 1984). *H. pylori* colonises the gastric mucosa of up to fifty percentage of the world's human population, with the vast majority of patients being asymptomatic (Pounder and Ng, 1995). The stomach mucosa usually becomes colonised with *H. pylori* during childhood. The prevalence varies between countries but infection rates are higher in developing countries. This is believed to be due to variation in living conditions and the widespread use of antibiotics during childhood (Peleteiro et al., 2014). The prevalence of *H. pylori* is declining on a global scale, in the US it is estimated 10% of those less than 20 year of age are infected compared to 40% of those over 60 (Grad et al., 2012). This is due to a birth cohort effect rather than acquiring the infection at a later age. In a few individuals it leads to serious complications such as peptic ulcer disease, distal gastric adenocarcinoma or primary gastric mucosa associated lymphoid tissue (MALT) lymphoma. *H. pylori* may also play a causative role in patients with unexplained iron deficiency anaemia (Qu et al., 2010). Other associated conditions include idiopathic thrombocytopenia purpura and atrophic gastritis. The infection has also been suggested to be a significant contributor to the hygiene hypothesis and the development of a healthy immune system (Atherton, 2006, Strachan, 1989). *H. pylori* has evolved with humans, and has multiple mechanisms to evade immune clearance, and thus persistently colonises the stomach unless treated (Blaser and Atherton, 2004). Its persistence may suggest that it offers a protective role in addition to its well documented adverse complications. Epidemiological studies have suggested protection against gastro oesophageal reflux disease, oesophageal adenocarcinoma, asthma, multiple sclerosis and inflammatory bowel disease (Arnold et al., 2012). This has been the subject of intense research but incomplete understanding remains due the complex host

pathogen interactions, a lack of mechanistic data and the paucity of interventional human studies (White et al., 2015).

1.2 *Helicobacter pylori*

The bacteria are spiral shaped with multiple flagella to allow for its rapid motility from the acidic gastric lumen and into the protective mucus layer. Some of the bacteria are mobile within the mucus whereas others are closely bound to the gastric epithelial surface. This association is vitally important for *H. pylori* pathogenesis (Terry et al., 2005). Another factor that is important for bacterial colonisation is the ability to acquire iron (Atherton, 2006). The gastric niche is dependent on acid resistance; there are a number of bacterial genes that are influenced by the acid environment (Wen et al., 2003, McGowan et al., 2003). An important *H. pylori* protein is urease which is involved in converting urea to ammonia and carbon dioxide. The effect of ammonia is to raise the pH helping to buffer the cytoplasmic membrane and allows *H. pylori* to survive hostile acidic conditions. The transport of urea into bacterial cells and the distribution of the bacteria are tightly regulated by channels that are acid dependent (Atherton, 2006). The combined effects of urease and urea channels help maintain an environment where *H. pylori* is able to survive in the normally acid stomach mucosa by creating a neutral pH (Weeks, 2000). Urease is highly expressed and is therefore is a good target for diagnostic tests(Laine et al., 1996). Urease production by *H. pylori* has revolutionised the diagnosis of the bacteria in clinical practice allowing for rapid testing (CLO test) at time of endoscopy and biopsy (Atherton, 2006).

Another interesting characteristic is the bacteria's ability to only inhabit the surface of the gastric mucosa and mucus secreting cells, but not the deep glands. Trefoil proteins are involved in maintaining the epithelial barrier and are located in mucus secreting cells. Work by Clyne *et al.* showed that *H. pylori* selectively binds to Trefoil factor 1 which acts as a receptor and explains its affinity to gastric mucosa and mucin molecules *in vivo* (Clyne et al., 2004).

O-glycans located in deeper gastric mucosa inhibit the synthesis of *H .pylori* cell walls, acting as the host's natural defence (Atherton, 2006). Human β-defensins also have antimicrobial properties that help to resist host colonization by *H. pylori* (Kawauchi et al., 2006).

1.2.1 Acute Infection

H. pylori strains have been successfully isolated from saliva, gastric reflux and vomitus in addition to gastric samples. The precise mode of transmission of *H. pylori* is not entirely clear, but it is likely to be a combination of the faecal to oral and oral to oral routes (Goodman and Correa, 2000). Initial infectious symptoms are variable and short lived but sometimes include dyspepsia, nausea and halitosis. Histological evidence has shown acute infection results in a severe gastritis, which is usually characterised by the persistence of inflammatory cells. This is accompanied by a decrease in acid secretion (Morris and Nicholson, 1987).

1.2.2 Chronic infection

A chronic infection usually leads to local inflammation (gastritis) in most individuals, and its distribution is dependent on multiple factors such as genetic, bacterial virulence, environment and the age of acquisition of infection. These interactions could explain why only a small number of those infected develop disease (Robinson et al., 2007). Majority of infected individuals remain asymptomatic with only 15% developing disease in their lifetime (Atherton, 2006).

H. pylori presence in the gastric mucosa leads to a strong immune response. It stimulates the expression of cytokines which attract inflammatory cells such as neutrophils and lymphocytes which induce release of reactive oxygen and nitrogen species (ROS/RNS) (Robinson et al., 2007, Sundquist and Quiding-Jarbrink, 2010). ROS and RNS mediated DNA injury is associated with the development of gastric cancer (Macarthur et al., 2004). The nature of the immune response is variable and influences disease risk.

Cell associated layer (MUC1) and secreted mucin (MUC5AC) acts as a protective cover of the gastric mucosa and influences the adhesion of *H. pylori* to the surface. Mucins also provide antibacterial properties for the host and the binding of *H. pylori* to MUC1 helps modulate the hosts inflammatory response (Park et al., 2012, White et al., 2015). In a similar protective mechanism, blood antigens interact with the bacteria limiting opportunities to bind with cell surface thus reducing bacterial numbers.

1.3 *H. pylori* mediated disease

1.3.1 Gastrointestinal disease

1.3.1.1 Peptic Ulcer Disease

Peptic ulcers are macroscopic breaks in the mucosa lining of the gastric or duodenal mucosa, NSAID use and *H. pylori* are the most common cause (White, 2016a). There is a well-established link between *H. pylori* colonisation and the development of peptic ulcer disease (Kandulski et al., 2008). Infection leads to chronic active gastric mucosal inflammation (gastritis) which leads to ulceration in approximately 15% of individuals (Atherton, 2006). Bacterial, host and environment factors influence this development. Those infected with *H. pylori* have a lifetime risk of developing peptic ulcer disease which varies from 3% to 25%. (Kandulski et al., 2008, Schlemper et al., 1996). The distribution of gastritis also influences the type of disease that develops. *H. pylori* is the leading cause of duodenal ulcers. Gastric antral predominant inflammation leads to an elevated gastric acid output. Resulting gastric metaplasia of duodenal mucosa enables *H. pylori* to colonise and eventual ulceration formation. In individuals with pangastritis the gastric acid output is normal or reduced, which prevents duodenal ulcer development but gastric ulcers, atrophic gastritis or adenocarcinoma may develop (Malfertheiner, 2011, Kusters et al., 2006, Correa, 1988). See Figure 1.1.

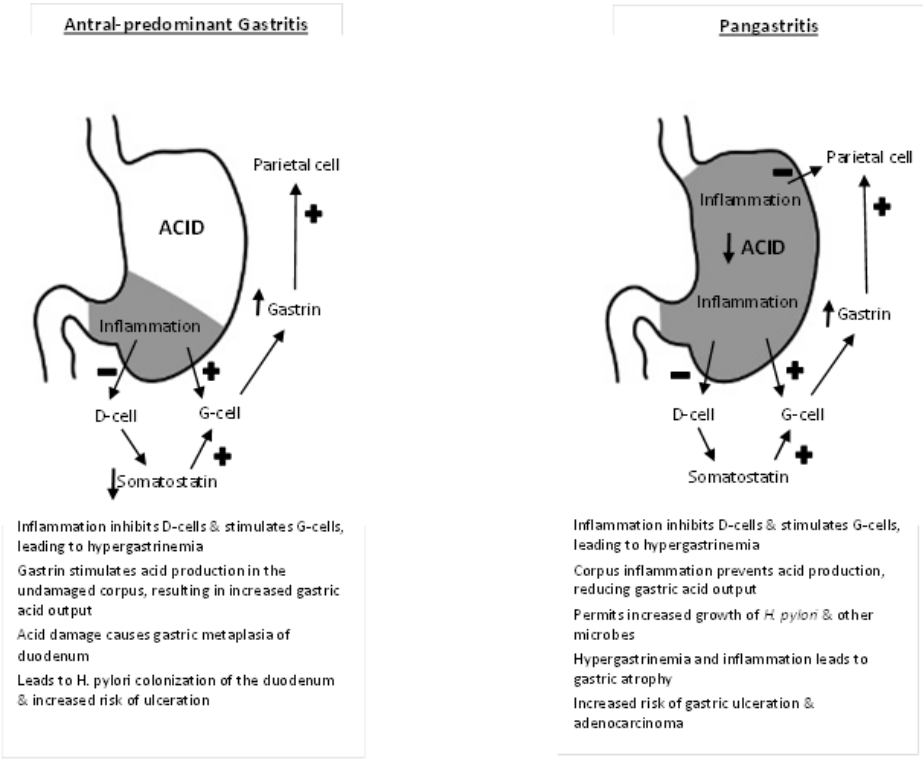


Figure 1.1 Gastritis pattern and gastric acid production

Figure adapted from (White et al., 2015).

NSAID use is the other important independent risk factor that contributes to ulceration and subsequent gastrointestinal haemorrhage - a common cause for hospitalisation. Although the prevalence of *H. pylori* has decreased secondary to treatment, the increasing use of NSAIDs continues to result in peptic ulcer disease related hospital admissions. *H. pylori* infection increases a patient's bleeding risk by 1.79 fold, and NSAIDs increase the risk by 4.85 fold. This becomes 6.13 fold when in combination (Huang et al., 2002).

Current guidelines from the 2012 Maastricht Report emphasises the importance of *H. pylori* eradication in a patient using long-term NSAIDs to prevent ulcer haemorrhage. Proton Pump Inhibitor (PPI) maintenance is also recommended treatment as it has been shown to be superior to eradication treatment in preventing recurrence of ulcer disease and haemorrhage in those taking long-term NSAIDs. Conversely, those who have suffered from ulcer haemorrhage who are found to be positive for *H. pylori* should also have the bacterium eradicated (Malfertheiner et al., 2012). *H. pylori* eradication is

also important to reduce PPI and *H. pylori* related gastric body gastritis, atrophy and reduction in specialised glands that may occur in those taking long-term PPI with coexistent infection (Schenk et al., 2000, Moayyedi et al., 2000b).

1.3.1.2 Dyspepsia

Dyspepsia is a symptom which may affect up to a quarter of the population and is estimated to cost the United Kingdom more than £1 billion annually (Ford et al., 2012). It has a variety of definitions but is often characterised by upper abdominal discomfort or pain. When investigated and no clinically significant cause is found, this is termed functional dyspepsia. The Rome IV criteria divide functional dyspepsia symptoms into two distinct syndromes: epigastric pain syndrome where patients describe epigastric discomfort and the postprandial distress syndrome. The later patients are either troubled with fullness after a normal sized meal or early satiety preventing meal completeness (Tack et al., 2006, Drossman, 2016). Up to 80% of patients investigated with endoscopy will have functional dyspepsia and the cause is likely multifactorial which makes treatment sometimes difficult (Ford and Moayyedi, 2013).

There are well documented guidelines on the management of uninvestigated dyspepsia which includes a trial of a PPI and investigating for *H. pylori* with eradication if positive. A gastroscopy is then second line for those whose symptoms remain unresolved or first line for those with sinister features. Using the test and treat strategy 12-15 patients need to be treated to cure one with dyspepsia (Moayyedi et al., 2000a). This is effective in areas where the prevalence of *H. pylori* is high (Kandulski et al., 2008). Eradication reduces the need for expensive gastroscopy or unnecessary long-term PPI therapy in many patients.

Dyspepsia associated with *H. pylori* may be related to its effects on gastric acid secretion, mediated by gastrointestinal hormones. Moss *et al*. showed *H. pylori* caused increases in gastrin levels after fasting and after meals (which

stimulates gastric acid production) and lower somatostatin levels (regulatory hormone) within the stomach mucosa. These abnormalities were corrected after the bacteria were eradicated (Moss et al., 1992). El-Omar *et al.* also showed that eradication of *H. pylori* had a significant effect on gastrin-mediated acid secretion. The numbers in this investigation were small but *H. pylori* positive and negative healthy volunteers and duodenal ulcer patients were studied. Patients with duodenal ulcers had increased levels of gastrin and showed an exaggerated response when compared to asymptomatic *H. pylori* and *H. pylori* negative patients. Both duodenal ulcer and asymptomatic *H. pylori* positive individuals had greater increases in acid secretion compared with *H. pylori* negative patients. Dyspepsia can therefore, be considered to be the first stage in peptic ulcer development (El-Omar et al., 1993, Suzuki et al., 2011).

In addition to the above, *H. pylori* infection has been shown to be involved in down regulation of a muscle specific microRNA which is essential for growth. This may cause gastric muscle hyperplasia and lead to abnormalities in gastric emptying that may account for pathogenesis of *H. pylori* associated gastric motility disorders (Saito et al., 2011).

1.3.1.3 Gastric Atrophy

Atrophic gastritis occurs when there is loss of the mucosal architecture as a result of *H. pylori* infection in the majority of individuals. This may progress from chronic active *H. pylori* gastritis to intestinal type gastric malignancy. However, there is a delay in this process which would suggest other factors exist to drive this progression. Gastric atrophy is therefore, considered a pre-cancerous condition and current guidelines recommend surveillance of these individuals to help pick up early cancerous lesions (Dinis-Ribeiro et al., 2012). More detailed information provided in Chapter 3.

1.3.1.4 Gastric adenocarcinoma

H. pylori is a strong risk factor for distal gastric adenocarcinoma. The 5 year survival is often poor because patients often are asymptomatic and when they present the tumour is advanced (Peek and Blaser, 2002, Herrero et al., 2014). Other factors such as autoimmune atrophic gastritis, host genetics, family history and environmental factors also play an important role in addition to *H. pylori* virulence in determining disease (Colquhoun et al., 2015). More detailed information provided in Chapter 3.

1.3.1.5 Mucosa Associated Lymphoid Tissue (MALT) Lymphoma

H. pylori is also a cause of MALT lymphoma. It is a form of B Cell Non-Hodgkin's Lymphoma. The stomach is a common site to be affected and up to 8% of lymphomas are due to MALT (Fischbach et al., 1992). There are a variety of prognostic factors and the lymphomas are categorised as low to high grade. Those with low grade commonly go into remission after *H. pylori* eradication therapy is given.

1.3.1.6 Iron Deficiency Anaemia

Iron deficiency anaemia (IDA) is a common problem, with prevalence in the developing world of up to 5% in adult men and postmenopausal women (Goddard et al., 2011). IDA has a variety of causes which include suboptimal dietary intake, blood loss, malignancy and chronic disease. *H. pylori* infection is another well-established cause for IDA and for some individuals eradication treatment resolves the anaemia. In a meta-analysis carried out by Qu HX et al using mainly observational studies, they found *H. pylori* and IDA were associated and that eradication improved haemoglobin and ferritin levels but the results were not significant (Qu et al., 2010). A meta-analysis published by Muhsen *et al*. looking at a small selection of interventional studies, case series, case reports and epidemiology studies suggested a positive association between *H. pylori* and reduced iron stores. The exact mechanism is not fully understood but is speculated to be related to chronic blood loss, reduced

absorption and increased uptake by the bacterium (Muhsen and Cohen, 2008). More detailed information provided in chapter 4.

1.3.2 Disease beyond the GI Tract

1.3.2.1 Thrombocytopenia purpura

It is believed that infection with *H. pylori* may be linked to development of idiopathic thrombocytopenia purpura (ITP), although the exact mechanism is not known. ITP is an autoimmune disorder where patients have an abnormally low platelet count. Antibodies are produced against platelets, the majority of patients are asymptomatic but others are at increased risk of haemorrhage and purpura. *H. pylori* infection has been implicated in ITP, since in many cases platelet count improves following eradication therapy. Those whose infection were not successfully eradicated did not show an improvement in platelet count (Franchini and Veneri, 2004).

1.3.2.2 Chronic Urticaria

Chronic urticaria is characterised by the appearance of itchy wheals over a period of longer than 6 weeks. The condition is thought to have a variety of triggers but often the main cause remains unknown. The exact mechanism of this allergic disease is unclear but ultimately it is the immune mediated release of histamine from mast cells that is responsible for the symptoms. It has been proposed that individuals with urticaria develop specific IgE against a *H. pylori* allergen triggering the above sequence of events. It has also been suggested that the patient may then be exposed to further gut allergens as *H. pylori* alters the permeability of the gastric mucosa. Clinically, eradication of this bacterium has improved urticaria symptoms in some studies but not in others. *H. pylori* has also been implicated in other skin conditions but there is insufficient evidence to prove these associations at present (Hernando-Harder et al., 2009).

1.3.2.3 Ischaemic Heart Disease

It has been proposed over a number of years that microbes may be involved in the inflammatory process of atherosclerosis, and some studies have linked *Chlamydophila pneumonie* to myocardial infarction (Kuo et al., 1993). The same has been suggested for *H. pylori*; however the results of several studies are conflicting. Multiple mechanisms have been proposed each with evidence to support or refute the theories. Some of the mechanisms proposed include alterations in lipid profile, systemic effects on immune mediators to alterations in aggregation of platelets. The detection of *H. pylori* DNA in atherosclerotic plaques also provides evidence for this association (Manolakis et al., 2007). Currently more research is needed to establish the exact role of *H. pylori* in the development of ischemic heart disease, but controlling for all the risk factors that are known to be associated with IHD is likely to be very difficult.

Other possible roles of *H. pylori* in extra gastric diseases including Parkinson disease, obesity and kidney disease have been suggested but evidence is lacking (Ekesbo et al., 2000, Weller et al., 2005).

1.4 *H. pylori* protective associations

1.4.1 Oesophageal Disease

There appears to be evidence supporting a protective association between *H. pylori* infection and oesophageal disease: reflux oesophagitis, pre-malignant Barrett's oesophagus and oesophageal adenocarcinoma (Vicari et al., 1998, Labenz et al., 1997). *H. pylori* infection has decreased but the incidence of oesophageal carcinoma has increased (el-Serag and Sonnenberg, 1998). Barrett's oesophagus is a well-recognised pre-malignant condition for adenocarcinoma. Individuals at risk in some developed countries are placed in surveillance programmes. This condition is more common in Caucasians than Asian individuals, in whom the incidence of *H. pylori* infection is lower. Studies have also shown a negative association between the presence of Cag+ strains

of *H. pylori* and the serious complications of oesophageal reflux disease (Chow et al., 1998, Sharma and Vakil, 2003). Some studies have not convincingly shown an increased risk of gastro-oesophageal reflux disease (GORD) after *H. pylori* eradication treatment (Moayyedi et al., 2001). Therefore there continues to be much debate in the literature on whether this is merely an association rather than actual causation.

1.4.2 Asthma, Allergy and Atopic conditions

The strongest evidence for *H. pylori* providing protection against extra gastric diseases comes from asthma and allergy data. Although this field is controversial it is important as the prevalence of *H. pylori* infections are decreasing but allergic conditions are becoming more prevalent (Robinson, 2015). Detailed information on this is provided in chapter 6.

1.5 Factors that determine gastric and duodenal disease development

The presence of *H. pylori* colonisation is insufficient on its own to lead to gastro-duodenal disease. Host genetics, immunity, environmental and bacterial virulence factors are all involved in determining which individuals will develop peptic ulcer disease.

1.5.1 Host Genetics

There are a variety of host factors including genetic factors that influence disease development and bacterial persistence. Pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs), and are expressed by gastric mucosa, activate the host's inflammatory gene expression by bacterial interaction (Table 1.1). Disruption of the expression of the TLR4 gene itself leads to atrophy and significantly reduced acid secretion. Patients with the genetic makeup to promote an inflammatory response in combination with reduced acid secretion help to create an environment for inflammation and cancer progression (Ricci et al., 2011, El-Omar et al., 2008).

NLR interaction promotes release of inflammatory cytokines (Vanaja et al., 2015).

Table 1.1 Pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) examples

Pattern recognition receptors (PRRs)	Pathogen-associated molecular patterns (PAMPs)
Toll-like receptors (TLRs)	Lipopolysaccharide (LPS)
Nucleotide-binding oligomerization domain (NOD)	Flagellins
NOD-like receptors (NLRs)	Cell wall peptides

In *H. pylori* associated gastritis, DNA repair is thought to be inhibited by causing more mutations, which lead to accumulation of damaged organelles which in turn promotes cancer development (Filomeni et al., 2015). The interaction of gastric mucosa and *H. pylori* leads to upregulation of pro-inflammatory cytokines and chemokines such as IL-8, IL-1β, TNFα, IL-6, IL-12, CCL2-5, CCL20, and CXCL1-3 (Peek et al., 1995, Cook et al., 2014).

Polymorphisms leading to upregulated expression of IL-1β, IL-6, IL-8 and TNFα (pro-inflammatory cytokines) increase risk of disease development (Backert and Clyne, 2011, Macarthur et al., 2004, Ramis et al., 2015, El-Omar et al., 2003). Polymorphisms leading to decreased expression of IL-10 (anti-inflammatory) also increase the risk of developing disease (Ramis et al., 2015). These cytokines in turn leads to recruitment of inflammatory cells, leading to more marked gastritis and increase disease risk (White et al., 2015).

1.5.2 Nature of the host's immune response

H. pylori induces a significant local and peripheral response from the immune system. The local effects include activation of neutrophils and lymphocyte penetration of gastric tissues. These local effects are, however, not adequate at clearing the infection. The bacteria also have mechanisms to reduce this

local inflammation and this will be discussed further in chapter 6. The bacteria also stimulate the acquired immune system. This response is led by T helper cells, a sub group of specialist lymphocytes. Where Th-1 and/or Th-17 activation is more marked, a greater severity of inflammation occurs and thus gastric cancer risk is increased. *H. pylori* has evolved to induce damaging Th subset responses but then further manipulates this response to avoid its destruction. The host's regulatory T lymphocytes (Tregs) are able to modulate this Th immune response too, to prevent damage to the host but consequentially, allow for bacterial persistence. The role of Tregs is currently being intensively studied (Atherton, 2006). See chapter 6 for more details.

1.5.3 Environmental factors

1.5.3.1 Smoking, *H.pylori* infection and physiological effects on the GI tract

H. pylori and smoking has been the subject of thorough research, yet evidence from epidemiological studies to suggest that the two are associated continues to be conflicting. Initially, measuring *H. pylori* serology in healthy patients did not show an association between *H. pylori* serology positivity and smoking (Graham et al., 1991). Stronger evidence later came from a large case controlled study of 4742 healthy adults which demonstrated smoking was an independent factor for *H. pylori* seropositivity: 63.6% in current smokers and 64.5% in ex-smokers when compared with 53.5% of non-smokers, fully adjusted for age, sex and social status (Murray et al., 1997).

Similar conflicting conclusions exist with regards to symptomatic patients. Bateson *et al* was the first prospective study to show a positive association (Bateson, 1993) but others later showed no association (Battaglia et al., 1993). However, the general consensus is that there is a clinically important association (Eastwood, 1997) (Parasher and Eastwood, 2000). Smoking is also thought to affect ulcer healing, as demonstrated by Korman *et al.* who measured the healing and relapse rate of duodenal ulcers, and found that failure to heal was greater in the smokers and correlated well with the number smoked (Korman et al., 1983).

Smoking has been hypothesised to affect the physiology of the upper GI tract in a number of ways.

In the first instance, the effect on gastric acid secretion is mixed which suggests that this unlikely to be the clinically relevant mechanism (Eastwood, 1997). Secondly, smoking inhibits gastric motility which accounts for the reflux of duodenal contents back into the stomach leading to an increase risk of peptic ulcer formation (Parasher and Eastwood, 2000).

Thirdly, smoking affects the mucosal protection mechanisms therefore allowing for the formation of peptic ulcers. One example of this is the effect on pepsin. This enzyme is released by chief cells in the stomach and has been shown to be important in peptic ulcer formation. Walker *et al.* showed smokers with PUD to have greater pepsin production after stimulation when compared with non-smokers with PUD (Walker and Taylor, 1979).

The production of damaging free radicals secondary to smoking is also increased when compared to non-smokers and may explain the increased disease activity often associated with smokers (Kalra et al., 1991). Similarly, smoking has been shown to lower levels of vitamin C and beta-carotene, which act as the host's natural anti-oxidants hunting down free radicals and preventing mucosal injury. Vitamin C secreted in the gastric environment by healthy tissue also affects the synthesis of nitric oxide, whose role in mucosal protection is unclear (Mackerness et al., 1989). It is hypothesised that a lower level allows the host to be more susceptible for infection, re-infection or failure to eradicate the bacteria (Schectman et al., 1989, Ruiz et al., 1994).

Prostaglandins also maintain the surface mucosa barrier by increasing mucus, pancreatic bicarbonate secretion and mucosal blood flow. These prostaglandins are decreased in smokers and in advanced age in multiple studies, although this was not reproduced in animal models (Cryer et al., 1992, Eastwood et al., 1993, Parasher and Eastwood, 2000).

Nicotine is thought to enhance the effects of VacA virulence factor toxin of *H. pylori* which could also lead to mucosa being more susceptible to ulceration (Cover et al., 1992).

Furthermore, smoking has been shown to lower serum immunoglobulins, regulatory T lymphocytes and other immune cells. It is still not clear whether these changes increase the risk of ulceration in individuals who smoke but the overall conclusion is that it is an important modifiable factor (Parasher and Eastwood, 2000).

Some studies have determined that smoking has no influence on ulcer healing after full eradication. (Kadayifci and Simsek, 1997). However, the evidence from using a variety of treatments regimes has shown that the eradication rate is different in individuals who smoke. Smoking has, however, been identified as an important factor in treatment failures when the regime including amoxicillin, clarithromycin and omeprazole is used (Kamada et al., 1999). Individuals who smoke who were then treated with regimes containing a PPI and an antibiotic used in combination either with metronidazole alone or combined with amoxicillin, had reduced success rates. Once treatment is successful, continued smoking does not seem a make a significant difference in the healing of ulcers or influences relapse.

In summary, the exact mechanism by which smoking contributes to ulcer formation and affects healing in patients infected with *H. pylori* is unknown. Smoking by itself is not an independent risk factor for peptic ulceration but in combination with *H. pylori* it seems to enhance the harmful complications (Parasher and Eastwood, 2000). Eradication treatment is the most important factor in healing and preventing recurrence in these patients.

1.5.3.2 Diet and *H.pylori* infection

Diet plays a role in the pathogenesis of gastric cancer and peptic ulceration. Kato *et al* carried out a large prospective study using a Japanese cohort of 7624 individuals. They showed a strong correlation between table salt and soy sauce in relation to gastric ulceration. They also proposed that a "Western diet" seemed to be protective in duodenal ulcer disease. Oriental diet has long being speculated as a possible risk factor for peptic ulceration but this study did not support this. Other dietary products such as coffee and pickles have been implicated but the evidence in favour of these is weak (Kato et al., 1992).

Adequate consumption of healthy food such as fruits and vegetables are thought to be protective in many serious diseases (Correa et al., 1985). For example vitamin E is believed to prevent the increased number of stimulated neutrophils which damage the gastric natural protective mechanism at time of *H. pylori* infection (Sugimoto et al., 2006, Izzotti et al., 2009).

Dietary products such as, N-nitroso compounds, may contribute to the harmful effects of *H. pylori* and the hosts protective mechanisms. These carcinogenic compounds are found in processed food such as bacon and ham, and have been suggested to increase the risk of gastric cancer (Correa et al., 1985). Presence of *H. pylori* causes increased nitric oxide production from macrophages in order to resist colonisation. This in turn leads to DNA damage (Shapiro and Hotchkiss, 1996). Continued infection leads to further types of tissue damage and the production of further toxin substances (Liu and Hotchkiss, 1995, Ziebarth et al., 1997). In addition to this, nitric acid produced from dietary sources adds to this low acidic environment where the bacterium can then go on to strive (Mowat et al., 1999).

Salt has also been implicated in leading to gastric cancer by inducing damage in the cell division process (Charnley and Tannenbaum, 1985, Yamaguchi and Kakizoe, 2001).This can lead to damage to the gastric mucosal protective barrier allowing for bacterial colonisation (Bartsch et al., 1988, Fox et al.,

1999). High environmental salt concentration increases vacA virulence factor expression (Amilon et al., 2015). Gancz *et al* showed that depending on specific strains, *vacA* expression could be increased in response to sodium chloride shock. Sodium chloride can therefore influence *H. pylori* growth and gene expression (Gancz et al., 2008).

1.5.4 Bacterial Virulence Factors

Bacterial virulence factors have been strongly implicated in *H. pylori* associated diseases and an increased gastric mucosa inflammatory response (Kuipers et al., 1995). The most important and extensively investigated virulence factors are CagA and VacA.

1.5.4.1 CagA and *cag* PAI

The most studied bacterial virulence factor associated with increased risk of disease is the cytotoxin associated gene A (*cagA*). *CagA* is present in 50-70% of *H. pylori* strains (Ching et al., 1996, Cover et al., 1995, Tummuru et al., 1993, Kusters et al., 2006). The gene is encoded on the *cag* pathogenicity island (*cag PAI*) (Censini et al., 1996). The *cag PAI* contains an estimated 30 genes and *cag+* strains need to have the whole PAI or are termed *cag*-. Some are intermediate and have parts deleted but have similar chemical and physical characteristics to *cag*- strains (Atherton, 2006, Censini et al., 1996). The c*ag PAI* encodes a type IV secretion system, which is important because it enables *H. pylori* and the host to interact.

Individuals are identified as cagA+ or cagA- by the presence or absence of antibody against the CagA protein. More marked inflammation and higher rates of gastric cancer are associated with CagA+ status, but cagA+ serology can also occasionally occur in individuals infected with strains that lack the *cag PAI*. Approximately 10% of strains have an incomplete *cag PAI* which in turn weakens its action. This mechanism of disease is poorly understood (Kusters et al., 2006). Epidemiological studies have estimated a strong association of cagA virulence strain with distal gastric cancer where the risk

increases by 1.64-fold (Ekstrom et al., 2001, Huang et al., 2003). The comB T4SS system is remarkable at acquiring new and advantageous genetic material. It is via this system that antibiotic resistance genes are transferred to the bacteria (Terradot and Waksman, 2011).

1.5.4.2 CagA delivery system

The *cag* type IV secretion system (T4SS) is a needle like structure which transfers bacterial components into the host cell cytoplasm. The *cag* T4SS comprises of a large complex structure which links the inner and outer surface of the bacteria. It is made up of proteins VirB1-11 and VirD4. This structure includes a pilus and multiple ATPases that help promote its function. The effector CagA protein is accurately transferred to host cells through the pilus (Rohde et al., 2003). This injected protein causes a cascade of signalling via tyrosine residues which ultimately leads to cellular disruptions.

Tyrosine phosphorylation of *cagA* (one of the important steps in signal transduction and control of enzyme activity) at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif is brought about by members of the Src family of kinases (SFKs) in the host cell cytoplasm. SHP-2 (Src homology 2-containing protein tyrosine phosphatase-2) is activated upon binding phosphorylated CagA, and this leads to a large number of effects on the cells. These are negatively controlled by inhibition by the phosphorylated CagA of the Src kinase. Gap junctions are also affected by CagA acting on zona occludens 1 protein (ZO-1). Epidermal growth factor receptors (EGFR) are activated influencing extracellular signal-regulated protein kinases (ERK) leading to enhanced proliferative properties. Phosphorylation of CagA also activates mitogen activated proteins (MAP) and other kinase pathways. Cell motility is affected by CagA acting directly on c-Met receptors. The actions on cells ultimately are the re-arrangements of cytoskeletons, disruption of cell junctions and pro and anti-apoptotic functions, alteration of cell polarity, release of limited pro-inflammatory cytokines and proliferation which enable the bacteria persistence (Figure 1.2). Some of these functions allow *H. pylori* not only to acquire the necessary

nutrients but lead to host damage in the process (Atherton, 2006, Higashi et al., 2005, Salama et al., 2013).

The physical interaction of the outer layer of *H. pylori* cell wall with the epithelial cells of the host via the T4SS also leads to pro-inflammatory response activation, such as expression of IL-8. Increased levels of this chemokine are associated with *cag+* strains (Fischer et al., 2001). These products are recognised by Nod1 molecules which mediate a series of reactions by activating signalling pathways via nuclear factor *k*B (NF*k*B) and biosynthesis (Viala et al., 2004). A number of genes that are essential for programming IL-8, human B-defensin 2 (human peptide with anti-microbial properties), anti-apoptotic factors and matrix metalloproteinases (proteases involved in apoptosis) are then activated by this process (Atherton, 2006).

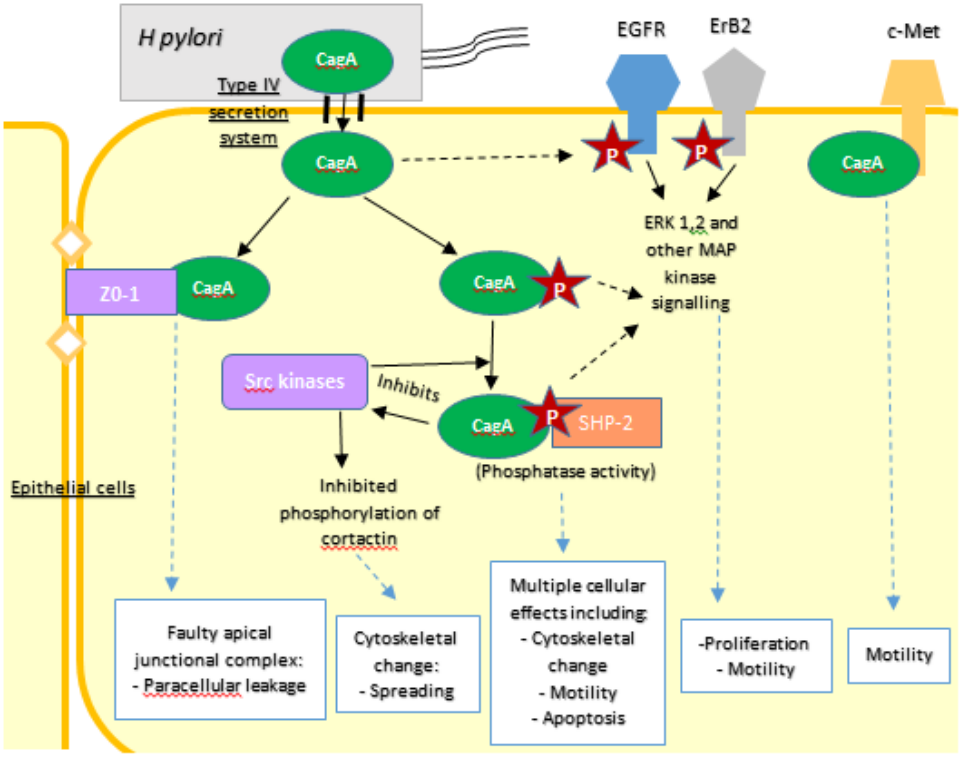


Figure 1.2: CagA signalling and cellular effects

Figure adapted from (Atherton, 2006). CagA protein=green oval, tyrosine phosphorylation= red P, Unclear pathway=dashed black line, effects CagA on cells=blue lines/boxes.

CagA also interferes with apoptotic pathways – programmed cell death. In animal models it has been shown to induce spermine oxidases (SMO) which cause oxidative stress and cell death, leaving a small surviving few with DNA damage. These cells are at significant risk to then become neoplastic (Piazuelo et al., 2008, Chaturvedi et al., 2011). CagA also interacts with proteins that reduces the p53 tumour suppressor ability and may escalate cancer formation (Buti et al., 2011). CagA is also implicated in affecting various other molecules which when overexpressed lead to precancerous lesions because normal cell transformation and growth are affected (Delahay and Rugge, 2012). These mechanisms lead to increased inflammation.

1.5.4.3 Disease association

cagA+ strains are more likely to be associated with the significant complications such as adenocarcinoma or peptic ulcer disease. *cagA*- strains can still cause peptic ulcer disease but it is more uncommon for it to be disease associated (Blaser et al., 1995). This strain dependence may be as a result of immune interactions, hosts surface proteins and the variation associated with the genetic diverse host (Salama et al., 2013).

High *H. pylori* prevalence generally is associated with higher risks for gastric cancer, but some geographical areas report lower gastric cancer rates despite the bacteria being highly prevalent with equivalent standards of healthcare and reporting. This is illustrated when looking at the Columbian population: those living in mountain areas have a twenty five-fold increased risk of gastric cancer when compared to a coastal region. These individuals originated from European ancestry where the virulence factors are associated with cell damage and gastric cancer risk (de Sablet et al., 2011). Along similar lines, the high occurrence of *cagA+* strains in Asian countries like Japan could be one of the explanations why adenocarcinoma of the stomach is increased in this region (Atherton, 2006).

The majority of strains express cagA protein but with varying related structures. cagA variates have up to five different tyrosine phosphorylation

sites; the greater this number, the greater the cagA phosphorylation and the more the severe is the cellular destruction. EPIYA regions are classified as A, B, C and D amino acid sequences. The Western pattern tends to be ABC and ABD pattern is seen in East Asia. Different EPIYA combination patterns can be associated with increasing degree of inflammation due to a greater degree of phosphorylation leading to more cellular damage. The risk of gastric cancer is dependent on the type of combination. In Western strains, gastric cancer risk is higher with the number of EPIYA-C regions (Basso et al., 2008).

More strain sites have also been associated with malignancy and pre-cancerous lesions (Atherton, 2006). Transgenic mice were created in the laboratory to express cagA, which later went on to induce malignancy. This study provided animal evidence of the cagA virulence factor ability as a cancer inducing protein (Ohnishi et al., 2008). However, the role of cagA in cancer may only be in the early stages because this protein is not found in cancer tissues. Specific alleles of another virulence factor, vacA, increase cagA cellular activity (Tsugawa et al., 2012).

1.5.4.4 Vacuolating Cytotoxin A (vacA)

The *vacA* gene differs from *cagA* in that it is present in all *H. pylori* strains, but this protein is only expressed in about 50% due to the presence of frameshifts and point mutations. *VacA* has many different forms, due to variation in three distinct areas of the gene: the signal (s), intermediate (i) and mid (m) region. It also has two different allelic variants for each regions, (s1/s2), (i1/i2), and (m1/m2) regions (Atherton et al., 1995, Rhead et al., 2007) . S1 and i1 regions are normally associated with an appreciable increase risk of peptic ulcer disease, atrophy and adenocarcinoma of the stomach, but *cagA* status and allele genetic relationship makes an estimation of each factor extremely difficult to determine (Rhead et al., 2007). VacA and cagA also have a functional relationship and can counteract the apoptotic process; cagA can prevent vacA entering host cells therefore protecting the host cell to which *H. pylori* is attached but allowing other cells to be affected by vacA(Oldani et al.,

2009, Akada et al., 2010). VacA and γ-glutamyl transferase have been shown in a mouse model to protect against asthma and promote Treg responses (Engler et al., 2014).

The s1/i1/m1 type strains are the most toxic, and expression of this form of vacA more common in strains from European or US patients with gastric adenocarcinoma (Letley et al., 2003). This could also account for the higher rates of gastric adenocarcinoma in Japan but this connection with the s1/m1 strain type has not been convincingly shown in Asian populations (Kusters et al., 2006). Mutations leading to inactivation of s1/m1 are the more likely cause of lower incidence of disease than the appearance of other types (Ito et al., 1998). VacA acts on the cell in a number of ways. VacA bound to the surface of the host is taken directly into the cell membrane. The main effects are as urea permease that promotes urea diffusion but vacA does not directly cause inflammation. It could also be taken up by the mitochondria to cause cellular apoptosis. Endocytosis of proteins may lead to the formation of vacuoles within the cell. A membrane channel could also be created to allow the passage of nutrients, necessary for survival. VacA mediated disruption of epithelial cell tight junctions could allow passage of the protein into the lamina propria to initiate T cells and their proliferation (Kusters et al., 2006, Cover and Blanke, 2005) (Montecucco and de Bernard, 2003). However, only a small amount of the secreted toxin is delivered to the host, with the majority remaining attached to the bacteria membrane (Ilver et al., 2004). As *H. pylori* comes into contact with host cells, toxins are released, presumably mediated by specific receptors, although they have not been successfully identified.

VacA expression may also be open to variation within the patient, owing to the constant adaption of the bacteria to allow persistence of infection. This could explain changes in toxicity of strains and why the appearance of ulcers varies (Atherton et al., 1995, Aviles-Jimenez et al., 2004, van Doorn et al., 1998, Kusters et al., 2006).

VacA has its effects on parietal cells, lowering the secretion of acid and therefore allowing for conditions to promote gastric cancer formation (Kusters et al., 2006). VacA protein also influences lymphocytes, reducing T lymphocyte activation by causing G1/S1 cycle programmed cell death, which is opposite to cagA which induces apoptosis of T lymphocytes (Gebert et al., 2003). S1 and i1 alleles are associated with greater disease risk (Rhead et al., 2007).

1.5.5 Other Virulence Factors

The majority of *H. pylori* is found within the gastric mucus layer but a small number are attached to epithelial cells, binding via expression of adhesins. Lewis b is an important antigen and is associated with higher *H. pylori* densities and the development of peptic ulcers (Boren et al., 1993, Atherton, 2006). BabA is a well characterised adhesion protein that mediates the binding to blood group antigens. It has been implicated in the development of peptic ulcer disease and gastric malignancy. However, it is not believed to be an independent disease marker (Gerhard et al., 1999, Rad et al., 2002). SabA acts on neutrophils and may have a role in inflammation and formation of gastric atrophy but it is not thought to be associated with early disease stages. Duodenal ulcer disease is present when the expression of SabB is not present (Mahdavi et al., 2002). OipA is a protein that forms the outer membrane of *H. pylori* so is normally present in all strains. It may be involved in IL-8 production and is associated with cagA; its presence is linked with peptic ulcer disease (Yamaoka et al., 2000, Yamaoka and Graham, 2014). DupA is a component of the T4SS and its role in disease is unclear (Shiota et al., 2010).

1.6 Established diagnostic tests for *H.pylori*

1.6.1 Urea Breath Test

One of the most used tests in clinical practice is the non-invasive Urea Breath Test (UBT). This test is very reliable; studies have shown it to have a sensitivity

and specificity of more than 95%. This high level of accuracy is due to the fact the UBT effectively involves testing the whole stomach. This test is used both to diagnose *H. pylori* and confirm that eradication treatment is successful. However, it does involve an overnight fast for the patients. They are asked to blow into a bag before drinking fluids labelled with ^{13}C urea. Approximately 30 minutes later they are asked to blow into the second. The test works on the principle that *H. pylori* hydrolyses the labelled urea to produce CO_2 which travels via the blood stream to the lungs where it is excreted and detected in the breath. UBT requires the use of a mass spectrometer to analyse the results. The test is very accurate because *H. pylori* is the most urease containing organism in the stomach, so a positive test is highly likely to be due to this bacterium (Gisbert and Pajares, 2004a).

1.6.2 Stool based tests

The stool antigen test is a protein based ELISA or immunochromographic test strip method which has a sensitivity of 91% and specificity of 93% (Malfertheiner et al., 2007). Unlike other tests, it does not require patients to fast and it is not influenced by other bacteria which are urease active. The tests can be successfully performed on stool samples stored up to a year (Sato et al., 2012). It is a cost effective test. There are also quick office stool tests available which provide immediate results (Gisbert and Pajares, 2004b). This test is mainly used to diagnose *H. pylori* infection in children pre-treatment because other techniques are less suitable. For example, gastroscopy may require a general anaesthetic and difficulties in getting children to follow the procedure required for a UBT. Stool antigen testing can also be used in epidemiological studies, is tolerable by patients and is quick. It detects alive, dead or degraded bacteria therefore cannot be completely reliable post treatment (Kabir, 2001). Proton pump inhibitor (PPI) use also affects sensitivity of the stool antigen test. There are many available kits for stool

antigen testing, some have published even better sensitivity and specificity when compared with UBT and serology (Makristathis et al., 2000).

Thomas *et al* were the first to successfully culture *H. pylori* from the faeces of African children in 1992 (Thomas et al., 1992). *H. pylori* was first successfully isolated and cultured from patients stool in the United Kingdom in 1990s (Kelly et al., 1994) but this is not done routinely because the extraction method can be time consuming and is unlikely to change initial clinical management.

General failure of PCR based stool testing may be due to problems with the reagents used to problems extracting DNA. Products of haemoglobin breakdown and complex polysaccharides present in faeces are also known to affect the PCR results (Monteiro et al., 1997). The many inhibitors present in human faeces must be removed for purification of DNA to allow good amplification during PCR. The first stage is the removal of inhibitor using the process of diffusion or filtration prior to the PCR. There are a variety of methods using different types of membranes. Embedded DNA is washed and the unwanted material diffuses through the membrane and the wanted DNA remained trapped. Alternatively the PCR inhibitor material can be disregarded by passing through specially designed filters. Paramagnetic beads coated with antibodies can also be used in the pre-PCR steps to remove inhibitor. Another method is the use of silica gel or other substances to absorb nucleic acid (Kabir, 2001).

There are commercial available stool PCR kit for the detection of *H. pylori* and clarithromycin resistance using fresh or frozen stool samples. ClariRes assay (Ingenetix, Vienna, Austria) has recently being developed and shown to have an accuracy in the low ninety percentage for detecting *H. pylori* and clarithromycin resistance (Scaletsky et al., 2011).

1.6.3 Biopsy based tests

1.6.3.1 Rapid Urease Test

The rapid urease test (or CLO test) is performed immediately after the biopsy obtained during upper GI endoscopy. It works on the basis that urease enzymes are produced by *H. pylori,* which are able to hydrolyse urea in the test to CO_2 and NH_4. This release of NH_4 correlates to a pH change which is seen as a colour change using in an indicator, such as phenol red. The unbuffered rapid urease test can give a result within one hour but CLO test which uses a solid media takes longer (up to 24 hours) for a test result. Both tests have similar sensitivity (over 90%) and specificity so the majority of clinical endoscopy units use the rapid urease test. Untreated patients with a positive test provide enough evidence for a clinician to start treatment. (Malfertheiner et al., 2007). However, after eradication treatment the sensitivity falls to 60% which is due to low numbers of the organism. (Nishikawa et al., 2000). The main advantages are the low costs and speed of the results. The main drawback is that PPI use effects the results and must be stopped within a minimum of two weeks prior to testing (Vaira et al., 2000).

1.6.3.2 Histological Examination

Information on the presence of bacteria, surrounding inflammation and evidence of atrophy is collected from biopsy samples. Histological staining helps identification of the bacteria. The distribution of *H. pylori* in the stomach may be patchy so a number of biopsies may be needed to confirm diagnosis. One of the widely used stains is Giemsa stain, which has a sensitivity (when compared to culture) of approximately 90% (Laine et al., 1997). Others include Genta's stain and Toluidine Blue which has a similar sensitivity to Giemsa. However, results may be affected by the experience of the pathologist, the staining used and the sites of the biopsy taken. If the biopsy is taken from areas of gastric atrophy or intestinal metaplasia, the bacterium is less likely to

be detected (Vaira et al., 2000). The updated Sydney classification is often used to grade gastritis (Dixon et al., 1996).

1.6.3.3 *H. pylori* Culture

The ability to cultivate *H. pylori* from gastric biopsies depends on: how rapidly it is processed, the experience of the person performing the test, and the culture media used. *H. pylori* are very slow to grow and may take up to seven days for the results to be available. *H. pylori* can be isolated from stool and saliva but this is not routinely done. Culture rates vary, depending on individual centres, but if the bacteria are cultured the specificity is 100% and this provides a definitive diagnosis of *H. pylori* colonisation (Vaira et al., 2000). Culturing also allows for the characterization of specific strains. In addition, antibiotic sensitivity testing allows for more focused treatment and provides information on local antibiotic resistance patterns. Thus more efficient use of resources and may reduce the risk of ulcer recurrence. PCR can be used to detect for mutations which lead to antibiotic resistance (Vaira et al., 2000).

1.6.7 Serology

Serology tests for *H. pylori* are very cheap and widely available. Serology is useful in situations where other tests may show a false negative such as GI bleeding due to PUD and the use of PPI. More detailed information is provided in chapter 2.

1.6.8 Other tests

Having easily obtainable samples is essential for developing a successful test. Saliva and urine are available in abundance and it is socially acceptable for patients to provide a sample for testing. However, assays using saliva have been tested as patients infected with *H. pylori* have been shown to have detectable *H. pylori* IgG levels in their saliva (Patel et al., 1994, Luzza et al.,

1995) but the results are disappointing. Saliva used to detect *H. pylori* antigen only had a sensitivity of 81% and specificity of 71%. Also although easy to perform the results do not match serum (Luzza et al., 2000).

It is thought the presence of other faster growing bacteria and proteins with antibacterial properties hinders the detection of *H. pylori* in saliva (Kabir, 2004). Approximately a quarter of those infected with *H. pylori* will have it detectable in saliva but the detection rate in studies ranges from 0-84% (Kabir, 2004). *H. pylori* antigens were first detected in urine in the early 1990's. Urine to detect *H. pylori* antigens has a sensitivity of 86% and specificity of 91% which is similar to serum but the results have not been successfully reproduced (Miwa et al., 1999, Vaira et al., 2002). Urine and saliva *H. pylori* antibody kits are available but their sensitivity is low and routine use in clinical practice is not recommended (Malfertheiner et al., 2007).

1.7 Non-invasive testing for *H. pylori* virulence factors and antibiotic resistance markers

1.7.1 Serum based tests

CagA+ strains are associated with more severe disease outcomes. The cagA molecule is highly immunogenic and this allows the detection of anti-cagA IgG in serum. The two main types of tests are ELISA and Immunoblot.

ELISA tests detect immunoglobulin G (IgG) *to H. pylori* cagA. More than 95% of *cagA+ H. pylori* patients have a positive serology but around 10% without documented infection still have positive serology. This could be due to past infection (Fusconi et al., 1999). CagA positive serology also correlates well to cagA positive strains grown by culture (Cover et al., 1995).

Immunoblotting uses electrophoresis to separate proteins which are then detected with specific antibodies. Some of the kits give a sensitivity of 74-100% and a specificity of 90-94% (Vaira et al., 2002, Basso et al., 1999). However, cagA serology may also remain positive in previously exposed individuals and those that are successfully treated. These tests are useful for research purposes but that cannot be recommended for use in clinical practice because of the false positives (Basso et al., 1999).

The accuracy of anti-cagA and vacA tests has been questioned in many studies. Figueiredo *et al.* in 2001 examined different assays to assess cagA status, the sensitivities for some were as low as 50% and as high as 100%, whilst others varied from 71% to 84.7%. They concluded that measuring cagA with the different available tests produced variable detection rates. Therefore, making it essential for researchers to quote which assay they used (Figueiredo et al., 2001). Yamaoka *et al*. also concluded that they could not recommend ELISA or Immunoblot as a reliable test (Yamaoka et al., 1998). In contrast to this, Kivi *et al.* compared immunoblot cagA status with PCR and found that serological methods were useful for assessing cag PAI status (Kivi et al., 2005). There are now commercially available anti-cagA IgG ELISA kits.

In conclusion, serological tests may provide useful information of virulence status but may produce variable results. These tests are also user dependent and are subject to cross reactivity. Nevertheless they provide rapid results and are useful for epidemiological studies.

1.7.2 Molecular Methods - PCR

Polymerase Chain Reaction (PCR) typing is excellent for characterising the virulence properties of different strains of *H. pylori* (Cirak et al., 2007). PCR tests for strain and virulence factors are only used in the research setting. PCR amplifies DNA from clinical samples to detect the presence of *H. pylori*. Primers are also available to detect *cagA, vacA*, other virulence genes and

specific *H. pylori* genes such as 16S RNA. This technique is very sensitive, specific with results approaching 100% (Ruzsovics et al., 2004) and allows the detection of *H. pylori* DNA directly from clinical samples. PCR provides more sensitive and specific results than traditionally used culture, histology and urease tests (Fabre et al., 1994). This method is vital when bacterial detection is difficult due to low organism numbers and those with slow growth. Samples used for PCR work include cultured cells, fresh or frozen cells or samples that are embedded in paraffin. Gastric biopsy DNA is extracted and purified from commercially available kits to enable the analysis of high quality DNA and the avoidance of inhibitors that will affect the final analysis. BioRobot EZ1 Qiagen kit produces high quality DNA with small amount of inhibitors leading to less DNA contamination and resulting in less false negatives (Ruzsovics et al., 2004). Other drawbacks of using PCR include the inability to establish whether the organism has died and that it is too expensive for routine use in clinical laboratories (Kabir, 2001).

There are a variety of PCR methods used. One method uses specific primers to detect single or multiple mutations or genes e.g. cagA. Gastric biopsy DNA can be analysed by this method using gel electrophoresis which takes about six hours. Another method detects restriction fragment length polymorphisms (RFLP), when DNA is broken up by restriction enzymes (endonucleases) and these fragments are separated by their length again by electrophoresis. The process takes approximately eight hours and can be used to examine gastric juice and biopsies. RFLP can be used for 23s rRNA characterisation. (Simala-Grant and Taylor, 2004). This identifies genes leading to clarithromycin resistance, an important antibiotic which is used in eradication (Vaira et al., 2002). Another method is PCR oligonucleotide ligation, which works by matching probes to DNA leading to ligation. This process can be used for the detection of antibiotic resistance. PCR dependent preferential homoduplex formation assay (PCR-PHFA) does not require *H. pylori* culture and be used to test stomach biopsies or its juices. Real time PCR uses the melting curves of the product and is a very fast test with results within two hours. Fluorescent

in situ hybridization (FISH) uses fluorescent probes to target areas of interest and results are obtained within three hours. PCR single strand confirmation polymorphism (SSCP) uses both PCR and gel electrophoresis. DNA enzyme immunoassay (DEIA) and Line probe assay (Li-PA) can be used for mass sample analysis. Other techniques include PCR in combination with sequencing (Simala-Grant and Taylor, 2004).

Chapter 2: Using a novel advanced endoscopic imaging technique to detect *H. pylori* associated disease in combination with serological markers

2.1 Introduction

2.1.1 White Light Endoscopy

Since the development of the endoscope to visualise the gastrointestinal tract by Basil Hirchowitz (Hirschowitz et al., 1957) there have been major advancements that now allow detailed visualisation and improved correlation with histology.

The routinely used endoscopy system design consists of a light source, image monitor, flexible endoscope and image processor. White light endoscopy (WLE) provides a detailed visualisation of the mucosa and the system consists of external arc lamp which causes the mucosa to illuminate via the visible full white light spectrum (400-700nm). The Charge-Coupled Device (CCD) is located at the tip of the endoscope. It is a photoactive region made up of a number of photo cells which receives this reflected light from the lens. An onscreen monitor displays digital images produced from the electrical information received from the CCD. The digital displayed image quality is determined by the CCD pixel density and monitor screen resolution (Beg and Ragunath, 2015). WLE endoscopes can produce of approximately 400,000 pixels compared to up to 1 million pixels seen with High Definition (HD) Endoscopes (Marvik and Lango, 2006) which boost the CCD and allowing for a more detailed assessment of mucosal pit pattern, vascular pattern and potentially aiding disease detection. This higher pixel density and more rapid monitor scanning produces a much sharper image (Subramanian and Ragunath, 2014) and are capable of magnifying images by factors of greater than 100 with resolution as small as 7.9µm which even enables the visualisation of capillaries and red blood cells (Yao et al., 2002, Anagnostopoulos et al., 2007). HD endoscopes are often used in combination with other image modalities to assist in disease detection. However, these improvements thus far have not removed the need for random biopsies.

2.1.2 Narrow Band Imaging

Narrow Band Imaging (NBI) was developed by the Olympus Corporation, Tokyo, Japan endoscopes (Gono et al., 2004, Yoshida et al., 2004). An additional filter is placed next to the white light source thus reducing the filtered wavelength bands (415-540nm) thus increasing the contribution of blue light (Asge Technology et al., 2008). Light penetration to tissue is dependent on the wavelength of light. Shorter wavelength blue light penetrates more superficially, whereas longer red light penetrates deeper. Blue light is also the wavelength absorbed by haemoglobin which therefore enables a sharper contrast between mucosal and vascular structures, appearing darker than the surrounding less vascular mucosa. NBI endoscopes enable the endoscopist to switch from WLE to NBI easily during the procedure to aid visualisation and optimise biopsy sampling. However, a major limitation of NBI is that after biopsies are taken the view is disturbed as blood strongly absorbs NBI light (Asge Technology et al., 2008). Other modalities often used in combination include computerized visual or dye chromoendoscopy, Autofluorescence (AFI) endoscopy, Confocal Laser Endomicroscopy and infra-red endoscopy.

2.1.3 WLE and NBI in identifying *H. pylori* associated disease

Standard WLE gastritis often correlates poorly with histology and the agreement among endoscopists is often not consistent (Laine et al., 1995, Redeen et al., 2003). There is also no consensus on the macroscopic appearance of gastritis and atrophy therefore clinicians are often reliant on histological findings (Bah et al., 1995, Redeen et al., 2003). However, the current gold standard use of random biopsies and use of the Sydney classification for the histological diagnosis of intestinal metaplasia fails to identify up to half of the proven cases (Dixon et al., 1996, El-Zimaity and Graham, 1999). Diagnosis of *H. pylori* associated gastritis, atrophy and intestinal metaplasia by histology is subject to a multitude of factors such as

biopsy area, biopsy number, pathologist experience and so is subject to sampling error (Gonen et al., 2009).

Anagnostopoulos *et al* described the magnification features of WLE (WLE-Z) and correlated these with histological findings and proposed a classification based on the premise that the normal mucosal surface of the gastric body once magnified was found to consist of a honeycomb type sub epithelial capillary network (SECN) with a regular arrangement of collecting venules and that the neck of gastric pit is surrounded by these sub epithelial capillaries, thus forming a regular honeycomb structure which drains the gastric mucosa. In contrast, a Type 1 pattern consists of regular pit and venules arrangement with a SECN pattern similar to a honeycomb. Type 2 is similar to type 1 but there is loss of collecting venules and sulci may or not be present. With Type 3, the normal SECN pattern and collecting venules are lost, surrounded pits are white, enlarged and encircled by redness. With Type 4, the normal SECN and pit pattern is lost with venules arranged in an irregular distribution, Figure 2.1. These patterns only apply to the corpus because collecting venules in antrum are difficult to visualise. The sensitivity, specificity, positive predictive and negative predictive values were as follows: Type 1 for predicting normal mucosa was 92.7%, 100%, 100% and 83% respectively, Type 2/3 for predicting *H. pylori* gastritis was 100%, 92.7%, 83.8% and 100% respectively. For Type 4 predicting atrophy was 90%, 96%, 85.7% and 97.3% respectively (Anagnostopoulos et al., 2007). This will later be referred to as the Nottingham Classification system. Similar results were seen in Yagi et al study using Japanese subjects (Yagi et al., 2002).

With regards to the performance of magnified NBI (NBI-Z) in distinguishing normal, *H. pylori* infection and atrophic mucosa the literature is good but the results vary due to study limitations (Curvers et al., 2009). In early studies NBI-Z has been shown to accurately identify *H. pylori* gastritis from the normal stomach with good agreement (Yagi et al., 2002, Yao et al., 2002). Later Pimentel *et al* proposed a more simplified classification using NBI with high accuracy rates: pattern A for predicting normal was 83%, pattern B for

predicting intestinal metaplasia 84%, pattern C 95% for dysplasia and variable vascular density (addition pattern +) 70% for *H. pylori* infection (Pimentel-Nunes et al., 2012). This will later be referred to as the Porto Classification system. Tahara et al was also able to accurately distinguish normal from *H. pylori* gastritis and atrophy using NBI-Z but one limitation was that there was only a single endoscopist and no observer agreement was carried out (Tahara et al., 2009). Another study showing similar findings was conducted on a Chinese population (Liu et al., 2014).

However, NBI without magnification was superior to WLE in detecting intestinal metaplasia and dysplasia in one European study with sensitivity and specificity of 71% and 58% (Capelle et al., 2010b). A larger study on an Asian population found a sensitivity and specificity of around 46.8% and 76.4% (Eshmuratov et al., 2010). The combination of NBI and magnification allows for clear visualisation of the gastric mucosa.

With regards to intestinal metaplasia, the light blue crest (blue-whitish patchy reflections sited on the epithelial margins) appearance in magnified NBI appearance of gastric mucosa was first described by Uedo *et al* and correlated well with an accuracy of 91% (Uedo et al., 2006). White Opaque Substance (WOS) is sometimes seen in certain epithelial tumours and intestinal metaplasia (Yao et al., 2012). Another feature associated with intestinal metaplasia is the ridge/villous pattern which also correlate well with histology (Bansal et al., 2008). A simplified classification system for precancerous and cancerous lesions described by Pimentel-Nunes *et al* reliably detected intestinal metaplasia with an accuracy of 84% (Pimentel-Nunes et al., 2012). Current European guidelines on the management of precancerous conditions and lesions recommends magnification chromoendoscopy with NBI to improve diagnosis and not standard WLE based on level 2++ evidence.

2.1.4 Serological markers of *H. pylori* associated disease

Serology detects *H. pylori* antibodies, so it is relatively cheap and non-invasive. Serology kits either measure specific immunoglobulin IgA, IgM and

IgG antibodies simultaneously, or IgA or IgG alone by ELISA (Laheij et al., 1998). IgG ELISA is the favoured serology test used. Accuracy is variable but some commercial kits have accuracy greater than 90% (Malfertheiner et al., 2012, Feldman et al., 1995). The diagnostic accuracy of *H. pylori* serology is lower at around 84%. However, it is important to note that the results are not affected by PPI treatment, like the other tests (UBT/CLO/histology/stool antigen). Serology tests can also detect antibodies against CagA (Malfertheiner et al., 2007). The main drawback with *H. pylori* serology is that a positive test cannot distinguish between a current or recently eradicated infection and may remain positive despite successful treatment. This will therefore lead to a number of false positives. Titre levels are not routinely tested in clinical practice. Commercial kits are available from several suppliers.

Serum pepsinogen levels can be measured in peripheral blood and can be used in clinical practice to predict the extent of gastric atrophy. Pepsinogen I (PGI) and Pepsinogen II (PGII) are released from secretory cells found in the gastric mucosa. PGII is also released from the duodenal mucosa. Inflamed gastric mucosa increases the level of PGI and PGII. This is usually greater in PGII so the PGI/II ratio decreases. With the progression to atrophy this causes the loss of these secreting cells and reduces pepsinogen levels. PGI is more affected by this and thus the ratio is decreased further. PGI/II ratio decreases with increase corpus atrophy, a ratio <3 and PGI <30µg/l signifies moderate to severe corpus atrophy (Varis et al., 1979, Varis et al., 2000). Therefore, low PGI or PGI/II ratios are good markers of the extent of atrophy in the corpus (Dinis-Ribeiro et al., 2012, Kuipers, 2003). Multiple studies have compared serum PG levels and correlated with histological findings, but due to the variation in populations studied, PG level cut off values are difficult to define. For estimating extent of atrophy, studies have described sensitivity and specificity from 9.4% to 92.3% and 9.9% to 100% respectively (Dinis-Ribeiro et al., 2012, Inoue et al., 1998, Graham et al., 2006, Leja et al., 2009). For intestinal metaplasia, sensitivity and specificity ranged from 15 to 75% and

92.2% to 97.8% respectively (Haj-Sheykholeslami et al., 2008, Urita et al., 2004, Dinis-Ribeiro et al., 2012).

Other biomarkers can accurately diagnose moderate to severe gastric atrophy when comparison is made with histology. A low fasting Gastrin-17 (G-17) level and *H. pylori* infection can indicate moderate to severe antrum atrophy or high acid secreting stomach. Also an abnormally raised G-17 level could be due to hypochlorhydria and may indicate gastric atrophy that is confined to the corpus (Vaananen et al., 2003, Storskrubb et al., 2008).

2.1.5 NBI in detecting villous atrophy (VA)

Coeliac disease is an immune mediated disease which affects individuals that are genetically predisposed to gluten allergy (Ludvigsson et al., 2013). The prevalence in Europe although variable is approximately around 1% and can affect individuals of any age. Patients can present with a varied number of symptoms and signs or can be asymptomatic (Ludvigsson et al., 2014). Diagnosis is confirmed by histological examination for architectural changes in the small bowel mucosa: increased number of intraepithelial lymphocytes (IELs), crypt hyperplasia and villous atrophy. However, serology tests are often used in children. The mainstay of treatment is avoidance of gluten in an individual's diet (Rostom et al., 2006).

Villous atrophy is the most characteristic finding in coeliac disease. Macroscopic endoscopy appearances include reduction in duodenal folds, scalloping and nodularity but the mucosa can also appear normal in early/patchy disease so macroscopic appearances can only guide biopsy sampling but not replace it (Dickey and Hughes, 2001, Oxentenko et al., 2002). Studies have shown NBI-Z endoscopy is useful in detecting villous atrophy (Banerjee and Reddy, 2009, Valitutti et al., 2014). Singh *et al* evaluated duodenal morphology and found a sensitivity and specificity for detecting and grading villous atrophy of 93.7%, 97.8%, 83.3% and 100% respectively (Singh

et al., 2010). Dutta *et al* investigated 100 subjects with NBI-Z and had sensitivity and specificity of 87.5% and 95.2% respectively (Dutta et al., 2014). De Luca *et al* compared standard WLE with NBI-Z and found that it was inferior with sensitivities of 41% vs 100%(De Luca et al., 2013). However, Badreldin *et al* found lower specificity of 62.9% of WLE-Z at detecting villous atrophy (Badreldin et al., 2005).

2.1.6 Serological markers of villous atrophy

Serological tests include IgA anti-tissue transglutaminase antibodies (TTG), endomysial antibodies (EMA) and/or IgA/IgG deamidated antigliadin antibodies (DGP) (Ludvigsson et al., 2014). EMA are very accurate but are less reliable and more expensive than TTG. The sensitivity and specificity for EMA range from 68-100% and 89-100% respectively. But for TTG these are lower at 38-100% and 25-100% respectively (Evans and Sanders, 2011).

2.1.7 Summary

No study has evaluated the utility of NBI-Z in the context of iron deficiency anaemia patients and only a limited number of studies have looked at advanced endoscopic imaging in detecting villous atrophy.

The potential clinical implications of having advanced endoscopy techniques to diagnose conditions that traditional white light endoscopy could not do without biopsies and that allow for real time diagnosis are huge. Minimising the number of biopsies needed would reduce associated costs and sampling error by targeting area of disease. (Dutta and Chacko, 2015) However, the main advantage with this technique is that if atrophy and *H. pylori* infection is detected, a more detailed examination of the stomach could then be done to attempt to identify early precancerous lesions. In this study we decided to investigate the detection of three conditions (*H. pylori* gastritis, gastric atrophy and villous atrophy/coeliac disease) that may be diagnosed by endoscopy features in patients with iron deficiency anaemia.

2.2 Aims

Assess the feasibility of magnification endoscopy with or without NBI and serology in identifying *H. pylori* gastritis, gastric atrophy and coeliac disease when compared to gold standard histology. To evaluate the inter observer agreement in assessment of magnified still images between endoscopists of varied experience.

Primary aims

- Assess the diagnostic accuracy of magnified endoscopy with NBI in diagnosing *H. pylori* gastritis, gastric atrophy and coeliac disease using the Nottingham and villous morphology classification.
- Compare the sensitivity, specificity and accuracy of magnification endoscopy with or without NBI and serology for detecting *H. pylori* gastritis, gastric atrophy and coeliac disease.

Secondary Aims

- Compare the sensitivity, specificity and accuracy of two classification systems (Modified Porto and Nottingham) using both WLE and NBI.
- Evaluate the inter-observer agreement of these classification systems between consultant and trainee endoscopist.

2.3 Hypotheses

We hypothesize that NBI with magnification will be superior to WLE and serology at detecting *H. pylori* gastritis, gastric atrophy and villous atrophy. Also that inter-observer agreement among consultant gastroenterologists is better than trainees.

2.4 Materials and Methods

2.4.1 Participants and clinical samples

The work was carried out after approval from the Derbyshire research ethical committee (REC Ref: 10/H0401/33) and informed consent from adult patients attending Nottingham University Hospitals NHS Trust for a diagnostic gastroscopy as part of their investigation into iron deficiency anaemia. Patients where no obvious cause for anaemia was found after full investigations were included in the final analysis. Adult patients between ages 18 to 85 years with a haemoglobin less than 130g/l for men and 120g/l for woman and either a low MCV or ferritin less than 25mcg/l men and 13 mcg/l for women were recruited. Patients on anticoagulants, with severe co-morbidity, peptic ulcer disease, oesophageal or gastric varices, malignancy or who had an obvious overt cause for the IDA found were not recruited. Patients with inadequate images or histology were also excluded. 150 patients were prospectively recruited, after exclusion, a total of 125 patients were included in the final analysis. The most common cause for exclusion after recruitment was that a malignancy was found or that no digital images or biopsies were obtained.

Dr JR White was responsible for the coordination, patient screening, recruitment and management of the study. All endoscopists were experienced in magnification endoscopy and NBI. Patient recruitment and sample collection was done by Dr JR White (35%), Dr S Sarmed (26%), Dr J Ortiz (15%), Dr E Telakis (12.5%), Dr J Mannath (2.5%), Prof K Ragunath (6%), Dr V Subramanian (3%). Fasting blood samples were collected for ELISA and laboratory analysis. Four biopsies were collected from the second part of the duodenum, two from the gastric antrum and corpus and immediately placed in three pots containing formalin for later histopathological scoring. One biopsy from the antrum was placed in urease medium for rapid urease detection, and another sample in iso-sensitest broth (Oxoid, Cambridge, UK)/10% glycerol for *H. pylori* isolation and culture.

2.4.2 Laboratory Investigations

Fasting blood samples were also collected for haematological and biochemical analysis by the local pathology department at Nottingham University Hospital. These tests included full blood count, B12, folate, coeliac serology (TTG), serum ferritin, transferrin saturation, transferrin, serum iron and iron binding capacity.

2.4.3 Histopathological analysis

Gastric and duodenal samples were placed in formalin and prepared and stained by the Histopathology department at the University Hospital in Nottingham. The samples were fixed in formalin, then embedded in paraffin and cut into approximately 4μm sections. Sections were stained with haematoxalin and eosin (H&E) to allow inflammation, atrophy and intestinal metaplasia scores to be carried out. Toluidine blue staining was also carried out for *H. pylori* density grading. For this grading scores were rated from zero to three, where 0=no *H. pylori*, 1=mild, 2= moderate, 3= high density. Villous atrophy was graded using Marsh criteria (Marsh, 1992). Three experienced GI histopathologists (Dr J. Davis/Dr A. Figgins/Dr P. Kaye) who were blinded to clinical and laboratory findings carried out the grading and classification using the updated Sydney scoring system (Dixon et al., 1996). Each biopsy was examined by two histopathologists (Dr J. Davis/Dr P. Kaye or Dr A. Figgins/Dr P. Kaye) and a consensus diagnosis was made.

2.4.4 ELISA assay

8mls of blood was collected in EDTA tube and centrifuged at 200 x *g* for 10 minutes at room temperature and three aliquots of plasma stored at -80°C. ELISA kits were followed according to manufacturer's instructions. Optical densities were measured with a microplate reader at 450nm with the reference wavelength of 620nm (See appendix 1 for an example of a typical calibration curve). *H. pylori* IgG (Biohit), Gastrin-17 (Biohit), Pepsinogen I

(Biohit) and Pepsinogen II (Biohit) ELISA were completed by Dr JR White (44 samples) and D. Reddiar (41 samples).

2.4.5 Endoscopy procedures

Procedures were done under pharyngeal local anaesthetic spray Xylocaine; AstraZeneca, Luton, UK) or conscious sedation and analgesia (midazolam/pethidine) depending on patient preference. A black soft rubber hood (MB46, MAJ-1990, Olympus) was attached to the endoscope tip; this was to allow a fixed distance of 2mm between the gastric mucosa and the end of the gastroscope. All procedures were done with high definition and magnification Gastroscopes (GIF-FQ260Z; Olympus Optical, Tokyo, Japan) and Lucera Elite CV290 video processor. The video images were viewed on a high definition video monitor (OEV-191H, Olympus). During the procedure the mucosa was washed with a mixture containing 100ml of water mixed with 2ml of acetylcysteine (200mg/ml, Parvolex, Celltech, UK) and 0.5ml (40mg/ml) dimethicone (Infacol, Forrest Laboratories, UK) to clear any additional mucus. Firstly, the duodenum was examined in overview mode, first in standard WLE, then NBI and still images captured. Then the duodenum was assessed in WLE-Z and NBI-Z modes. After a careful examination, still digital images were recorded in both WLE-Z and NBI-Z. Biopsies were taken from the areas where the digital images were recorded as previously described in section 2.4.1. The same examination was completed for both the gastric corpus and antrum. A minimum of 12 images were recorded for each patient. All still images were transferred to an external hard drive.

2.4.6 Post endoscopy image production

Consecutive images, with corresponding histology were included. Out of focus or duplicates images were excluded. Images were stored as JPEG files (200-300 kilobytes, 1093x948 pixel array and 32 bit colour. Approximately 900 images were anonymised, given random numbers generated in Excel from

Office 2010 (Microsoft Corporation, Redmond, Washington, USA) and transferred into evaluation set folders. The folder contained the randomised, anonymised images placed in 8 folders according to the area and classification system.

- A) WLE-Z Corpus-Nottingham Classification
- B) NBI-Z Corpus Nottingham Classification
- C) WLE-Z Corpus- Modified Porto Classification
- D) NBI-Z Corpus-Modified Porto Classification
- E) WLE-Z Antrum-Modified Porto Classification
- F) NBI-Z Antrum- Modified Porto Classification
- G) WLE-Z D2 Classification
- H) NBI-Z D2 Classification

A training set was created containing a PowerPoint presentation (Microsoft; Redmond, Washington, USA) containing detailed descriptions of gastric and duodenal classifications. Also a word document which detailed study instructions and a data recorded Excel spreadsheet which recorded both image grade and quality according to a 10 point Visual Analogue Scale (VAS) (Likert, 1932). The quality of images were on a scale of 0-10 (0=worst clarity/unclear views hindering diagnosis with confidence, 10=most clear ever/excellent views allowing for diagnosis with utmost confidence). All post endoscopy image randomisation, editing, evaluation folder organisation and creation of training presentation was done by Dr JR White.

2.4.7 Image assessment

Two principal endoscopists (Prof K Ragunath/Dr S Sarmed) experts in advanced endoscopy agreed on the magnified appearance of each selected images of the corpus, antrum and duodenum in WLE-Z and NBI-Z according to

specific criteria. Both endoscopists performed more than 500 NBI endoscopies. Both endoscopists were blinded to clinical and histological findings. Approximately 1000 anonymised, randomized images were reviewed and graded according to mucosal morphology using the classifications described below. These scores were used to assess magnification endoscopy performance in terms of sensitivity, specificity, accuracy, positive and negative likelihood ratios.

2.4.8 Image classification

The classification of the gastric corpus (the Nottingham Classification, see Figure 2.1) was evaluated in Prof Ragunath's previous research group's study and is widely used in our centre (Anagnostopoulos et al., 2007). Pimentel-Nunes *et al* simplified NBI classification of gastric lesions (Pimentel-Nunes et al., 2012) was modified to include gastric atrophy (class D) and termed the Modified Porto classification (see Table 2.1 and Figure 2.2 to 2.3). The endoscopic features for duodenal mucosa were classed according to histological grades (see figure 2.4): Normal (Marsh 0) = regular finger shaped projections, Partial villous atrophy (Marsh 3a) = villi have a shorter and stunted pattern, Total villous atrophy = surface was flat featureless with no identifiable villi (Singh et al., 2010, Marsh, 1992).

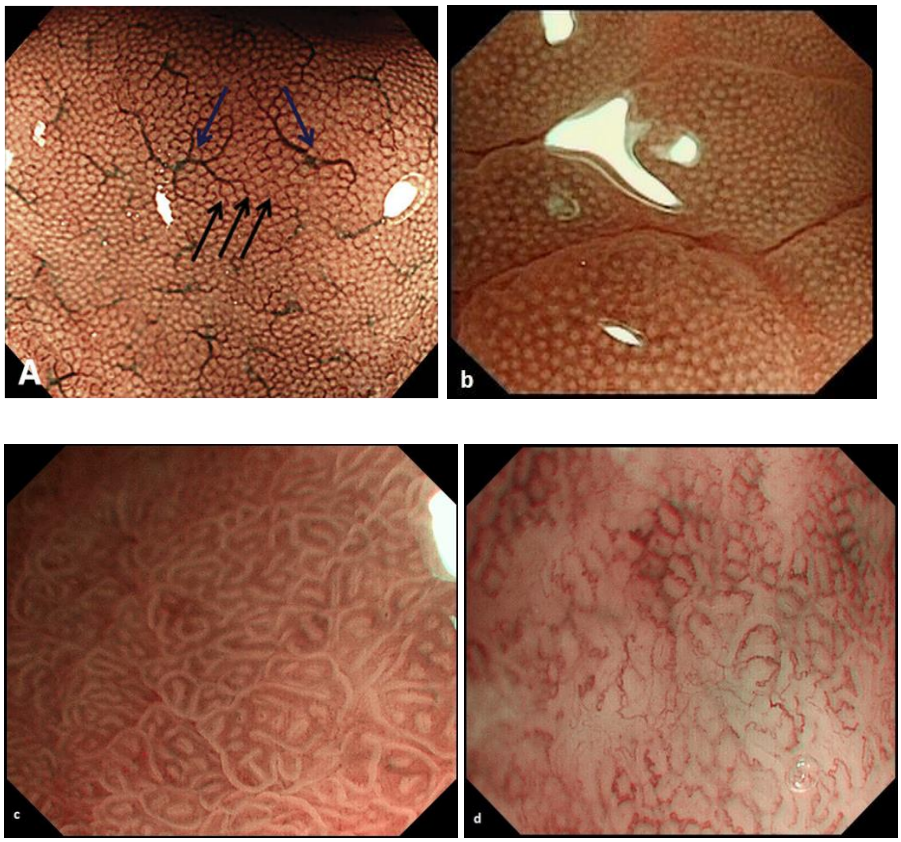


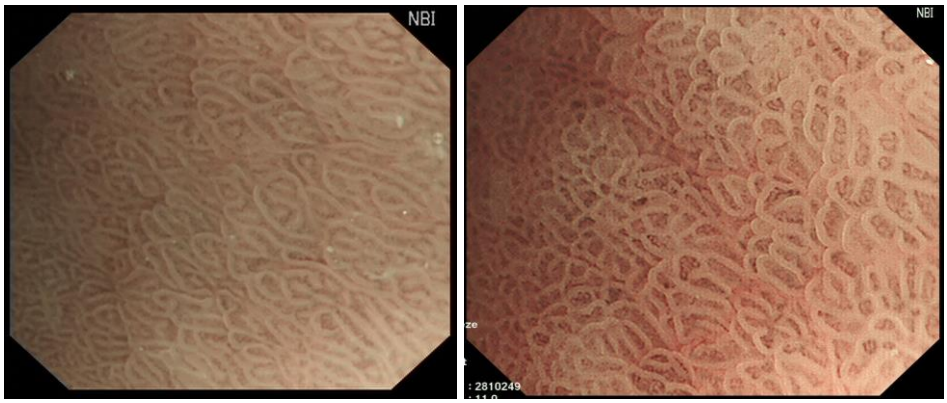
Figure 2.1 Nottingham classification in gastric corpus

Type I (A) normal gastric body microvasculature consists of a honeycomb type subepithelial capillary network (SECN) and collecting venules (CV) in a regular arrangement. Type II (b) comprises a honeycomb−type SECN with regular round pits, either with or without sulci, but with loss of collecting venules. Type III (c) there is loss of the normal SECN and collecting venules, with enlarged white pits surrounded by erythema. Type IV (d) is characterized by loss of the normal SECN and round pits, with irregular arrangement of the collecting venules (Anagnostopoulos et al., 2007). Magnification 115X.

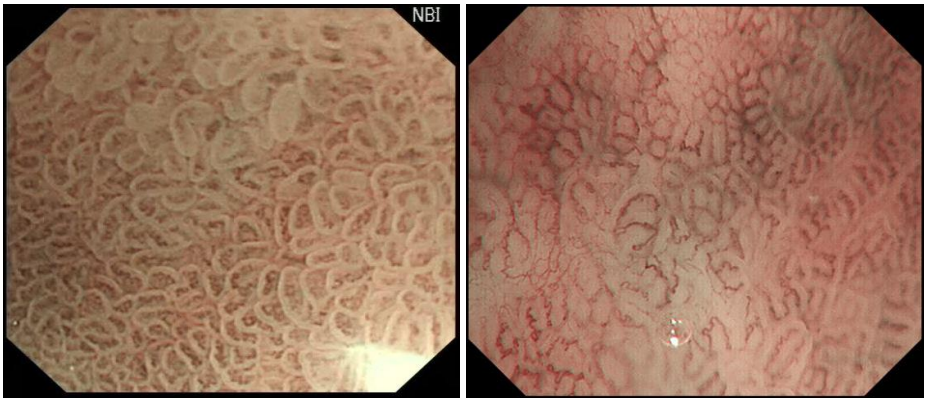
Table 2.1 Modified Porto classification

	A*	**B***	***H. pylori* +**	**C***	**D***
Mucosal pattern	Regular circular/oval	Regular Ridge/tubulo-villous with or without light blue crest	Regular	Irregular/ absent with or without white opaque substance	Absent
Vascular pattern (SECN)	Regular mucosa and SECN Vascular pattern central in antrum/peripheral in corpus of gland	Regular	Regular/ variable vascular density	Irregular	Absent
Expected outcome	Normal	Intestinal metaplasia	*H. pylori* infection	Dysplasia	Atrophic gastritis

Proposed simplified classification (Pimentel Nunes et al) was modified by addition of class D for gastric atrophy. The letter (a) for antrum or (b) for corpus is added to the class type to signify the sites. For *H. pylori* positive pattern, the symbol (+) is added to end of the pattern class site. For e.g. *H. pylori* positive pattern in corpus would be classed as Ab+. Adapted from (Pimentel-Nunes et al., 2012).



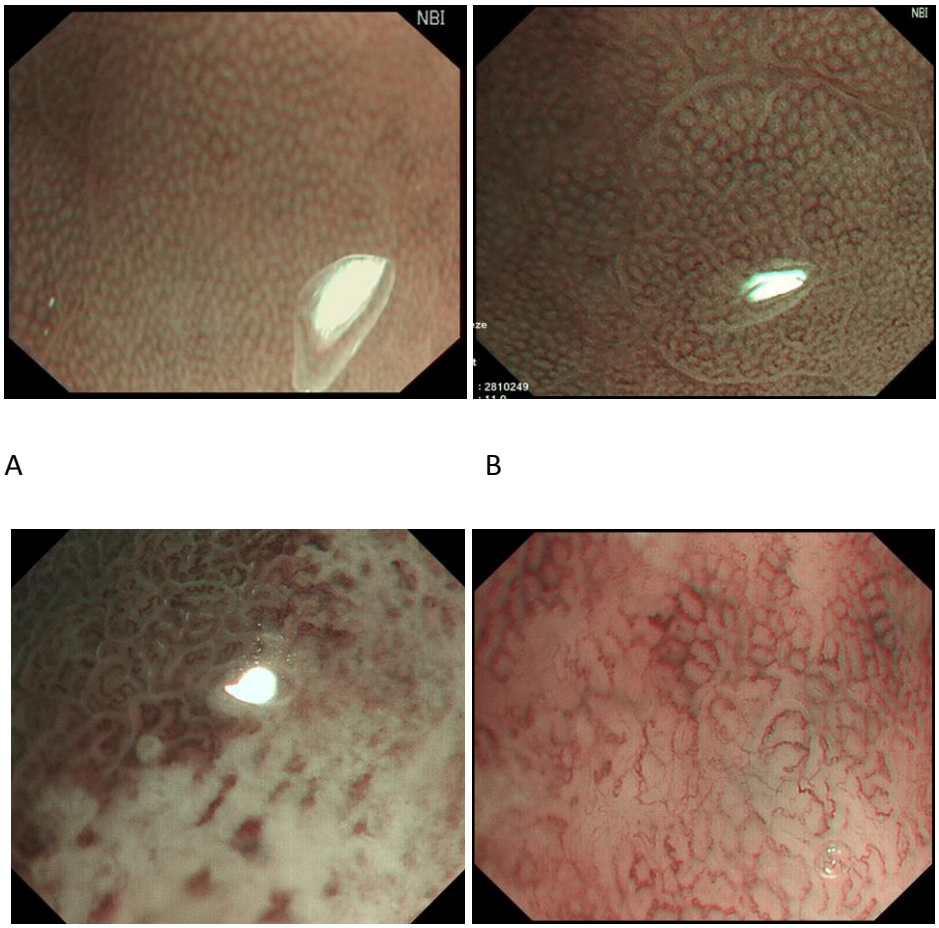
A B



C D

Figure 2.2 Modified Porto classification in antrum

(A) Normal antrum (Aa), (B) *H. pylori* gastritis antrum (Aa+), (C) intestinal metaplasia (Ba) and (D) atrophy in antrum (Da). Magnification 115X.

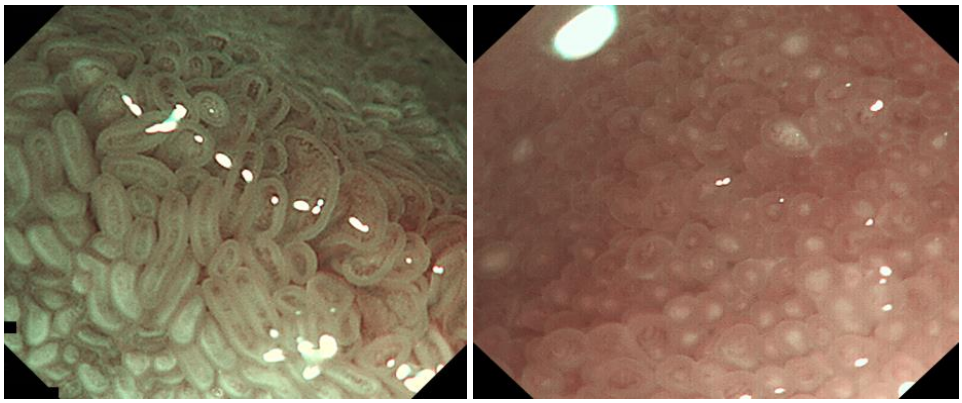


A B

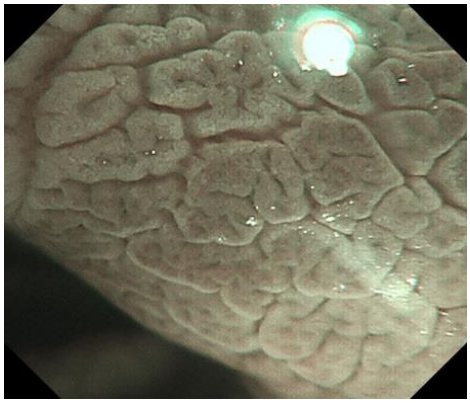
C D

Figure 2.3 Modified Porto classification in corpus

(A) Normal corpus (Ab), (B) *H. pylori* gastritis corpus (Ab+), (C) Intestinal metaplasia/ light blue crest corpus (Bb) and (D) atrophy in corpus (Db). Magnifications 115X.



A B



C

Figure 2.4 Magnified images of duodenal mucosa

Normal duodenal villi (A), partial villous atrophy (B) and total villous atrophy (C). Magnifications 115X.

2.4.9 Inter-observer variability

Eight endoscopists who were blinded to histological and clinical findings were invited to assess the endoscopy still digital images. Four expert endoscopists were consultant gastroenterologists: Dr J Mannath, Dr S Ahmad, Dr Telakis and Dr A Parra-Blanco. Four gastroenterology trainees: Dr M Kasi, Dr P Thiagarajan, Dr R Scott and Dr S Begg. All endoscopists reviewed the same Powerpoint training set and evaluated the images independently and in a randomised fashion. Each endoscopist viewed all 8 folders with approximately 900 images over an unrestricted length of time. Scores for each image and

VAS were recorded and captured into an excel spreadsheet. Each assessor recorded scores according to Modified Porto, Nottingham and villous morphology from a drop down menu of pre-defined options. VAS was graded 0 to 10.

2.4.10 Statistics

Sensitivity, specificity, accuracy, positive and negative likelihood ratios along with 95% confidence intervals (CI) for magnification endoscopy appearances and serological tests were compared with gold standard histological findings separately. Chi-squared test was used to compare diagnostic accuracies.

Kappa (k) scores were calculated to determine chance adjusted agreement for endoscopy images among experts and trainees. Interpretation of k values was as follows: <0 indicated no agreement; 0–0.20 = slight agreement; 0.21–0.40 = fair agreement; 0.41–0.60 = moderate agreement; 0.61–0.80 = substantial agreement; and 0.81–1 = almost perfect agreement (Landis and Koch, 1977). Image quality was assessed using the Mann Whitney U test. P values less than 0.05 were considered as statistically significant. Stata version 14 (Stata Corporation, College Station, Texas) were used for the statistical analysis. All statistical analysis was carried out by Dr J.R White.

2.5 Results

2.5.1 Demographics

125 patients were included in the final analysis, 53 male and 72 female with a median age of 65 years (range 19 to 85 years). 66% of procedures were performed under conscious sedation with intravenous midazolam (median dose 3.25mg) and pethidine (median dose 25mg). See Table 2.2 for demographic and clinical data. 22% patients had histological evidence of *H. pylori* infection, 33% gastric atrophy and 7.2% coeliac disease.

Table 2.2 Patient demographics and clinical data

Number of patients	125
Male: Female	53:72
Median age (range)	65 (19-85)
≥1 co-morbidities	36%
Aspirin (75mg)	20%
Haemoglobin males (130-180g/L)	117 (8.5 -14.8)
Haemoglobin females (115-165g/L)	108 (3.8-14.5)
MCV (84-102fl)	83 (56-111)
Ferritin (25-350µg/L)	13 (1-474)
Iron (9-31µmol/L)	11 (1-83)
Folate micro g/L (3.9-14)	9.4 (1.5-24)
B12 ng/L (190-800)	347 (16-980)
TTG +ve	7.2%
% Positive *H pylori* IgG	42%
Median Pepsinogen I µg/l	112 µg/l (2.8-367)
% Pepsinogen I <30µg/l	12.5%
Median Pepsinogen II µg/l	20.4 µg/l (3-55)
Median PG I/II ratio	5.9 (0.3-36)
% Pepsinogen I/II Ratio <3	36%
Sedation	66%
Median midazolam (mg)	3.25mg (1-5mg)
Median Pethidine (mg)	25mg (25-100mg)

Demographic and clinical data represented as median with range. Serological markers of *H. pylori* gastritis and atrophy represented as median values and percentages.

2.5.2 Primary endpoints

A) Diagnostic accuracy of NBI-Z in detecting *H. pylori* gastritis, gastric atrophy and coeliac disease

NBI-Z was reliable at predicting histology for *H. pylori* gastritis, gastric atrophy and coeliac disease using the Nottingham classification and villous morphology. The diagnostic accuracy of NBI-Z in predicting normal corpus (Type I) was 0.87 (95% CI 0.82-0.92), *H. pylori* gastritis (Type II/III) 0.65 (95% CI 0.55-0.75), gastric atrophy (Type IV) 0.88 (95% CI 0.81-0.94) and coeliac disease 1.0 (95% CI 1-1). Sensitivities, specificity, positive and negative likelihood ratios are demonstrated in Table 2.3.

Table 2.3 Sensitivity, specificity and likelihood ratios with 95% CI of NBI-Z for detecting *H. pylori* gastritis, gastric atrophy and coeliac disease using Nottingham classification and villous morphology

	Sensitivity (95% CI)	**Specificity (95% CI)**	**Positive Likelihood ratio (95% CI)**	**Negative Likelihood ratio (95% CI)**
NBI-Z Type I	75.6% (64.6-84.7)	98.6% (92.2-100)	52.2 (7.4-366.8)	0.25 (0.17-0.37)
NBI-Z Type II/III	62.1% (42.3-79.3)	68.1% (58.8-76.4)	1.95 (1.32-2.87)	0.56 (0.34-0.9)
NBI-Z Type IV	75.6% (59.7-87.6)	100% (96.6-100)	-	-
Villous atrophy	100% (63-100)	100% (94.9-100)	-	0

B) Comparing NBI-Z and WLE-Z using the Nottingham classification

NBI-Z was superior to WLE-Z for detecting normal and atrophic corpus. NBI-Z had a statistically significant increased accuracy for detecting normal corpus (Type I) NBI 0.87 (95% CI 0.82-0.92) vs WLE 0.82 (95% CI 0.76-0.87), $p<0.0001$.

For detecting atrophy (Type IV), NBI-Z 0.88 (95% CI 0.81-0.94) vs WLE-Z 0.81 (95% CI 0.73-0.89), p<0.0001. WLE-Z performed better than NBI-Z for detecting *H. pylori* gastritis, NBI-Z 0.65 (CI 0.55-0.75) vs 0.69 (95% CI 0.6-0.79), p<0.0001. Sensitivities, specificity, positive and negative likelihood ratios are demonstrated in Table 2.4.

C) Comparing NBI-Z Nottingham classification and serology

H. pylori IgG had a numerically higher accuracy for detecting *H. pylori* gastritis but this did not achieve statistical significance. NBI-Z 0.65 (95% CI 0.55-0.75) vs *H. pylori* IgG 0.82 (0.73-0.9), p=0.078. NBI-Z was superior to serology for detecting gastric atrophy. NBI-Z 0.88 (95% CI 0.81-0.94) vs PG I/II ratio <3 0.74 (95% CI 0.62-0.85), p<0.0001, NBI-Z 0.88 (95% CI 0.81-0.94) vs PG<30µg/l 0.75 (95% CI 0.64-0.87), p<0.0001 and NBI-Z 0.88 (95% CI 0.81-0.94) vs Gastrin-17 >7pmol/l 0.7 (95% CI 0.58-0.82), p=0.01.

D) Detecting villous atrophy with NBI-Z, WLE-Z and serology

Both NBI-Z and WLE-Z were accurate at detecting villous atrophy. NBI-Z performed better, NBI-Z 1.0 (95% CI 1-1) vs WLE-Z 0.93 (95% CI 0.81-1), p<0.0001. NBI-Z also performed better than serology, NBI-Z 1.0 (95% CI 1-1) vs TTG 0.90 (95% CI 0.8-1), p<0.0001. Table 2.4 illustrates sensitivity, specificities and likelihood ratios for villous atrophy.

Table 2.4 Sensitivity, specificity and likelihood ratios with 95% CI for WLE-Z, NBI-Z and serology for detecting *H. pylori* gastritis, gastric atrophy and coeliac disease using Nottingham classification and villous morphology

	Sensitivity (95% CI)	**Specificity (95% CI)**	**Positive Likelihood ratio (95% CI)**	**Negative Likelihood ratio (95% CI)**
WLE Type I	66.2% (54.6-76.6)	96.8% (89-99.6)	20.9 (5.3-82.4)	0.35 (0.25-0.48)
NBI Type I	75.6% (64.6-84.7)	98.6% (92.2-100)	52.2 (7.4-366.8)	0.25 (0.17-0.37)
WLE Type II/III	74% (53.7-88.9)	64.9% (55-73.7)	2.11 (1.5-2.95)	0.4 (0.21-0.77)
NBI Type II/III	62.1% (42.3-79.3)	68.1% (58.8-76.4)	1.95 (1.32-2.87)	0.56 (0.34-0.9)
***H. pylori* IgG**	90% (68.3-98.8)	73.9% (61.9-83.7)	3.45 (2.26-5.27)	0.14 (0.04-0.51)
WLE Type IV	64.1% (47.2-78.8)	98% (93-99.8)	32.4 (8-130)	0.37 (0.24-0.56)
NBI Type IV	75.6% (59.7-87.6)	100% (96.6-100)	-	-
Gastrin-17 >7pmol/l	77.8% (52.4-93.6)	62.3% (49-74.4)	2.06 (1.37-3.10)	0.36 (0.15-0.87)
PG I <30µg/l	52.6% (28.9-75.6)	98.4% (91.2-100)	32.11 (4.4-235)	0.48 (0.3-0.77)
PG I/II ratio <3	73.7% (48.8-90.9)	73.8% (60.9-84.2)	2.81 (1.71-4.63)	0.36 (0.17-0.77)
WLE-Z VA	87.5% (47.3-99.7)	98.6% (92.4-100)	62.1 (8.7-443)	0.13 (0.02-0.79)
NBI-Z VA	100% (63-100)	100% (94.9-100)	-	0
TTG	80% (44-98)	99% (95-100)	80 (7.7-325)	0.2 (0.02-0.8)

2.5.3 Secondary Endpoints

A) Comparing diagnostic accuracy of Modified Porto classification in the antrum

The performance of NBI-Z was superior to WLE-Z using the modified Porto classification for detecting normal, intestinal metaplasia and an atrophic antrum. Normal antrum (Aa) NBI-Z 0.78 (95% CI 0.7-0.86) vs WLE-Z 0.7 (95% CI 0.63-0.78), p<0.0001. Intestinal metaplasia (Ba) NBI-Z 0.77 (95% CI 0.52-0.82vs WLE-Z 0.73 (95% CI 0.59-0.87), p=0.006. Gastric atrophy (Da) NBI-Z 0.72 (95% CI 0.64-0.83) vs WLE-Z 0.69 (95% CI 0.57-0.77), p<0.0001. For detecting *H. pylori* gastritis (Aa+) in the antrum there was no statistically significant difference in the accuracy, NBI-Z 0.82 (95% CI 0.66-0.96) vs WLE-Z 0.64 (95% CI 0.54-0.75), p=0.61. Table 2.5 illustrates sensitivity, specificities and likelihood ratios for the modified Porto classification in the antrum.

Table 2.5 Sensitivity, specificity and likelihood ratios with 95% confidence intervals of WLE-Z and NBI-Z using the Modified Porto classification in the antrum

	Sensitivity (95% CI)	**Specificity (95% CI)**	**Positive Likelihood ratio (95% CI)**	**Negative Likelihood ratio (95% CI)**
WLE Aa	54.8% (43.5-65.7)	86.1% (70.5-95.3)	3.94 (1.71-9.1)	0.53 (0.4-0.69)
NBI Aa	76% (65.5-84.4)	80% (63-91.6)	3.8 (1.93-7.44)	0.3 (0.2-0.45)
WLE Aa+	62.1% (42.3-79.3)	66.7% (55.9-76.3)	1.86 (1.24-2.8)	0.57 (0.35-0.93)
NBI Aa+	80% (44-98)	83% (73-90)	4.71 (2.53-6.77)	0.24 (0.16-0.55)
WLE Ba	50% (23-77)	96.2% (90.6-99)	13.3 (4.4-39.6)	0.52 (0.31-0.88)
NBI Ba	57% (18-90)	97.3% (92.3-99.4)	21 (12.4-52.5)	0.44 (0.32-1)
WLE Da	43% (22-66)	94.9% (88.6-98.3)	8.6 (3.8-14.9)	0.6 (0.59-1.1)
NBI Da	50% (27-73)	93% (86-97)	7.1 (3.3-70.4)	0.54 (0.44-0.95)

B) Comparing diagnostic accuracy of Modified Porto classification in the corpus

The accuracy of NBI-Z was superior to WLE-Z for detecting normal, *H. pylori* gastritis and atrophy in the corpus. Normal corpus (Ab) NBI-Z 0.81 (95% CI 0.74-0.87) vs WLE-Z 0.79 (95% CI 0.72-0.87), p<0.0001. *H. pylori* gastritis (Ab+) NBI-Z 0.77 (95% CI 0.67-0.87) vs WLE-Z 0.62 (95% CI 0.51-0.74), p=0.01. Gastric atrophy (Db) NBI-Z 0.85 (95% CI 0.72-0.93) vs WLE-Z 0.81 (95% CI 0.46-0.93), p<0.0001. Although numerically accuracy was higher for intestinal metaplasia using NBI-Z, this did not achieve statistical significance. Intestinal metaplasia (Bb) NBI-Z 0.79 (95% CI 0.66-0.9) vs WLE-Z 0.7 (95% CI 0.55-0.83), p=0.28. Table 2.6 illustrates sensitivity, specificities and likelihood ratios for the modified Porto classification in the corpus.

Table 2.6 Sensitivity, specificity and likelihood ratios with 95% confidence intervals of WLE-Z and NBI-Z using the Modified Porto classification in the corpus

	Sensitivity (95% CI)	**Specificity (95% CI)**	**Positive Likelihood ratio (95% CI)**	**Negative Likelihood ratio (95% CI)**
WLE Ab	77.8% (66.4-86.7)	81% (65.9-91.4)	4.1 (2.2-7.7)	0.27 (0.17-0.43)
NBI Ab	70.5% (59.8-79.7)	90.9% (78.3-97.5)	7.75 (3-19.9)	0.33 (0.23-0.45)
WLE Ab+	43.5% (23.2-65.5)	81.3% (71.8-88.7)	2.33 (1.24-4.4)	0.7 (0.48-1.01)
NBI Ab+	75% (53-90.2)	78.7% (69.8-86)	3.52 (2.3-5.4)	0.32 (0.16-0.64)
WLE Bb	50% (26-74)	90.1% (82.5-95.1)	5 (2.89-8.53)	0.56 (0.36-1.17)
NBI Bb	61% (36-83)	95.8% (90.4-98.6)	15.3 (9.78-29.6)	0.41 (0.29-0.99)
WLE Db	61% (45-76)	100% (95.7-100)	-	0.4 (0.27-0.79)
NBI Db	70% (52-81)	99% (94.5-100)	70 (10.74-307)	0.3 (0.23-0.78)

C) Inter observer agreement

When we tested the reproducibility of Nottingham, Porto and villous atrophy endoscopy classifications for NBI-Z after a training session, the mean kappa values were higher among consultant gastroenterologists than trainees. The mean kappa value among consultants using NBI-Z for detecting villous atrophy was 0.5 (95% CI 0.24-0.69), p<0.0001 and for trainees this was 0.25 (95% CI 0.12-0.39), p<0.0001. Agreement was higher with Nottingham than Modified Porto classification in the corpus for both consultants and trainees. The mean kappa for NBI-Z among consultants Nottingham classification for the corpus was 0.63(95% CI 0.51-0.73), p<0.0001 but for Modified Porto it was 0.33 (95% CI 0.21-0.43), p<0.0001. The mean kappa for NBI among trainees Nottingham classification for the corpus was 0.5 (95% CI 0.39-0.62), p<0.0001 but for Modified Porto it was 0.2 (95% CI 0.12-0.28), p<0.0001. (Table 2.7 to 2.9). The overall image quality was significantly higher for NBI-Z when compared with WLE-Z in most image sets (7 vs 6 p<0.0001). See Figure 2.5.

Table 2.7 Kappa values and confidence intervals for inter- agreement for endoscopy images of the corpus

	Observer	Kappa	95% CI	P value
NBI Nott Corpus	Consultant	0.63	0.51-0.73	<0.0001
NBI Nott Corpus	Trainee	0.50	0.39-0.62	<0.0001
NBI M. Porto Corpus	Consultant	0.33	0.21-0.43	<0.0001
NBI M. Porto Corpus	Trainee	0.20	0.12-0.28	<0.0001

The mean kappa values for assessing endoscopy images for all consultants (four) and trainees (four).

Table 2.8 Kappa values and confidence intervals for inter-agreement for endoscopy images of the antrum

	Observer	Kappa	95% CI	P value
NBI M. Porto Antrum	Consultant	0.30	0.22-0.48	<0.0001
NBI M. Porto Antrum	Trainee	0.15	0.08-0.21	<0.0001

The mean kappa values for assessing endoscopy images for all consultants (four) and trainees (four).

Table 2.9 Kappa values and confidence intervals for intra agreement for endoscopy images of the duodenum

	Observer	Kappa	95% CI	P value
NBI D2	Consultant	0.59	0.3-0.92	<0.0001
NBI D2	Trainee	0.32	0.15-0.47	<0.0001

The mean kappa values for assessing endoscopy images for all consultants (four) and trainees (four).

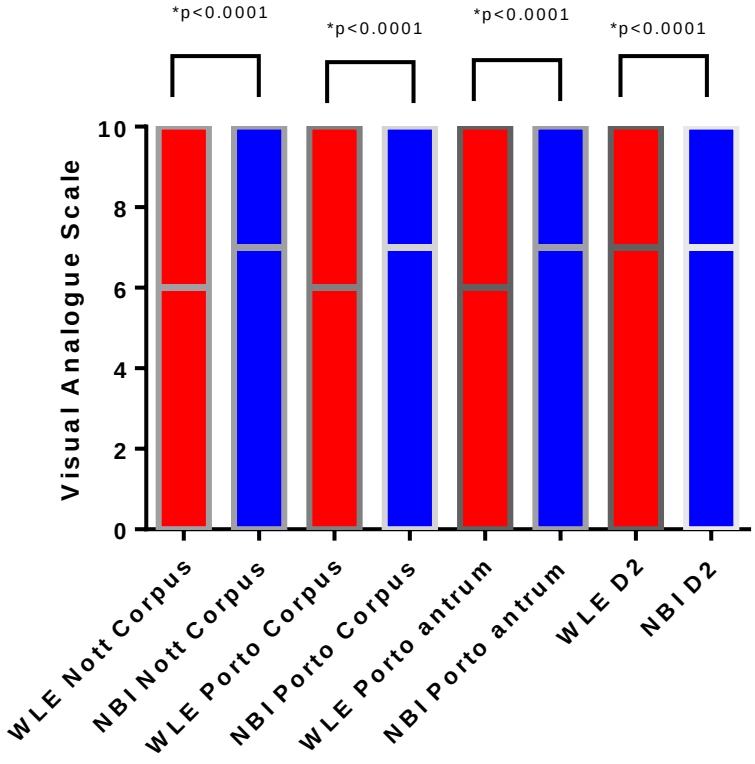


Figure 2.5 Observers independent assessment on image quality using Visual analogue scales

Image quality rated using VAS for all observers (principal endoscopists/consultants/trainees), horizontal line represent medians and Mann Whitney U test was carried out for statistical significance.

2.6 Discussion

Only a small number of studies have investigated the endoscopic appearance of *H. pylori* gastritis, describing nodular mucosal changes and gastric fold hypertrophy as specific features of *H. pylori* infection. However, these features are often associated with low sensitivities when compared with histological findings (Laine et al., 1995, Yela et al., 1997). The exact features that describe *H. pylori* gastritis are not known which makes description and interpretation difficult. We found that using NBI-Z the Nottingham classification Type I pattern had a sensitivity of 75.6% and specificity of 98.6% for predicting normal histology. For predicting *H. pylori* gastritis this was lower

with sensitivity and specificity of 62.1% and 68.1% respectively. Gastric corpus atrophy detection was better with a sensitivity of 75.6% and 100%.

These findings are lower than when the initial classification was described using WLE (Anagnostopoulos et al., 2007). A potential reason why these values are lower than we would expect could be due to the fact that in our study the principal endoscopists who scored the images were blinded to clinical findings. In Anagnostopoulos *et al* the image appearance was decided by the endoscopist performing the procedure which may have influenced the picture by including clinical findings. Anagnostopoulos *et al* results are a better reflection of clinical practice because both clinical information and macroscopic endoscopic appearance are available that aid endoscopic diagnosis.

The main benefit of endoscopy is to detect pre-cancerous lesions to improve patient outcomes. *H. pylori* is a human carcinogen so detecting the organism is vitally important and in a climate where health resources are limited being able to adequately detect this in a way which may reduce pathology associated costs is vitally important (Plummer et al., 2015). The evidence for magnified endoscopy is inconsistent as shown in our data. Eshmuratov *et al* found that patients had lower accuracy for the detection of mucosal inflammation, atrophy and intestinal metaplasia especially in those who were younger or who had mild histological scores. Their sensitivity and specificity for detecting gastric atrophy was 46.8% and 76.4% respectively (Eshmuratov et al., 2010), but in our data the patient's median age was 71 years with a Sydney atrophy score of 3. Therefore, this is an unlikely explanation for these lower values. However, others have found good correlation (Anagnostopoulos et al., 2007, Yan et al., 2010, Tahara et al., 2009, Liu et al., 2014).

It is also worth noting that 22% of patients had taken a proton pump inhibitor (PPI) in the preceding two weeks. All patients were asked to discontinue the PPI two weeks prior to the procedure but due to the fact that the majority of patients were fast tracked to endoscopy to exclude a GI malignancy this was

not always possible. This high proportion of patients could explain why some of the results were not as accurate as in the previously published data as PPIs suppress *H. pylori* bacteria which could lead to increased false negatives.

The training of western endoscopists is also likely to be different compared to Asian countries where the incidence of gastric cancer is higher so they are more experienced (de Vries et al., 2007). Also at present the lack of uniform classification criteria within the literature further adds to variability seen within the results. The current classification criteria used have not been tested in well controlled trials with the large numbers needed and in a range of population settings to ensure validity. In our study we compared accuracies between two classification systems that were commonly used in our centre. Although overall both classifications produced similar sensitivities and specificities for normal and atrophic mucosa the specificity differed in the detection of *H. pylori* gastritis. Specificity for the modified Porto and Nottingham classification was 78.7% and 64.9% respectively. Although the modified Porto classification required more time to examine the images it offered more details in terms of the presence of intestinal metaplasia and dysplasia. The modified Porto classification would be useful in deciding whether to take biopsies. The specificity was 78.7-99%, so this would enable the endoscopists to confidently not take biopsies in the corpus.

Based on our findings, NBI-Z could in the future replace the current practice of random biopsies for villous atrophy as sensitivities were close to 90% for WLE-Z and 100% for NBI-Z. This recommendation is based on The American Society for Gastrointestinal Endoscopy PIVI guidelines which state imaging technique with targeted biopsies should achieve 90% sensitivity in order to replace standard random biopsies (Sharma et al., 2012). Previous studies have described similar findings. Singh *et al* described a sensitivity and specificity of 93.3% and 97.8% for detecting the present or absent of villous atrophy using NBI-Z (Singh et al., 2010). Although smaller numbers were examined Cammarota *et al* also found similar results (Cammarota et al., 2008). Newer techniques such as confocal images are expensive, with limited centre

experience and research is still ongoing to determine their role in clinical practice. These data show that endoscopic diagnosis can complement histology as the gold standard but ultimately that much larger patient numbers are needed to establish the true benefits of these advanced techniques.

Endoscopy was better than serology for the prediction of villous changes; NBI-Z correctly identified all normal and cases of villous atrophy. The results were similar to previous studies. Even though endoscopy was better it is unlikely be used as alternative screening tool for coeliac disease. Normally in clinical practice if the clinical suspicion for coeliac disease was low and patient had negative serology they would unlikely be referred for a gastroscopy and duodenal biopsies. Due to the variability in previous studies at the moment endoscopy findings cannot replace the gold standard. Endoscopy would miss early stages of coeliac disease (Marsh 1 and 2) because the villi would be normal (Marsh, 1992). However, in patients who test positive for coeliac serology, using NBI-Z to demonstrate villous atrophy may be sufficient to diagnose coeliac disease without taking biopsies. Our results suggest NBI-Z could be used instead of WLE-Z which was originally proposed by Cammarota *et al*. This approach would provide cost savings by reducing sampling and processing costs (Cammarota et al., 2005).

H. pylori serology, PGI/II ratio and coeliac serology had a sensitivity and specificity of 90%, 73.7%, 80%, 73.9%, 73.8% and 99% respectively. Our data are similar to previous studies (Leffler and Schuppan, 2010, Malfertheiner et al., 2012, Dinis-Ribeiro et al., 2012). *H. pylori* serology is commonly used in clinical practice but in the UK serological markers for atrophy are not routinely used. Our results show that endoscopy was better than serology in predicting atrophy in terms of accuracy. Our results do not suggest serology could replace endoscopy for the surveillance or detection of gastric atrophy. One benefit of serological tests, if these are negative and a patient is referred to endoscopy this may reduce the need for biopsies for gastric atrophy or coeliac disease. The specificity for PGI<30 and coeliac serology was 98.4% and 99%

respectively. Serology tests were reliable at excluding *H. pylori* infection, corpus atrophy and coeliac disease. Having serology tests available to the endoscopist may influence whether biopsies are needed.

Inter-observer agreement among consultants was fair to substantial but in trainees this was more variable but lower. Using still images does not accurately represent the total assessment of upper GI mucosa. Endoscopy assessment is dependent on experience and training, so these techniques perform better in the hands of experts. Classification systems must be reproducible to be relevant in clinical practice. VAS median scores were statistically higher for NBI when compared to WLE suggesting the observers felt NBI-Z images provided more clarity. These results along with improved sensitivity and specificity results suggest NBI-Z was better than WLE-Z for disease detection. NBI requires a certain skill level and is not available in all centres so is therefore not always practical in the clinical setting. For the use of NBI to be effective like other endoscopy modalities training in mucosal pattern recognition is very important (Basford et al., 2015).

Strengths

This was a large, prospective study investigating anaemic patients with the aid of two endoscopy classification criteria in addition to serological markers.

In contrast to previous studies, bias was reduced by blinding of both the endoscopists and histopathologists. The same gastroscope was also used consistently for all procedures.

The classification systems tested provides useful information on the GI mucosa but needs to be tested in different centres before clinical usefulness can be fully recommended.

Limitations

As only patients with IDA were included, this makes the study prone to selection bias. Also 20% were taking aspirin for cardiovascular prevention and as NSAIDs are known for causing erosions so this would be a confounding factor. IgA levels were also not measured so if a patient had suffered with both coeliac disease and IgA deficiency this may have detrimentally affected the results by increasing the number of false negatives.

Also the time spent for the inter-observer study was not specified so the time duration observing the still images is likely to have influenced the decision making. The images were also all captured by experienced endoscopists and in real clinical practice it is unlikely a general endoscopist would capture such high quality images during the procedure. This may enable diagnosis or allow for better classification of mucosal pattern.

We also did not measure inter-observer agreement before and after training sessions to ascertain the level of training required to master the use of NBI in our group of observers.

Finally, the majority of patients with coeliac disease were Marsh 3 so we could not assess the use of magnification endoscopy in those with early disease.

Planned future work

Our centre has recently acquired a Confocal Laser Endomicroscopy (CLE) which enables in vivo histology subcellular level magnification and resolution of GI mucosal layer in real time (Subramanian and Ragunath, 2014). This new research tool is currently being evaluated but little work has been published in the literature with regard to detection of gastric atrophy, *H. pylori* gastritis and villous atrophy. We plan to set up a new study investigating the utility of this new technique in the diagnosis of upper GI disorders.

Our results show good inter-observer agreement with consultants but not trainees so increased training is likely to improve detection. We plan a future

study to assess this learning curve by recording endoscopist image scores before and after training.

2.7 Conclusions

- NBI-Z was very accurate at detecting gastric atrophy, 0.88 (95%CI 0.81-0.94) and villous atrophy 1.0 (95% CI 1-1).
- Accuracy using NBI-Z was lower for *H. pylori* gastritis 0.65 (95% CI 0.55-0.75).
- NBI-Z performed better than WLE-Z and serology for detecting gastric and villous atrophy
- Modified Porto classification NBI-Z performed better than WLE-Z in both the antrum and corpus.
- Inter-observer agreement was better among consultants and higher for the Nottingham classification.

Chapter 3: Use of NBI and new endoscopic classification system in detecting pre-cancerous lesions

3.1 Introduction

3.1.1 Gastric Atrophy

Gastric atrophy occurs as a result of a chronic inflamed gastric mucosa, leading to loss of specialised glandular cells and replacement with an intestinal type surface and fibrous tissue (Gao et al., 2009, Rugge et al., 2002). This may then progress to intestinal metaplasia then dysplasia and finally intestinal type gastric cancer. The development from normal mucosa to gastric cancer is gradual and in a stepwise manner (Correa, 1995).

The main causes of atrophy are the long term consequence of infection with *H. pylori*, autoimmune conditions which cause destruction of glandular structures, and some environmental triggers. Intestinal metaplasia affects *H. pylori* colonisation and may permit other bacteria to colonise the gastric mucosa. Intestinal metaplasia may also affect gastric pH. *H. pylori* is a type I carcinogen (Herrero et al., 2014) affecting the whole stomach leading to pan gastritis. A marked increase in gastrin is seen leading to expansion of enterochromaffin like cells. Also parietal cells are lost leading to reduced acid secretion, both of which predispose to carcinoid tumour development. However, there is a delay in this process which would suggest other factors exist to drive progression. As gastric atrophy is a pre-cancerous condition, high risk patients with markers of severe atrophy are routinely offered endoscopic surveillance and endoscopic resection of appropriate lesions or surgery (Correa, 1992, Bordi et al., 1995, Vannella et al., 2012, Dinis-Ribeiro et al., 2012).

Gastric atrophy is more common in Asian communities and in those of increased age. The incidence is variable ranging from 0-10.9%. Time to diagnosis is affected by whether patients have a past history of *H. pylori* treatment or gastric surgery and whether they are symptomatic. (Vannella et al., 2012). *H. pylori* infected subjects with atrophy and intestinal metaplasia are up to six times more at risk of developing cancer, compared with fifteen times for pan-gastritis and thirty four times for gastric body predominant

inflammation. Eradication may improve or prevent progression of less severe forms of atrophy but intestinal metaplasia cannot be reversed (Malfertheiner et al., 2007). It is estimated that approximately 10% of individuals with moderate to severe atrophy requiring surveillance will develop gastric malignancy over a follow up period of under eight years (Dai et al., 2011, Uemura et al., 2001).

3.1.2 Intestinal Metaplasia

Normal gastric mucosa is replaced with intestinal type mucosa, which has characteristic mucus secreting goblet cells. The annual incidence of progression from intestinal metaplasia to gastric cancer varies from 0.1% to 0.9%, which has a similar progression rate to other GI conditions that currently have an established surveillance programme(O'Connor et al., 2013). The annual incidence increases to 6% in dysplasia (de Vries et al., 2008, den Hoed et al., 2013). Additional risk factors for intestinal metaplasia include alcohol, bile acid reflux, high intake of salt and smoking (Leung et al., 2004, Kneller et al., 1992). After the initial trigger that stimulates the development of intestinal metaplasia, the foci develop usually at the incisura angularis. Over time these foci enlarge and extend to other areas of the mucosa (Correa et al., 1970). Small areas of dysplasia eventually appear within the intestinal metaplasia and progression may be influenced by *H. pylori* virulence factors, host and environmental factors (Correa et al., 2010).

3.1.3 Gastric adenocarcinoma

Gastric cancer is ranked the fifth most common malignancy worldwide (Colquhoun et al., 2015). The majority of cases are found in Asia and since most cases are discovered at an advanced stage gastric cancer is now the third most common cause of cancer related death(Herrero et al., 2014). Declining prevalence of *H. pylori* could explain the recent fall in gastric cancer (Peleteiro et al., 2014).

In terms of anatomical location, cardia cancer originates from the gastro-oesophageal junction and shares similar risk factors to oesophageal adenocarcinoma. Non-cardia gastric cancers, which arise from the distal stomach, are thought to be attributable to *H. pylori* infection in up to 89% of cases (Plummer et al., 2015) but other factors such as autoimmune atrophic gastritis, host genetics, family history and environmental factors are also important in determining disease. The lifetime risk of an *H. pylori* infected individual developing gastric cancer is between 1-2% (Kusters et al., 2006, Colquhoun et al., 2015).

Adenocarcinoma is the most common type of gastric cancer but others include carcinoid tumours, soft tissue sarcomas (GIST) and Mucosa Associated Lymphoid Tissue (MALT) lymphoma. Gastric adenocarcinoma has two histological types: intestinal (differentiated) and diffuse (undifferentiated) (Lauren, 1965).

The intestinal type is driven by *H. pylori* which starts the process of inflammation of the normal gastric mucosa. Adenocarcinoma is the final step in this stepwise progression (Correa, 1995). These stages have been replicated in animal models (Correa, 1988, Dixon, 2001, Honda et al., 1998, Watanabe et al., 1998). The diffuse type differs in that it mainly affects younger individuals and is not associated with intestinal metaplasia but (although the exact mechanism is unknown) it may also be triggered by *H. pylori* infection (Peek and Blaser, 2002).

Despite the health burden associated with gastric cancer few countries offer a screening programme to detect early pre-cancerous lesions or offer national *H. pylori* eradication. In theory, mass *H. pylori* eradication therapy should reduce gastric cancer incidence but randomised controlled trials have not shown a significant reduction (Mera et al., 2005, You et al., 2006) and benefits remain unproven (Fuccio et al., 2009, Lee et al., 2013). Multiple reasons for not adopting this eradication approach include the long-term physiological

and immunological detrimental effects being unknown, antibiotic resistance and the role of other factors in cancer development. (Herrero et al., 2014).

3.1.4 Operative link for gastritis assessment

Due to the gastric cancer risk associated with gastric atrophy, a classification system was developed called the operative link for gastritis assessment (OLGA) staging system (Rugge et al., 2008). OLGA grades I-IV represented increasing stages of atrophy assessed by histology (Table 3.1). The later OLGA stages have correlated with serological markers assessing the severity of atrophic gastritis (Rugge et al., 2010).

Table 3.1: The OLGA classification

Atrophy Score		**Corpus**			
		No Atrophy (Score 0)	Mild Atrophy (Score 1)	Moderate Atrophy (score 2)	Severe Atrophy (score 3)
Antrum	No Atrophy (Score 0)	Stage 0	Stage I	Stage II	Stage II
	Mild Atrophy (Score 1)	Stage I	Stage I	Stage II	Stage III
	Moderate Atrophy (score 2	Stage II	Stage II	Stage III	Stage IV
	Severe Atrophy (score 3)	Stage III	Stage III	Stage IV	Stage IV

The OLGA staging system calculated from biopsies taken from gastric antrum including incisura angularis and corpus. Adapted from (Rugge et al., 2008).

OLGA staging system was modified to OLGIM where histological assessment of atrophy was replaced with intestinal metaplasia alone as this is less difficult to assess histologically (Capelle et al., 2010a, Rugge et al., 2011).

3.1.5 Endoscopic diagnosis

Gastroscopy is vitally important for diagnosis of *H. pylori* infection, gastric atrophy, pre-cancerous lesions and gastric cancer. Macroscopic endoscopic features of *H. pylori* infection include: haemorrhagic gastritis, fundic gland polyps, mottled patchy erythema, rugal hyperplasia, xanthoma and loss of corpus ruggae. (Watanabe et al., 2013). Gastric atrophy features seen during conventional endoscopy include mucosal atrophy, diffuse areas of grey intestinal type epithelium and visible blood vessels at the mucosal surface (Dai et al., 2011). Magnification endoscopy can visualise mucosal surface and vascular pattern with magnification up to 200 times (Lee et al., 2006). Mucosal surface epithelium structure, arrangement, mucosal oedema, blood vessel morphology and distribution are reflected in the pit pattern. These gastric pits are surrounded by grooves. The pit pattern changes as atrophy develops or lesions form (Dai et al., 2011).

Endoscopy with high definition magnification modalities can identify normal mucosa, *H. pylori* gastritis and gastric atrophy with good accuracy in western subjects (Anagnostopoulos et al., 2007). Narrow band imaging (NBI) uses light of blue and green wavelengths to help enhance certain aspects of the gastric mucosa allowing for better visualisation. The absorption offered by this form of imaging is similar to that of haemoglobin, so blood vessels and the mucosal surface is more distinct (Singh et al., 2011). The combination of NBI and magnification allows for clear visualisation of the gastric mucosa.

Other techniques to improve visualisation of gastric mucosa include chromoendoscopy, which is the use of specific dyes during the endoscopy procedure. Also autofluorescence (AFI) is used, mainly in combination with

magnification (zoom) and NBI. In the case of AFI, the mucosa is exposed to short wavelengths of light which leads to the emission of larger wavelengths. Depending on the tissue type, the fluorescence patterns vary allowing identification of different types and of possible abnormal, dysplastic areas (Singh et al., 2011). An area of gastric atrophy tends to produce a green pattern when compared to normal mucosa which is purple (Inoue et al., 2010).

3.1.6 Surveillance of pre-cancerous lesions

The correlation between white light endoscopy (WLE) and histology is considered inadequate for detection of *H. pylori* gastritis and lesions so it cannot reliability be used without histology. NBI and chromoendoscopy sometimes have greater accuracy at detecting gastric lesions (Dinis-Ribeiro et al., 2003). European guidelines recommend the use NBI or chromoendoscopy with magnification to aid the detection of lesions. Individuals with severe atrophy with or without severe intestinal metaplasia should be offered endoscopy surveillance on a three yearly interval. Those with evidence of dysplasia or visible gastric lesions should be offered this more frequently on a six to twelve month basis. There is wide variability on the exact NBI gastric mucosa pattern which best describes precancerous lesions so there a number of classification systems (Dinis-Ribeiro et al., 2012). A classification system validated in 2012 was able to accurately detect intestinal metaplasia and dysplasia up to 90% but was expertise dependent (Pimentel-Nunes et al., 2012, Dias-Silva et al., 2014). These classification systems used to describe lesions have not always been reproduced accurately in clinical practice (Kikuste et al., 2013).

3.2 Aims

The aims of the study were as follows:

- Assess the real time diagnostic accuracy of high-resolution endoscopy with and without NBI in the diagnosis of gastric premalignant conditions.
- Derive a new endoscopic classification for grading gastric intestinal metaplasia (EGGIM).
- Compare the accuracy of NBI and WLE for the detection of *H. pylori* gastritis, intestinal metaplasia and dysplasia.
- Compare the sensitivity, specificity of NBI and WLE for detecting intestinal metaplasia.
- Compare EGGIM grading with gold standard histology.

3.3 Hypotheses

We hypothesize that NBI with magnification will be superior to WLE at detecting intestinal metaplasia and dysplasia (Kato et al., 2010). The new EGGIM would be highly accurate in identifying severe intestinal metaplasia.

3.4 Materials and methods

3.4.1 Participants and clinical samples

This multicentre study was conducted and coordinated by the Portuguese Oncology Institute of Porto, Portugal. Five centres were involved: Portuguese Oncology Institute of Porto (Portugal); Hospital Sant'Andrea, University Sapienza Roma (Italy); Los Angeles County Hospital, Keck School of Medicine, University of Southern California, Los Angeles (United States); University of Medicine and Pharmacy TG., Mures (Romania); Nottingham University Hospitals NHS Trust and the University of Nottingham, Queen's Medical Centre, Nottingham (United Kingdom).

Consecutive UK patients were prospectively recruited between January 2014 and March 2015 after approval from Nottingham Research Ethical Committee 2 (REC Ref: 08/H0408/195). Informed consent was obtained from adult patients attending Nottingham University Hospitals NHS Trust for a diagnostic gastroscopy. Main exclusion criteria included previous gastric malignancy, gastric surgery, anticoagulation therapy or no biopsy samples. A total of 35 (15%) patients were recruited from Nottingham and total of 238 from all 5 centres. Patient recruitment, endoscopy and data collection for Nottingham was done by Dr JR White.

3.4.2 Endoscopic procedures

All procedures at Nottingham were done with high definition and magnification gastroscopes (GIF-FQ260Z; Olympus Optical, Tokyo, Japan). Procedures were done under pharyngeal local anaesthetic or conscious sedation. Representative areas from anatomical landmarks were examined with magnification after standard WLE and magnification without NBI. Images were then captured from the incisura and the lesser and greater curvature of the antrum and corpus. The images were recorded and then the macroscopic appearance described as 'normal', 'intestinal metaplasia' or 'dysplasia' according to Pimentel-Nunes *et al* classification (Pimentel-Nunes et al., 2012, Pimentel-Nunes et al., 2016) (See appendix 2). The same process was then repeated for the NBI with magnification. Biopsies were taken from each area the images were captured from. A total of 5 areas had images captured. Each centre had one or more endoscopists with more than 100 high resolution NBI experience per year.

3.4.3 Histology

Gastric samples were placed in formalin and prepared and stained by the Histopathology department at the University Hospital in Nottingham. Samples were fixed in formalin, embedded in paraffin and cut into approximately 4µm sections. Sections were stained with haematoxalin and eosin (H&E) to allow

inflammation, atrophy and intestinal metaplasia scores to be carried out. Toluidine blue staining was also carried out for *H. pylori* density grading. Grading scores were rated: 0=absent, 1=mild, 2= moderate, 3= severe. Histopathologists who were blinded to clinical and endoscopy findings carried out the grading and classification using updated Sydney scoring system(Dixon et al., 1996). For the remaining four centres, gastric samples were processed in a similar way but modified Giemsa (2%) stain was used for *H. pylori* detection. If possible all centres carried out OLGIM grading for intestinal metaplasia (0-IV) (Capelle et al., 2010a).

3.4.4 Data recording

All data was transferred to an online resource created by the Portuguese Oncology Institute of Porto. This included anonymised demographic details, gastric endoscopy appearances details of superficial lesions and whether targeted or random biopsies were taken. Details were recorded for both WLE and NBI. Endoscopic images were uploaded alongside corresponding descriptive gastric mucosa details and histology. Once all centres had uploaded all the information the online resource was blocked to prevent further editing.

3.4.5 NBI endoscopic grading of gastric intestinal metaplasia (EGGIM)

The Portuguese Oncology Institute of Porto, Portugal created the new grading system for estimating intestinal metaplasia. They concentrated on five areas: two areas in the antrum, two in the corpus and one in the incisura. Each area was then scored: 0 (no intestinal metaplasia), 1 (focal intestinal metaplasia or less or equal to 30%) or 2 (extensive intestinal metaplasia or more than 30%). A maximum score was 10 (Table 3.2). A single observer Pimentel-Nunes, who was blinded to histology, graded all patients from 5 centres according to the new proposed EGGIM classification. This grade was then correlated with

OLGIM. Grading was carried out in the same order per patient using high resolution still images with normal magnification images.

Table 3.2 Endoscopic Grading of Gastric Intestinal Metaplasia (EGGIM) classification

Endoscopic IM scale	Antrum		Incisura Angularis	Corpus	
	Lesser Curve	Greater Curve		Lesser Curve	Greater Curve
No IM	0	0	0	0	0
Focal IM (≤30%)	1	1	1	1	1
Diffuse IM (≥30%)	2	2	2	2	2
IM Score	0-4		0-2	0-4	

Endoscopy images were collected from five centres and one observer Pimentel-Nunes, who was blinded to histology, graded all the images according to the new EGGIM classification. Adapted from (Pimentel-Nunes et al., 2016).

3.4.6 Statistics

SPSS 20.0 Package Facility, SPSS Inc., Chicago, Illinois, USA) and Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, USA) was used for data analysis. Sensitivity, specificity, global accuracy with 95% confidence intervals (CI) was calculated for each centre and combined globally. Sensitivity and specificity values were used to calculate likelihood ratios (LR). Chi-squared was used to detect differences in dichotomous variables. Diagnostic accuracies were compared using McNemar's Chi-square test and Student t-

test were used for comparison of normally distributed data. A receiver operating characteristic (ROC) curve was used to assess accuracy and optimal cut off of the EGGIM scale for detection of extensive metaplasia. ROC curve is a representation of the relationship between sensitivity and specificity and helps determine the best threshold of a cut off value. Histology was considered the gold standard. Statistically significance was considered if $p<0.05$. Local data in Nottingham was collected by DR JR White but all statistical analysis was done by the Pimentel-Nunes and the Portugal team unless stated otherwise.

3.5 Results

3.5.1 Demographics

3.5.1.1 Nottingham University Hospital demographics

35 patients were recruited and a total of 170 biopsies were collected. Male to female ratio was 0.49 with a mean age of 72 years (standard deviation 12). 23% were taking a PPI at the timing of endoscopy.71% had a diagnostic procedure due to symptoms and the rest due to gastric atrophy surveillance. 37% of patients had normal gastric histology, 40% had focal intestinal metaplasia and 23% had extensive intestinal metaplasia. 29% were infected with *H. pylori*. 1 patient had evidence of dysplasia (Pimentel-Nunes et al., 2016). 3% of patients had a family history of gastric adenocarcinoma. 72% were consciously sedated and 28% of the procedures were done under pharyngeal local anaesthetic. The mean procedure time was 26.3 minutes (Table 3.3). All UK demographic data was collected and analysed by Dr J White.

Table 3.3 Nottingham University hospital patients' demographics

	Nottingham University Hospital (UK)	Global Total (Portugal, Italy, USA, UK, Romania)
Patients' n (%)	35 (15%)	238
Biopsies n (%)	170 (15%)	1123
Male: Female	0.49	0.42
Mean age (standard deviation)	72 (12)	60 (14)
PPI (%)	23	32
Gastroscopy indication **Symptoms (%)** **Surveillance (%)**	 71% 29%	 77% 23%
Histology **Normal (%)** **Focal intestinal metaplasia (%)** **Extensive intestinal metaplasia (%)**	 37% 40% 23%	 42% 35% 21%
***H. pylori* infection**	29%	23%
Dysplasia (numbers)	1	23
Sedation (midazolam/Pethidine)	72%	n/a
Mean procedure time	26.3 minutes	n/a

Each centre supplied demographic details, gastroscopy details and histology details to the coordinating centre. The UK data and combined global data are represented in the table. Data is represented as a mean with standard deviation, ratio or percentage. Table adapted from (Pimentel-Nunes et al., 2016).

3.5.1.2 International demographics

238 patients were recruited and 1123 biopsies included from all five centres. The male to female ratio was 0.42 with an average age of 60 (standard deviation 14). 32% of patients were taking a PPI and the majority of endoscopies were done for symptoms (77%) with the remaining 23% for surveillance. Both the Romanian and Portuguese centre had a slightly higher proportion of patients recruited who had the gastroscopy for surveillance purposes (Table 2.3). The histological features were significantly more

advanced when compared to the other three centres (p<0.001) (Pimentel-Nunes et al., 2016).

3.5.2 Diagnostic accuracy

3.5.2.1 Nottingham University Hospital diagnostic accuracy

The Nottingham data showed a numerical difference but this did not show a statistical difference. NBI is more sensitive than WLE in providing a real time diagnosis of intestinal metaplasia (76% vs 59%). Specificity is similar between NBI and WLE (99% vs 98%). Both NBI and WLE are sensitive and specific at detecting dysplasia (100% vs 100%) and (100% vs 99%) respectively. Sensitivity for both NBI and WLE is low for detecting *H. pylori* gastritis (46% vs 37%) but higher specificity (88% vs 88%). NBI has superior positive LR compared to WLE for intestinal metaplasia and *H. pylori* gastritis, (82.2 vs 32.8) and (3.6 vs 2.9) respectively (Deeks and Altman, 2004). See Table 3.4. UK accuracy with NBI was higher than WLE but not statistically significant (88% vs 84%) (Pimentel-Nunes et al., 2016).

Table 3.4: Sensitivity, specificity, LR+ and LR- for diagnosis of intestinal metaplasia, dysplasia and *H. pylori* gastritis

	UK WLE	UK NBI	Global WLE (95% CI)	Global NBI (95% CI)	P value
Intestinal Metaplasia					
Sensitivity	59%	76%	53% (47-58%)	87% (83-91%)	p<0.001
Specificity	98%	99%	98% (97-99%)	97% (95-98%)	
IM LR+	32.8	82.2	28.8 (17-49)	27.8 (19-41)	-
IM LR-	0.42	0.5	0.48 (0.43-0.53)	0.13 (0.1-0.17)	-
Dysplasia					
Sensitivity	100%	100%	74% (52-90%)	92% (73-99%)	ns
Specificity	99%	100%	99% (98-100%)	99% (98-100%)	ns
LR+	n/a	n/a	407 (100-1658)	512 (128-2058)	
LR-	n/a	n/a	0.26 (0.13-0.52)	0.08 (0.02-0.31)	
H. pylori					
Sensitivity	37%	46%	57% (43-69%)	69% (55-81%)	ns
Specificity	88%	88%	79% (72-85%)	67% (60-74%)	ns
***H. pylori* LR+**	2.9	3.6	2.7 (1.9-3.9)	2.1 (1.6-2.7)	-
***H. pylori* LR-**	0.73	0.62	0.55 (0.41-0.74)	0.46 (0.31-0.69)	-

Each centre used same classification system to grade images (Pimentel-Nunes et al., 2012) to allow sensitivity, specificity, LR+ and LR+ calculations to be carried out. Data represented as percentage with 95% confidence intervals where appropriate. Table adapted from (Pimentel-Nunes et al., 2016).

3.5.2.2 International diagnostic accuracy

NBI showed a significant increase in the sensitivity for detection of intestinal metaplasia 87% (95%CI 83-91) vs 53% (95% CI 47-58), p<0.001. Specificity for intestinal metaplasia with NBI and WLE were 97% and 98% respectively. Although not statically significant there was a higher sensitivity for the detection of dysplasia with NBI 92% (95% CI 73-99) vs 74% (95% CI 52-90) with similar specificity 99% (95% CI 98-100) vs 99% (95% CI 98-100). There was no significant difference in accuracy for detecting *H. pylori* gastritis 69% (95% CI 55-81) vs 57% (95% CI 43-69) but there was a slightly higher specificity for WLE 67% (95% CI 60-74) vs 79% (95% CI 72-85) but non-significant. NBI has a superior positive likelihood ratio to WLE suggesting a superior diagnostic yield (Deeks and Altman, 2004). See Table 3.4.

Accuracy was significantly higher using NBI when compared with WLE across all five centres 94% (95% CI 92-96) vs 83% (95% CI 80-85) p<0.01. When stratifying by Operative Link on Gastric intestinal metaplasia stage (Rugge et al., 2008), focal intestinal metaplasia OLGIM I/II diagnostic accuracy was statistically higher in NBI when compared to WLE 88% (95% CI 85-92) vs 74% (95% CI 70-78), p<0.001. For extensive metaplasia OLGIM III/IV NBI diagnostic accuracy was also statistically higher than WLE 93% (95% CI 88-96) vs 67% (95% CI 60-75), p<0.001. See Table 3.5. (Pimentel-Nunes et al., 2016).

Table 3.5 Global diagnostic accuracy and according to extent intestinal metaplasia

	Global WLE (95% CI)	Global NBI (95% CI)	P value
Diagnostic accuracy	83% (80-85%)	94% (92%-96%)	p<0.01
Extensive Intestinal metaplasia (OLGIM III/IV)	67% (60%-75%)	93% (88%-96%)	p<0.001
Intestinal Metaplasia (OLGIM I/II)	74% (70%-78%)	88% (85%-92%)	p<0.001
No Intestinal metaplasia (OLGIM 0)	99% (98%-100%)	97% (95-99%)	ns

Accuracy was calculated for WLE and NBI according each OLGIM stage and represented as a percentage with 95% confidence intervals. Table adapted from (Pimentel-Nunes et al., 2016).

3.5.2.3 Diagnostic accuracy of EGGIM

The diagnostic accuracy using the EGGIM classification was investigated with a ROC curve. The AUC of ROC curve for extensive intestinal metaplasia was excellent at 0.983 (SE 0.007, 95% CI 0.969- 0.997). The optimal cut off value for extensive intestinal metaplasia (OLGIM III/IV) was an EGGIM score of 5 with a sensitivity and specificity of 94.2% and 95.2% respectively. While an EGGIM 4 indicated moderate intestinal metaplasia with a sensitivity and specificity of 98.1% and 86% respectively (Pimentel-Nunes et al., 2016). EGGIM correlated with OLGIM classification and the majority of patients who scored 0 on both OLGIM and EGGIM had absent intestinal metaplasia. Also the majority of patients with an OLGIM I/II or EGGIM 1-4 had focal or moderate intestinal metaplasia and those with OLGIM III/IV or EGGIM 5-10 had extensive intestinal metaplasia. See Table 3.6. (Pimentel-Nunes et al., 2016).

Table 3.6 Correlation between OLGIM and EGGIM

% total patients		EGGIM				
		0	1-2	3-4	5-7	8-10
OLGIM	0	**37%**	5%	0%	0%	0%
	I	3%	**10%**	4%	1%	0%
	II	1%	4%	**10%**	3%	0%
	III	0%	1%	1%	**9%**	0%
	IV	0%	0%	0%	2%	**9%**
Intestinal metaplasia		Absent	Focal/moderate		Extensive	

A single observer Pimentel-Nunes, who was blinded to histology, graded all patients from 5 centres according to new proposed EGGIM classification. This grade was then correlated with OLGIM. Data represented as percentage of global patients. Table adapted from (Pimentel-Nunes et al., 2016).

3.6 Discussion

In the international cohort, NBI was more accurate than WLE in detecting intestinal metaplasia or dysplasia (94% vs 83% $p<0.001$). In the UK cohort, there was no significant difference in accuracy between NBI and WLE. In the international cohort and single UK centre, sensitivity, specificity and likelihood ratio were superior in NBI when compared to WLE. EGGIM grading system correlated well with histology and OLGIM stages.

Previous studies have found NBI improves diagnostic yield of detecting pre-cancerous lesions. Capelle *et al* calculated sensitivity, specificity, positive and negative predictive values and differences between NBI and WLE using ROC curves on a smaller patient numbers. Sensitivity, specificity, positive predictive values for NBI were 71%, 58%, 65% and 65% respectively, slightly better than WLE (Capelle et al., 2010b). NBI has been shown to increase

detection in pre-cancerous lesions of the GI tract (East et al., 2008b). Our study global sensitivity and specificity produced similar results to a Japanese study which produced sensitivity and specificity values of 89% and 93% respectively (Uedo et al., 2006). A multi-centre study done in Asia also showed a significant difference between NBI and WLE of 10% for detecting intestinal metaplasia (Ang et al., 2015). NBI international accuracy data was over 90% and suggests this method could be used to target biopsies instead of random sampling. However, previous studies using combination of NBI and WLE although producing promising results are not enough to suggest replacing histology (Nakayoshi et al., 2004). Other studies have not shown an advantage of using NBI over WLE (Xirouchakis et al., 2013).

Detection of *H. pylori* gastritis was low with both NBI and WLE in both UK and combined global results. These findings are similar to other studies (Pimentel-Nunes et al., 2012). The exact mucosal changes that occur with *H. pylori* infection are not known so this makes it difficult to make an endoscopic diagnosis. PPI use may heal inflammatory changes induced by *H. pylori* gastritis and hence heal the gastritis. Up to 32% of international patients were taking a PPI which may affect results by limiting the histological detection of *H. pylori* infection and potentially healing endoscopic features.

NBI had higher sensitivity than WLE for detecting dysplasia (92% vs 74%), so if only WLE was used these lesions may have been missed. NBI international accuracy was 94% and equally high with more extensive intestinal metaplasia. WLE international accuracy for extensive metaplasia was 67%, so a significant proportion of patients would be missed without taking biopsies.

The current gold standard for diagnosis of intestinal metaplasia and dysplasia is still histology. Currently no endoscopic modality has replaced histology despite combining NBI and WLE so the best way of detecting early gastric cancer remains unclear (Kato et al., 2007). Chromoendoscopy or confocal endomicroscopy are other modalities that could be used for early detection of pre-cancerous lesions but these may be too complex to be used in routine

practice. Magnification endoscopy has been shown to have a high sensitivity and specificity but most published studies are from Japanese centres where expertise is high and results may not be applicable to western practice (Capelle et al., 2010b).

Strengths

The reproducibility of gastric atrophy histological classification is inadequate and there is not a universally accepted endoscopic classification. However, the classification of intestinal metaplasia is easier to reproduce both histologically and endoscopically (Offerhaus et al., 1999) but maybe patchy. Therefore, relying on random biopsies without the use of NBI may miss important areas. EGGIM correlated well with histology: score of 5 = OLGIM III/IV. The implications of this allow a more detailed endoscopic examination report, an adjunct to histology in grading intestinal metaplasia and provide guided instead of random biopsies. EGGIM would provide an adequate tool to select individuals at higher risk who may benefit from surveillance (Karim-Kos et al., 2008). Other future implications include producing a detailed estimate of intestinal metaplasia to allow for immediate decisions on surveillance once endoscopy is complete, without awaiting histology confirmation. It may ultimately remove the need for histology thus reducing surveillance costs. Having a classification system which gives an estimation of the total area affected during endoscopy may also be a good research tool in estimating regression or progression. Current intestinal metaplasia detection is dependent on the updated Sydney system but this system may not always be reliable (El-Zimaity and Graham, 1999). Combining histology with serology and clinical parameters can improve detection of extensive intestinal metaplasia (de Vries et al., 2009) so having an EGGIM classification that examines the whole stomach could help augment histology that suffers from sampling error. EGGIM could also be used as a tool to help define disease risk because it correlates well with OLGIM classification. This study investigated an average to high risk population, the individuals that are most likely to benefit from surveillance.

Limitations

A single endoscopist examined the mucosa with WLE followed by NBI. Although this represents real practice this may have introduced bias because the observations of WLE may influence NBI findings, termed the "second pass effect"(East et al., 2008a). This could lead to an overestimation of the NBI detection rate because more time will have been spent on scrutinising the mucosa. However, this second pass effect is less applicable with still images. Also even though NBI was superior to WLE, in everyday practice they are used in combination with WLE. Other limitations include the fact that the histology was reviewed by two experienced GI pathologists at each centre. Variability between centres would have been reduced if one dedicated site reviewed all the histology slides. In contrast, only one experienced endoscopist applied the new EGGIM classification system meaning that expert evaluation may lead to better correlation with histology and an overestimation of the performance of the study. If the classification system was validated using endoscopists of varying experience, this would be more applicable to clinical practice. Study was carried out in tertiary centres where each endoscopist had significance experience with advanced endoscopy imaging. Dias-Silva *et al* have shown that there is a learning curve for mucosal pattern recognition (Dias-Silva et al., 2014). So results can only be generalised to tertiary centres and not the vast majority of centres without this experience.

In addition, 23% of recruited patients were on a surveillance programme. This could improve the sensitivity, specificity and accuracy because of the bias of already being aware of the diagnosis. Patients were not randomised to WLE or NBI therefore this study was unable to completely control for confounders or selection bias. Also the gastroscopes were not the same in each centre, thus the findings could be influenced by the subtle technological difference in addition to modality used.

Planned future work

A follow up study has been planned to assess the incidence of superficial lesions, progression of intestinal metaplasia endoscopically and histologically and also to validate the EGGIM classification. A retrospective multi-centre study is planned with approximately 200 patients from five centres followed over a 3 year period. Patients with intestinal metaplasia who had an endoscopy between 2011 and 2013 will be recruited. Clinical and histological data will be collected during the follow up period on progression by assessing repeated endoscopy images and histology.

3.7 Conclusions

- In the UK centre, NBI was more sensitive and specific than WLE in detecting intestinal metaplasia but was not statistically significant.
- NBI on an international scale increased diagnostic accuracy for intestinal metaplasia and this was statistically significant
- NBI on an international scale increased the diagnostic accuracy of dysplasia but this did not achieve statistical significance.
- Combined international centres showed low sensitivity, specificity for detecting *H. pylori* gastritis which was similar to previous studies.
- EGGIM classification correlated well with OLGIM.
- Detailed endoscopic evaluation of intestinal metaplasia can occur without the need for biopsy using Pimentel-Nunes *et al* classification.

Chapter 4: Assessment and treatment of anaemia in patients with *H. pylori* infection and gastric atrophy

4.1. Introduction

4.1.1 Iron Deficiency Anaemia and *H. pylori*

Iron, which is essential for cell function, is absorbed from the duodenal and upper jejunal enterocytes. The lifespan of these cells is determined by regulatory iron transporters. Iron taken up by these cells either enters the blood stream to be stored in the liver or is shed in faeces. Acidic conditions aid the process of iron absorption. Iron concentration is closely regulated to keep up with demand and prevention of toxicity (Ganz, 2011).

Iron deficiency anaemia (IDA) affects up to two billion people worldwide (Ramakrishnan, 2002). IDA prevalence in post-menopausal women and men in the west is up to 5% (Guralnik et al., 2004, Sayer and Long, 1993). The most likely cause is GI blood loss, so it is often necessary for patients to have invasive upper and lower endoscopies to detect possible malignant GI lesions (Hardwick and Armstrong, 1997). Markers of anaemia include low serum haemoglobin, hypochromia, microcytosis, low ferritin, low transferrin saturation, low iron or raised total iron binding capacity.

Causes of IDA include low dietary intake of iron, chronic blood loss or malabsorption. However, after negative endoscopic investigations, up to 35% of patients do not have a cause identified (McIntyre and Long, 1993). It is routine clinical practice to look for coeliac disease in the investigation of IDA but it is not routine to take gastric biopsies during upper GI endoscopy to look for atrophy or *H. pylori* colonisation. Therefore, the contribution of these conditions to IDA may be underestimated (Goddard et al., 2011, Kaye et al., 2008, Qu et al., 2010). The evidence for the association with *H. pylori* comes from case reports, observational studies, animal studies and limited randomised controlled trials. The main drawback to the results is that there are multiple confounders. Prior to *H. pylori* discovery, it was noted that there was an association between gastritis, IDA and low gastric acid production. In 1955, Davidson *et al* examined the gastric mucosa of patients with IDA and

those without. They found that only a quarter of IDA cases had a normal mucosa compared to three quarters of the controls. The mucosal abnormalities were more pronounced in individuals with low gastric acid secretion, but they failed to show there was an association with iron stores and the extent of gastric damage (Davidson and Markson, 1955, DuBois and Kearney, 2005). In a retrospective case control study, Kaye *et al.* found that gastric atrophy was strongly associated with IDA, and may therefore have a causative role. (Kaye et al., 2008).

Limited evidence suggests PPI use reduces absorption of iron and that they are sometimes used in the management of haemochromatosis. However, PPI use does not normally result in IDA, but in patients with established IDA requiring increased iron absorption PPI use may retard the response to iron supplements. PPI also lowers vitamin C in gastric juice and this is more pronounced in individuals infected with *H. pylori*. Finally, PPI use also reduces B12 absorption (McColl, 2009).

4.1.2 Case Reports

One early case report of note is from Dufour *et al.* of a child with iron deficiency anaemia refractory to oral supplementation and normal investigations apart from *H. pylori* gastritis. Once the *H. pylori* was eradicated the iron indexes returned to normal (Dufour et al., 1993).

4.1.3 Epidemiology studies

Multiple studies have been carried out to provide evidence for the association between *H. pylori* and a reduction in iron indexes. However, not all the studies agree (Muhsen and Cohen, 2008). The main problems with these studies are the variation in diagnostic criteria, sampling size, analysis, absence of controls and failure to account for confounding variables. This makes it difficult to ascertain a causal relationship.

A few studies that have shown a difference in iron parameters in asymptomatic individuals with evidence of positive *H. pylori* serology are as follows:

- Milman *et al.* used serology as a marker of infection and found ferritin was reduced in individuals with IgG antibodies to *H. pylori*. The subjects were 2794 Danish people from public register. The differences were greater in men and post-menopausal women and ferritin of less than 30µg/L was more likely in those with positive serology (Milman et al., 1998).

- Berg *et al.* also demonstrated that ferritin was reduced in individuals with *H. pylori* positive serology by 17%. The researchers made adjustment for confounders and found the difference was not related to individuals CagA status (Berg et al., 2001).

- In an intervention study where eradication treatment was given without iron supplementation individuals ferritin levels increased. It also increased in those without evidence of IDA (Kurekci et al., 2005).

- A randomised controlled trial conducted showed that patients who had eradication treatment with iron treatment had a more rapid improvement in blood parameters compared to those who just received iron treatment. The three groups examined were group A with oral iron supplement and eradication treatment. Group B were placebo and eradication treatment and Group C oral iron supplement and placebo. Group A showed the most marked increase in parameters followed by Group B. The major drawbacks were that the numbers studied were too small and subjects were all adolescents (Choe et al., 1999).

- A meta-analysis by Muhsen *et al.* including only studies that were deemed “well designed” and accounted for confounders as above,

estimated a 2.8-fold significant increase in risk for IDA subjects with *H. pylori* (Muhsen and Cohen, 2008).

- A meta-analysis of eight RCTs conducted in Asia concluded that *H. pylori* eradication can improve iron parameters in IDA (Huang et al., 2010).

The only study to not show a difference between oral iron supplementation and *H. pylori* eradication was one conducted by Gessner but this may have been underpowered. (Gessner et al., 2006, Muhsen and Cohen, 2008).

Evidence suggests that *H. pylori* infected individuals are more likely to have depleted iron stores, and the addition of eradication treatment to iron therapy may increase ferritin and haemoglobin levels (Hudak et al., 2016).

4.1.4 Potential Mechanisms

H. pylori may have an impact on iron absorption, thus leading to reduced iron stores by changing the gastric physiology. Gastric pH is known to influence the transformation of iron to a ferrous form to allow the transportation of iron. Reduced gastric ascorbic acid and higher pH has been observed in patients with *H. pylori* and IDA. Zhang *et al* also observed that this was related to the degree of inflammation present (Zhang et al., 1998). The effects on ascorbic acid were however overcome after eradication treatment (Ruiz et al., 1994). By measuring iron absorption in humans specifically, a study by Ciacci showed that this improved after eradication treatment. However, this only included small numbers and there was some variation in pH measurements (Ciacci et al., 2004). Raised gastric pH and lower ascorbic acid absorption was also shown in another study evaluating gastric pH and ascorbic acid (Annibale et al., 2003). An acidic pH is needed for iron absorption and *H. pylori* infection can reduce acid levels affecting iron absorption. Low iron induces virulence factors expression, damaging the epithelial cells and enhancing the ability of *H. pylori* to acquire iron from these damaged cells (Merrell et al., 2003).

Another proposed mechanism is that discrete bleeding from peptic ulcers or severe gastritis could lead a state of chronic blood loss then ultimately IDA. The evidence for this is less convincing because case reports did not identify any bleeding lesions or evidence of blood loss in faeces. But there have been case reports of haemorrhagic gastritis with IDA and raised haemoglobin levels in faeces but there was no follow up of these patients after receiving eradication treatment to provide concrete evidence for this theory (Yip et al., 1997, DuBois and Kearney, 2005, Kostaki et al., 2003).

A different theory is that *H. pylori* sequesters iron, therefore becoming in direct competition with the host (Otto et al., 1992). Some bacteria use sideraphores (iron chelators) to extract iron from transferrin or lactoferrin. In *H. pylori,* iron absorption from lactoferrin seems to be mediated by receptors. *H. pylori* is able to grow in iron reduced environments via expression of lactoferrin binding protein. This expression is also more marked in *H. pylori* gastritis and IDA individuals (Husson et al., 1993, Choe et al., 2003, Nakao et al., 1997, DuBois and Kearney, 2005). Mice subject to an iron deficient diet, thus leading to reduced iron stores, had their stores lowered even further when infected with *H. pylori* (Keenan et al., 2004).

Iron and *H. pylori* binding occur via outer membrane proteins (iron repressible outer membrane proteins). The expression of these proteins has been measured in an iron reduced environment and after additional iron supplementation. The strains obtained from patients with IDA showed greater expression of these proteins. This provided more evidence that *H. pylori* reduces iron stores in iron depleted states leading to IDA in individuals (Lee et al., 2009). The few studies done to investigate the relationship with *cagA+* virulent strains have established there is no link with IDA (Ciacci et al., 2004, Berg et al., 2001).

In conclusion*, H. pylori* is thought to be a risk factor for IDA, especially in individuals with lower iron stores. However, the interventional studies that have shown an improvement have all evaluated IDA in children. The causal

relationship in adults, therefore, remains unproven. The only way to add substance to these findings is a well-designed randomised control trial, but unfortunately there has not been any recently published data of this sort. Thus the general consensus from clinicians is to test and eradicate *H. pylori* if IDA persists (Malfertheiner et al., 2007).

4.1.5 Iron transporter regulators

Hepcidin is a small anti-microbial-like peptide and is essential for iron metabolism as it regulates iron absorption and distribution by inhibiting the release of intracellular iron into plasma via feedback mechanisms (Rivera et al., 2005).

Ferroportin is present in all cell types and is involved in the transfer of iron (Donovan et al., 2000). This iron exporter is located at the cell surface membrane of these cells (McKie et al., 2000). Ferroportin is the molecular target for hepcidin. When hepcidin binds to ferroportin it leads to its engulfment by the cell membrane and degradation. This reduces the export of iron from enterocytes and macrophages into plasma. Ferroportin is therefore re-localised from the cell surface membrane to the cytoplasm (Figure 4.1). The exact structure of the ferroportin transmembrane molecule has not been fully discovered and thus the exact mechanisms as to how iron is transferred is unknown (Ganz, 2011).

On entering the plasma iron is bound to the transporting protein transferrin to enable its eventual delivery to all cells. Transferrin receptor 1 allows cells to take up iron where it is used for metabolic activities or stored inside ferritin. The vast majority of iron transferrin is utilised in haemoglobin synthesis in the bone marrow (Ganz, 2011).

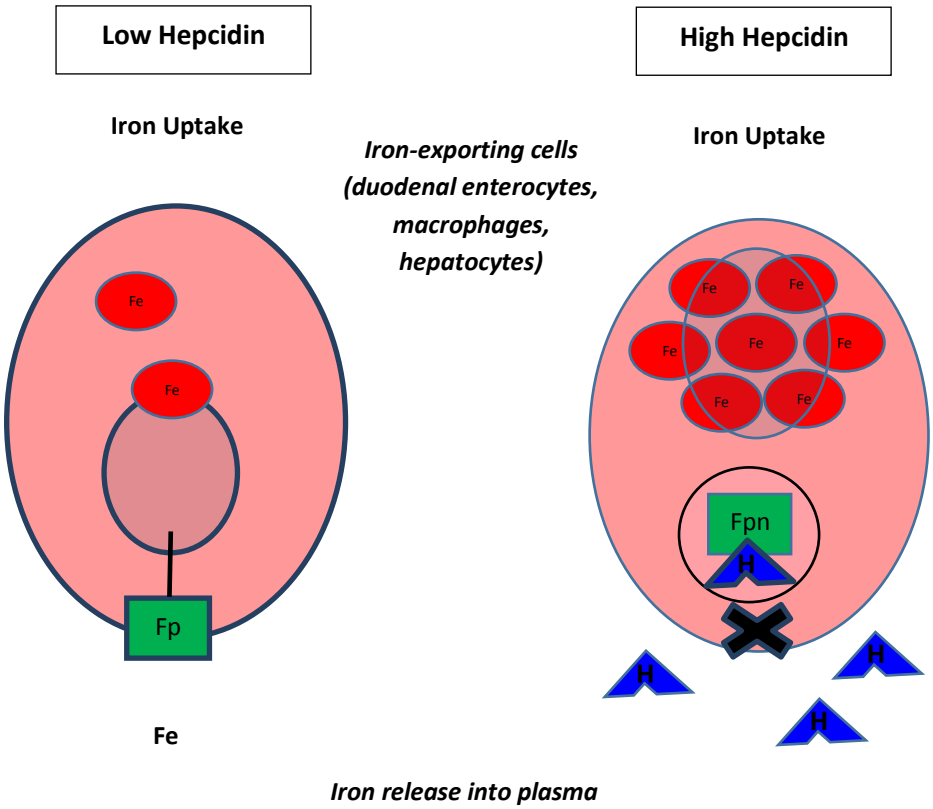


Figure 4.1 Mechanism of hepcidin-mediated cellular iron regulation

Figure adapted from (Lemos Ados et al., 2010). In response to increased iron levels the liver increases hepcidin (H) synthesis. Absorbed iron binds to ferritin (Fe) as it migrates to cell surface membrane. This binds to ferroportin (Fpn) located on the cell membrane preventing export (from enterocytes and hepatocytes) into plasma, reducing serum iron levels. Hepcidin is also influenced by inflammation, hypoxia and erythropoiesis. Excessive hepcidin leads to reduced serum iron and anaemia while deficiency of hepcidin leads to iron overload.

4.1.6 Systemic iron homeostasis

During normal iron concentrations, hepcidin inhibits the flow of iron into plasma cells. In states when iron is plentiful, hepcidin release is increased to limit iron absorption from dietary sources and release from iron storage sources. When iron is needed, however this leads to a decrease in hepcidin release which subsequently allows iron to re-enter plasma meeting this demand. Hepcidin production is regulated by red blood cell production and inflammation, via IL-6 and activing B (Nemeth et al., 2003, Besson-Fournier et al., 2012).

Gene mutations can lead to pathological conditions such as iron overload in the case of hepcidin deficiency which is seen in haemochromatosis. During the low hepcidin states, increased absorption of dietary iron and release from macrophages raises the plasma iron levels and deposition of iron in organs (Griffiths, 2007).

Iron deficiency seen in some chronic disease/infectious states (e.g. *H. pylori* infection) is thought to be driven by inflammation and excess hepcidin production. Hepcidin prevents efflux of iron from storage source and absorption at the duodenum. This results in the iron-limited reduction of red blood cells and subsequent anaemia (Arezes and Nemeth, 2015, Ganz, 2011).

4.1.7 Iron deficiency anaemia, hepcidin and *H. pylori*

Sata *et al*. found serum prohepcidin levels to be higher in *H. pylori* infected individuals. They also found a statistically significant negative correlation between serum iron and prohepcidin. They suggested hepcidin overproduction as a possible mechanism for iron deficiency anaemia in *H. pylori* infected individuals (Sato et al., 2015). One case control study showed that raised serum hepcidin was associated with *H. pylori* infection in children, and was also associated with a reduced response to oral iron therapy. In the *H. pylori* infected children there was a negative relationship between anaemia markers and hepcidin (Azab and Esh, 2013).

Lee *et al*. performed a prospective study measuring serum prohepcidin in IDA patients before and after standard eradication treatment. They showed that serum prohepcidin levels decreased after *H. pylori* eradication and iron replacement therapy, leading to subsequent improvement in anaemia parameters but that there was no difference in the baseline prohepcidin levels between *H. pylori* infected and uninfected anaemic adults. Serum prohepcidin levels seem to be related to the extent of iron deficiency anaemia rather than *H. pylori* infection indicating that the prohepcidin level is likely to be a functional measure of anaemia (Lee et al., 2010). Ulukol *et al.* assessed pro-hepcidin levels in young children and concluded there was no statistically

significant difference between IDA and healthy individuals (Ulukol et al., 2007).

More recent research from the Crabtree research group in 2012 investigated the haematological and molecular changes in gastric mucosa associated with hypergastrinemia in INS GAS mice. These mice overexpress the gastrin gene, leading to excessive gastrin production and spontaneous development of adenocarcinoma of the stomach by 2 months. Groups of *H. felis* infected and uninfected mice were sacrificed at month 3, 6 and 9 and blood and stomach tissue was collected for laboratory analysis. They found that the infected group had a reduction in the number parietal cells during the later months of the experiment. As expected the inflammation was greater and more gastric lesions and atrophy were seen with the longer periods of infection duration. The infected group also had lower serum ferritin and iron as the infection duration increased compared with controls. Total iron binding capacity also increased and transferrin saturation decreases with time. The hepcidin upstream regulators Bmp4/6 were reduced and ferroportin Fpn1 increased over time. This reduction in haematological markers signified poor iron absorption in the infected mice. The reduction in parietal cells contributed to poor iron absorption and may have led to a reduction in hepcidin expression (Thomson et al., 2012). Their hepcidin data are however, different to a human study that was published that same year, which found an increase in gastric hepcidin expression but no systemic correlation (Schwarz et al., 2012).

Schwarz *et al.* established that the stomach, in addition to the liver, is a hepcidin-secreting tissue. They used quantitative RT-PCR, *in situ* hybridisation and immunofluorescence to confirm hepcidin expression and localisation in gastric tissue. They analysed both *in vitro* and *in vivo* expression, and animal models were used to assess hepcidin impact on bacterial overgrowth. Hepcidin was found to be expressed in the gastric antrum and corpus, located in parietal cells, and was essential for the secretion of gastric acid. Hepcidin deficiency in mice is associated with reduced acid secretion, lower gastrin secretion and bacterial overgrowth. Therefore, hepcidin may contribute to the

formation of peptic ulcers. In humans, iron and *H. pylori* both upregulate hepcidin expression and this normalized after *H. pylori* eradication treatment. In conclusion, increased hepcidin production associated with *H. pylori* may contribute to peptic ulcer development (Schwarz et al., 2012).

Cherian *et al.* investigated this relationship from a different angle, looking at serum inflammatory cytokines, IDA and urine hepcidin. They investigated individuals with IDA to see if they had a reduction in urine hepcidin but there were no correlations with serum inflammatory cytokines or helicobacter or malarial infections. Urine hepcidin, however, seemed to be positively correlated with ferritin (Cherian et al., 2008).

In addition to serum iron, hepcidin synthesis is regulated by the innate immune system where it is more dominantly expressed in the liver. Inflammation and infection stimulates hepcidin production via IL-6 (Nemeth et al., 2003). The binding of hepcidin to ferroportin leads to this exporter protein being removed from the cell surface. This prevents the delivery of iron to the hepatic portal and systemic circulation. Following the iron regulatory function, iron overload leads to hepcidin expression, while iron deficiency leads to reduced hepcidin levels (Schwarz et al., 2012, Knutson, 2010).

To summarise, although data are limited *H. pylori* seems to be associated with increased serum hepcidin and gastric mucosa hepcidin expression via a variety of mechanisms.

4.2 Aims

IDA is a common problem in clinical practice with significant associated costs to the health care budget. Eradication resolves IDA in some also infected with *H. pylori* but due to multiple confounders in the literature causality is unproven. Mouse studies have demonstrated that *H. pylori* infection has important effects on iron transporters and induces IDA, but human data are currently lacking. This is a pilot study to further our understanding of iron transport in humans. Ultimately it may be possible to allow targeted

investigation and adopt new treatment strategies that may be more effective in those with *H. pylori* infection.

The aim of this study was to characterise local and systemic iron transporter expression in three groups of patients with:

1. *H. pylori* gastritis and IDA (HP+IDA)
2. *H. pylori* negative dyspepsia (Controls)
3. IDA in the absence of *H. pylori* infection (IDA)

4.3 Hypotheses

1. IDA is more severe in those with *H. pylori* infection compared to those without
2. Gastritis and gastric atrophy is more severe in IDA patients with *H. pylori* infection, compared to those without
3. Hepcidin expression is increased more in those with *H. pylori* infection
4. The cellular localisation of ferroportin is different in IDA patients with *H. pylori*

4.4 Materials and Methods

4.4.1 Participants and clinical samples

The work was carried out after approval from Derbyshire Research Ethical Committee (REC Ref: 10/H0401/33) and written informed consent from adult patients attending Nottingham University Hospitals NHS Trust for a diagnostic gastroscopy as part of their investigation into iron deficiency anaemia. Patients for whom no obvious cause for anaemia was found were included in the final analysis. Patients without IDA or *H. pylori* were also prospectively

recruited to act as controls after approval from Nottingham Research Ethical Committee 2 (REC Ref: 08/H0408/195) and informed consent from adult patients attending Nottingham University Hospitals NHS Trust for a diagnostic gastroscopy. 109 patients who attended routine endoscopy appointments were recruited. After exclusions a total of 76 patients were included in the final analysis (*H. pylori* and IDA=18, Controls=18, IDA=40). The most common reason for exclusion in individuals with anaemia was that a cause for the anaemia had been found (e.g. malignancy) or having an incomplete set of research samples. In the control group the most common reason for exclusion was incomplete diagnostic investigations. Dr JR White was responsible for the coordination, patient screening and management of the study. Patient recruitment and sample collection was done by Dr JR White (59%), Dr S Sarmed (23%) and Dr J Ortiz (18%).

Fasting blood samples were collected for plasma and serum, duodenal and gastric biopsies were snap frozen in liquid nitrogen and stored at -80°C for later analysis. Biopsies were collected from the second part of the duodenum, gastric antrum and corpus and immediately placed in formalin for later histopathological scoring. One biopsy from the antrum was placed in urease medium for rapid urease detection, and another sample in iso-sensitest broth (Oxoid, Cambridge, UK)/10% glycerol for *H. pylori* isolation and culture. *H. pylori* negative patients had all three negative tests.

4.4.2 ELISA assay

6mls of blood was collected in vacutainers (non-additive serum tube, Becton Dickinson). The time that the blood was taken was recorded and the bottle inverted several times and left standing at room temperature for 1 hour then centrifuged at 200 x *g* for 10 minutes. The supernatant (serum) was transferred to three 1.5ml cryotubes and stored at -80°C. 8mls of blood was collected in EDTA tube and centrifuged at 200 x *g* for 10 minutes at room temperature and 3 aliquots of plasma stored at -80°C. The remaining blood

was sent to clinical laboratory for full blood count, ferritin, iron studies and coeliac serology.

CagA IgG ELISA kit (Genesis) was followed according to manufacturer's instructions to determine *cagA* status from stored plasma. Optical densities were measured with a microplate reader at 450nm with the reference wavelength of 620nm. CagA IgG ELISAs were completed by Dr JR White (109 samples). The concentration of *H. pylori* IgG (Biohit), Pepsinogen I (Biohit) and Pepsinogen II (Biohit) was determined using ELISA kits according to manufacturer's instructions. *H. pylori* IgG, Pepsinogen I and Pepsinogen II ELISAs were completed by Dr JR White (66 samples) and D. Reddiar (43 samples). A commercial ELISA assay was used to quantify serum Hepcidin-25 (Peninsula Laboratories International, Inc.) Hepcidin-25 ELISAs were completed by Y. Falcone and M. Lingaya according to the manufacturer's instructions.

4.4.3 Laboratory Investigations

Fasting blood samples were also collected for haematological and biochemical analysis by the local pathology department at Nottingham University Hospital. These tests included full blood count, B12, folate, coeliac serology, serum ferritin, transferrin saturation, transferrin, serum iron and iron binding capacity.

4.4.4 Histopathology

Gastric and duodenal samples were placed in formalin and prepared and stained by the Histopathology department at the University Hospital in Nottingham. The samples were fixed in formalin, then embedded in paraffin and cut into approximately 4µm sections. Sections were stained with haematoxalin and eosin (H&E) to allow inflammation, atrophy and intestinal metaplasia scores to be carried out. Toluidine blue staining was also carried out for *H. pylori* density grading. For this grading scores were rated from zero to three, where 0=no *H. pylori*, 1=mild, 2= moderate, 3= high density. Two

experienced GI histopathologists (A. Figgins/P. Kaye) who were blinded to clinical and laboratory findings carried out the grading and classification using the updated Sydney scoring system (Dixon et al., 1996).

4.4.5 Immunohistochemistry

Paraffin sections were incubated at 95-100°C in WCAP reagent (Surgipath) for 30 minutes then washed and incubated in 10:1 methanol: H_2O_2 for another 10 minutes. Sections were incubated with the appropriate antibody Human Hepcidin-25 peptide (Abcam) or Ferroportin Anti-SLC40A1 (Abcam) for 60 minutes. Immunoreactivity was then visualised with an Envision assay kit and DAB reagent (DakoCytomation). Then counterstained with haematoxylin and mounted. Positive controls were normal liver sections and negative controls included the omission of primary antibody and liver sections (Brookes et al., 2006). Immunostaining was carried out by N. Worton and A. Deacon.

Slides were then evaluated and scored by two independent blinded observers H. Omar and M. Mangalika. One evaluator was a senior scientist and the other an experienced GI pathologist. Slides were evaluated to ensure staining was specific and occurred at certain locations (nuclear, cytoplasmic, cell surface membrane) and the predominant expression cell type. Staining intensity product score was determined by two factors:

1. Scale of 0 to 3 for intensity (0 = negative expression, 1=weak expression, 2=moderate expression 3 = intense expression)
2. Percentage of epithelial cells within the microscopic field of view positively stained (0 = no cells, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100% of cells)

These two scores were multiplied to give a total score between 0-12 (Di Martino et al., 2006). This score allowed for a semi-quantitative evaluation of protein expression to be determined.

4.4.6 Statistics

Statistical analysis was carried out using Prism 6.0 (GraphPad Software 6 USA), unless otherwise stated horizontal lines within bars represent median values. Mann Whitney U tests were used for unpaired group data. A statistical significant difference was considered if p values were ≤ 0.05. Kappa (k) statistical test was calculated using SPSS (version 21) to calculate agreement between observers after accounting for chance agreement. Interpretation of k values was as follows: <0 indicated no agreement; 0–0.20 = slight agreement; 0.21–0.40 = fair agreement; 0.41–0.60 = moderate agreement; 0.61–0.80 = substantial agreement; and 0.81–1 = almost perfect agreement (Landis and Koch, 1977). Statistical Package for Social Sciences (SPSS 21.0 Package Facility, SPSS Inc., Chicago, Illinois, USA) was used for data analysis of Kappa values. All statistical analysis was carried out by Dr J.R White.

4.5 Results

4.5.1 Demographics

The male to female ration for the HP+IDA, controls and IDA groups were 0.5, 0.44 and 0.53 respectively. The median age and range for HP+IDA, IDA and control group were 74.5 (34-85), 57.5 (32-79) and 67 (42-85) years respectively (Table 4.1).

Table 4.1: Haematological and serological data in three groups of patients, *H. pylori* and IDA, controls and IDA only

	Hp+ IDA	Controls	IDA
Median Hb Males (130-180g/L)	112 (85-129)	144 (124-178)	111 (80-153)
Median Hb Females (115-165g/L)	106 (85-129)	136 (122-161)	111 (78-153)
Median MCV (84-102fl)	82 (73-91)	88.5 (84-99)	83 (65-106)
Median MCH (28-33pg)	26.4 (21.5-30.3)	29.9 (27.5-33.5)	25.9 (20-37.1)
Median Ferritin (25-350µg/L	25.5 (5-264)	93.5 (13-199)	15 (3-126)
Median Serum Iron (9-31µmol/L	9.5 (4-22)	15 (8-22)	9 (3-23)
Median Transferrin g/L (2-3.6)	3.35 (2.1-4.3)	2.6 (2.3-3.4)	3.15 (1.7-4)
Median Transferrin saturation 20-50%	15.5 (4-30)	27 (14-38)	13.5 (3-35)
Median Serum Folate micro g/L (3.9-14)	13.9 (4.9-24)	10 (5.7-24)	9.75 (3.8-24)
Median B12 ng/L (190-800)	323.5 (82-960)	405 (130-618)	340.5 (170-980)

Anaemia parameters of the three groups expressed as the median and range.

4.5.2 Anaemia parameters

Anaemia parameters were not more severe in those with *H. pylori* infection when compared with those without, which is opposite to the hypothesis. The anaemia parameters were however significantly lower in both the HP+IDA and IDA groups when compared to controls ($p<0.0001$). See Figure 4.2.

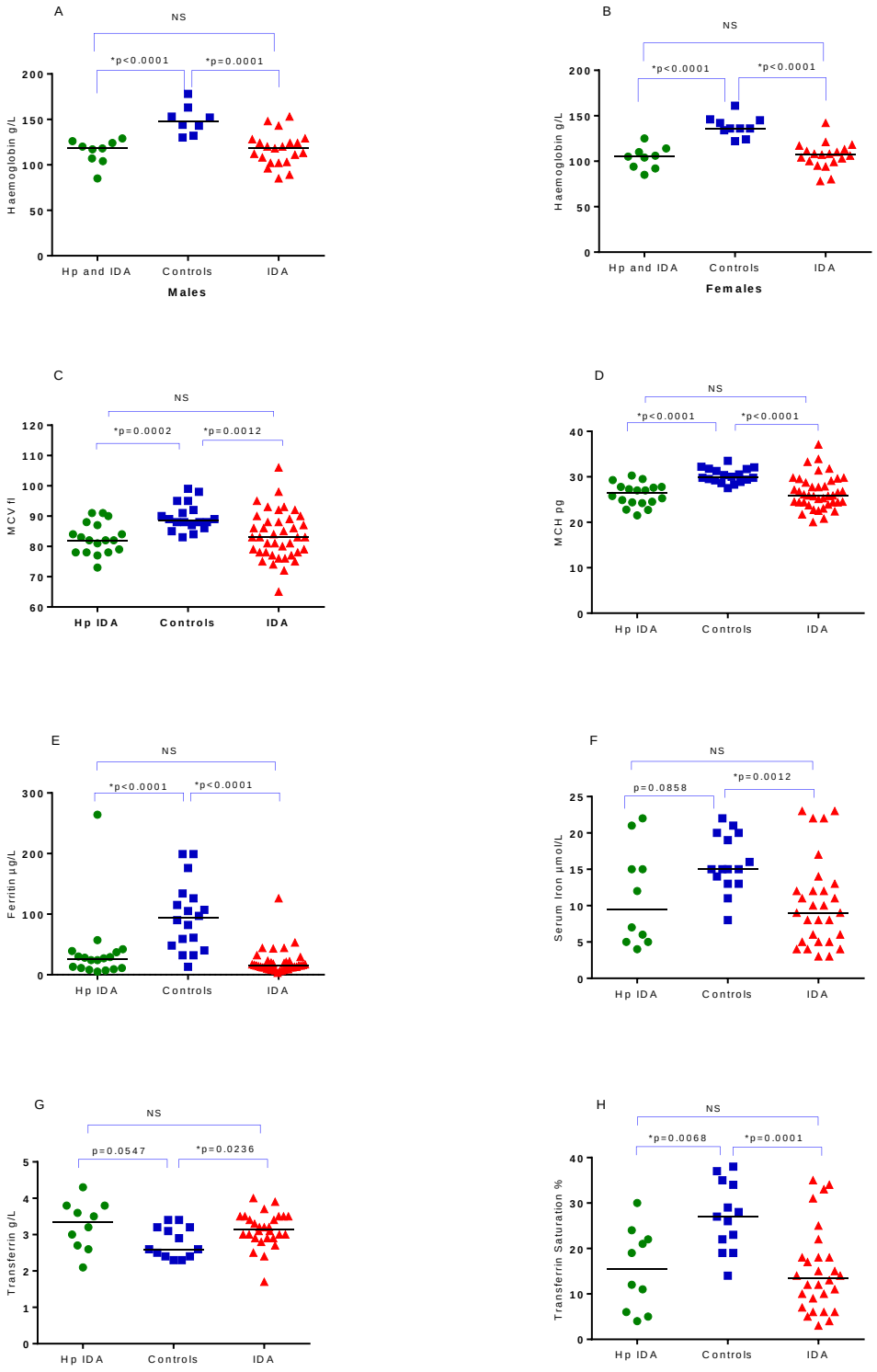


Figure 4.2: Differences in anaemia parameter between the HP+ IDA, Controls and IDA groups

Haemoglobin in males (A), haemoglobin in females (B), MCV (C), MCH (D), serum ferritin (E), serum iron (F), transferrin (G), transferrin saturation (H). Patient numbers, HP and IDA=18, Controls=18 and IDA=40. Mann Whitney U test was carried out for statistical significance, horizontal line represent the median and NS=non-significant.

4.5.3 Gastritis and gastric atrophy

As predicted, the HP+IDA group had significantly higher gastric inflammation, atrophy and intestinal metaplasia than the controls and IDA group (Table 4.2, 4.3, Figure 4.3 and 4.4). Statistically significant lower PGI/II ratios were observed in the HP+IDA group when compared to the controls and the IDA group (3.6-fold p=0.0005 and 3.5-fold p=0.02 respectively).There was no significant relationships between atrophy score and *cagA* status or hepcidin levels and *H. pylori* density (data not shown).

Table 4.2 Histopathological variables in the gastric antrum of the HP+ IDA, Controls and IDA groups

Parameter		Hp IDA (N=18)	Controls (N=18)	IDA (N=40)
Antral Active Inflammation	**0**	9 (50%)	18 (100%)	35 (87.5%)
	1	3 (16.7%)	0	3 (7.5%)
	2	3 (16.7%)	0	1 (2.5%)
	3	3 (16.7%)	0	0
Antral Chronic Inflammation	**0**	0	18 (100%)	26 (65%)
	1	9 (50%)	0	9 (22.5%)
	2	9 (50%)	0	3 (7.5%)
	3	0	0	1 (2.5%)
Antral Atrophy	**0**	8 (44.4%)	18 (100%)	36 (90%)
	1	9 (50%)	0	3 (7.5%)
	2	1 (5.6%)	0	0
	3	0	0	0
Type Atrophy	**Metaplastic**	5	n/a	1
	Non Metaplastic	3	n/a	n/a
	Both	0	n/a	n/a
Antral Intestinal Metaplasia	**0**	12 (66.7%)	18 (100%)	35 (87.5%)
	1	1 (5.6%)	0	2 (5%)
	2	4 (22.2%)	0	1 (2.5%)
	3	1 (5.6%)	0	0
Antral *H. pylori*	**0**	6 (33.3%)	18 (100%)	37 (92.5%)
	1	5 (27.8%)	0	0
	2	6 (33.3%)	0	0
	3	1 (5.6%)	0	0

Histopathological variables were scored using the updated Sydney scoring system, by two experienced GI pathologists who were blinded to the group designations. The number of patients and percentage per group for each parameter is shown.

Table 4.3 Histopathological variables in the gastric corpus of the HP+ IDA, Controls and IDA groups

Parameter		Hp IDA (N=18)	Controls (N=18)	IDA (N=40)
Corpus Active Inflammation	**0**	2 (11.1%)	18 (100%)	36 (90%)
	1	6 (33.3%)	0	1 (2.5%)
	2	8 (44.4%)	0	0
	3	2 (11.1%)	0	2 (5%)
Corpus Chronic Inflammation	**0**	0	18 (100%)	29 (72.5%)
	1	5 (27.8%)	0	1 (2.5%)
	2	10 (55.6%)	0	6 (15%)
	3	3 (16.7%)	0	3 (7.5%)
Corpus Atrophy	**0**	9 (50%)	18 (100%)	30 (75%)
	1	2 (11.1%)	0	1 (2.5%)
	2	4 (22.2%)	0	3 (7.5%)
	3	3 (16.7%)	0	5 (12.5%)
Type of Atrophy	**Metaplastic**	4 (50%)	n/a	2 (5%)
	Non Metaplastic	2 (25%)	n/a	0
	Both	2 (25%)	n/a	4 (10%)
Corpus Intestinal Metaplasia	**0**	14 (77.8%)	18 (100%)	37 (92.5%)
	1	1 (5.6%)	0 (100%)	2 (5%)
	2	3 (16.7%)	0	0
	3	0	0	0
Corpus *H. pylori*	**0**	2 (11.1%)	18 (100%)	38 (95%)
	1	10 (55.6%)	0	0
	2	5 (27.8%)	0	0
	3	1 (5.6%)	0	0

Histopathological variables were scored using the updated Sydney scoring system, by two experienced GI pathologists who were blinded to the group designations. The number of patients and percentage per group for each parameter is shown.

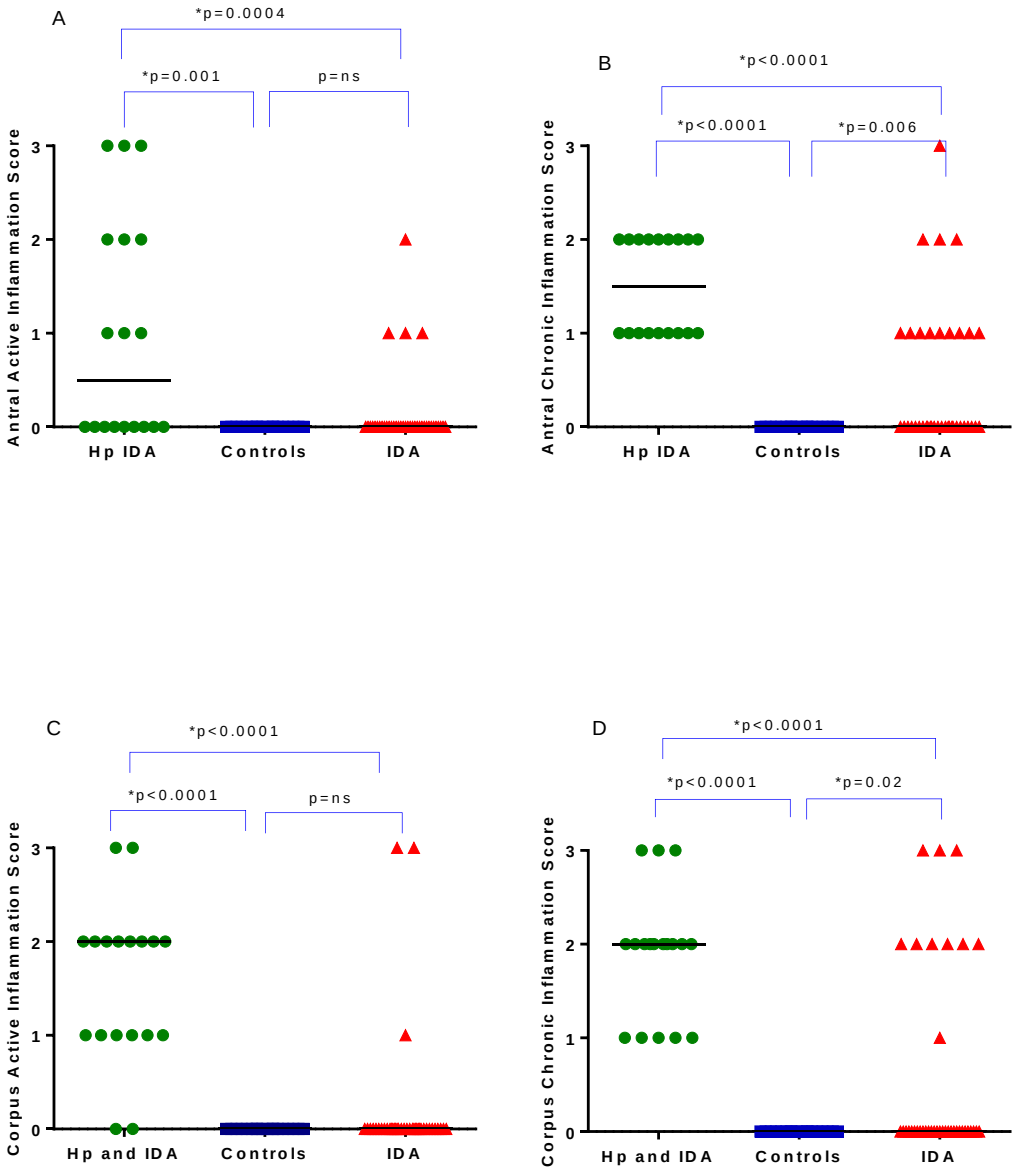


Figure 4.3 Inflammation scores in the antrum and corpus of the HP+ IDA, Controls and IDA groups

The HP+IDA group had significantly higher scores for gastric inflammation than IDA and control groups. (A) Antral active inflammation. (B) Antral chronic inflammation. (C) Corpus active inflammation and (D) Corpus chronic inflammation. Patient numbers, HP and IDA=18, Controls=18 and IDA=40. Mann Whitney U test was carried out for statistical significance, horizontal line represent the median and ns=non-significant.

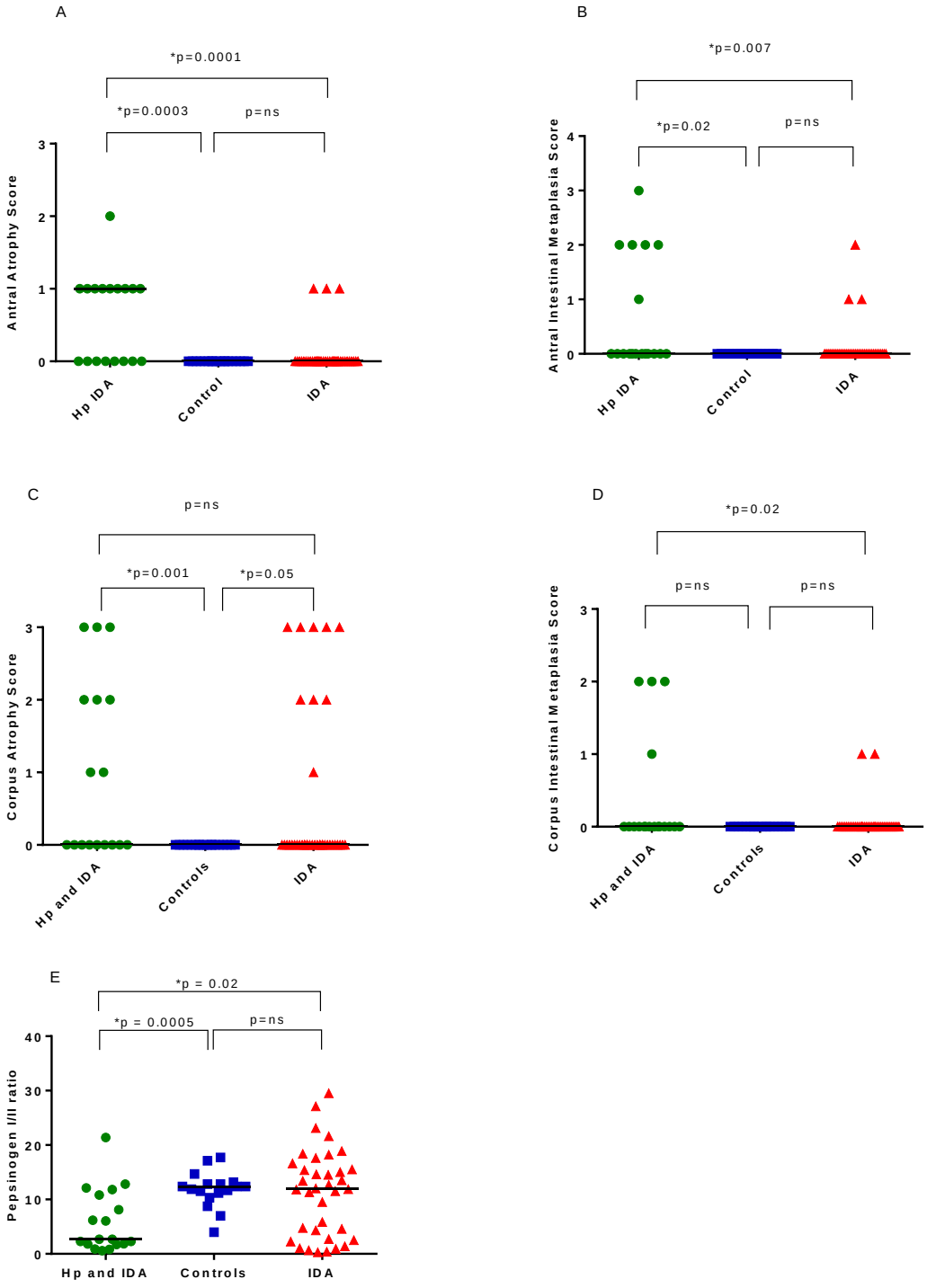


Figure 4.4 Atrophy, intestinal metaplasia scores and PGI/II ratios in the HP+ IDA, Controls and IDA groups

Antral atrophy score (A), antral intestinal metaplasia score (B), corpus atrophy score (C), corpus intestinal metaplasia (D) and pepsinogen I/II ratio (E). Patient numbers, HP and IDA=18, Controls=18 and IDA=40. Mann Whitney U test was carried out for statistical significance, horizontal line represent the median and ns=non-significant.

4.5.4 Serum and mucosal hepcidin levels

Serum hepcidin concentrations were significantly reduced in the HP+IDA and IDA groups compared to the controls (9-fold, p=0.009 and 5-fold, p<0.0001 respectively). See Figure 4.5. Gender, *cagA* status, PPI or iron therapy did not lead to a significant change in serum hepcidin within each group (data not shown).

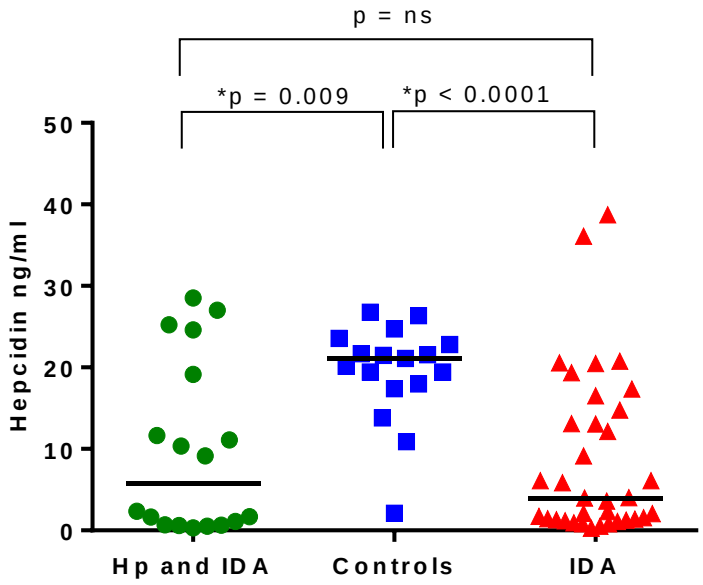


Figure 4.5: Serum hepcidin in the IDA, controls and IDA group

Patient numbers, HP and IDA=18, Controls=18 and IDA=40. Mann Whitney U test was carried out for statistical significance, horizontal line represent the median and ns=non-significant.

Mucosal hepcidin levels were not significantly higher in the HP+IDA group. Hepcidin expression was highest in the corpus in all three groups. Duodenal scores were higher in the IDA group than HP+IDA (p=0.002) and controls (p=0.007) (medians: HP+IDA=5.5, Controls=5.0, IDA=8.0). Corpus scores were slightly higher in HP+IDA compared to the others but this did not achieve statistical significance (medians: HP+IDA=12.0, Controls=9.8, IDA=10.5, see figure 4.6). Figure 4.7 shows examples of immunohistochemistry slides that were scored according to intensity and percentage of positively stained cells.

Hepcidin was predominately localised in the cytoplasm in all groups and areas. The predominant cell type was enterocytes in the duodenum and parietal cells in the corpus.

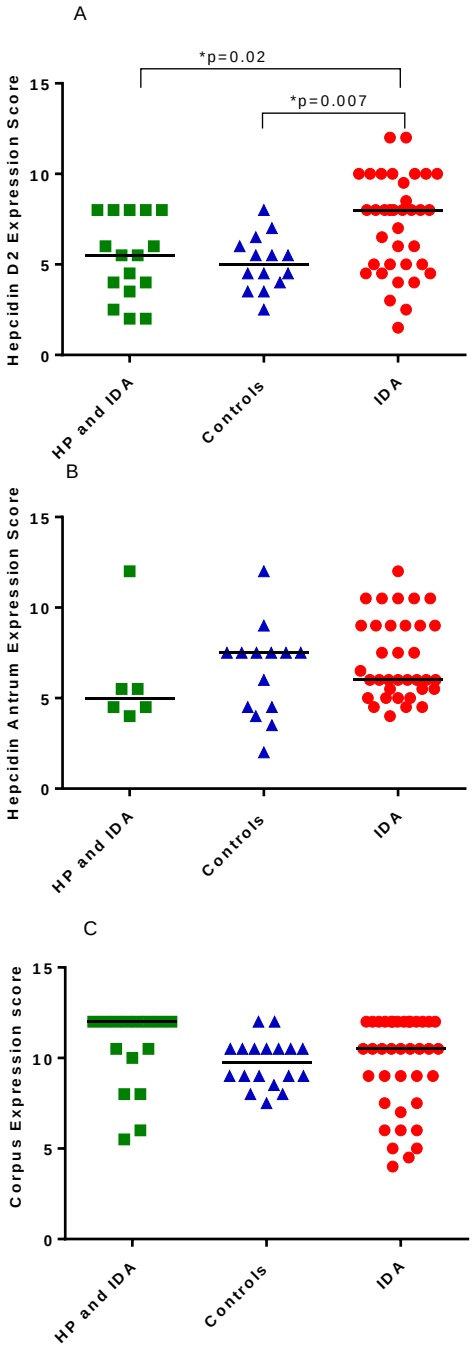
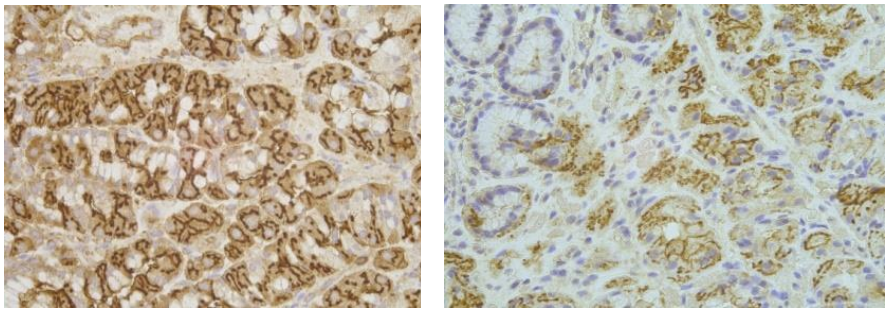
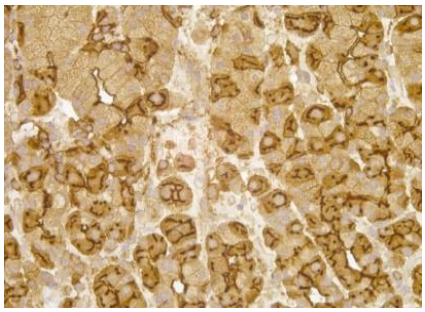


Figure 4.6 Hepcidin expression scores in the HP+IDA, controls and IDA group

Graph (A) represents duodenal hepcidin expression intensity score, (B) antrum and (C) corpus. Patient numbers, HP and IDA=18, Controls=18 and IDA=40. Mann Whitney U test was carried out for statistical significance, horizontal line represent the median and ns=non-significant



A B



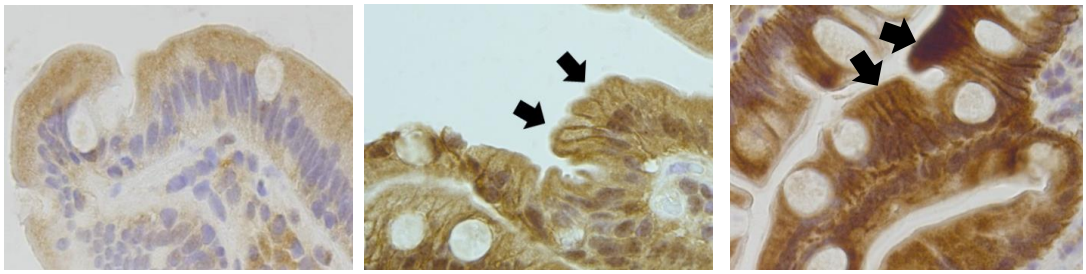
C

Figure 4.7 Examples of hepcidin staining on sections from corpus of HP+IDA, control and IDA groups

Gastric corpus of HP+IDA (A) controls (B) and IDA (C) group. Images were taken at a magnification of x40. Corpus hepcidin staining (brown) is more intense in HP+IDA (A) and IDA (C) compared to controls (B). Hepcidin staining was predominantly localised in the cytoplasm. Blue circular objects represent cell nucleus.

4.5.5 Ferroportin cellular localisation

Duodenal ferroportin was localised to the cytoplasm of enterocytes in the HP+IDA group in 78% of cases. For the controls and IDA group, ferroportin was mainly localised to the cell surface membrane with only 33% and 40% localised to the cytoplasm respectively (Figure 4.8). The predominant cell type in all three groups was enterocytes.



A B C

Figure 4.8: Duodenal ferroportin cellular localisation

Images represent duodenal mucosa magnified 40 times and arrows represent high cell surface membrane staining intensity (brown). Staining was localised to the cytoplasm in 78% cases in HP+IDA group (A), 33% in the controls (B) and 40% in IDA only (C) group. Blue circular objects represent cell nucleus.

4.5.6 Inter-observer agreement on immunohistochemistry scores

Inter observer agreement for intensity, percentage positive epithelial cells stained, predominant cellular location and predominant cell type was moderate, fair, fair and almost perfect ($p<0.0001$) respectively (Table 4.4).

Table 4.4 Inter observer agreement among assessors

	Number cases	Kappa value	p value	Agreement Interpretation
Intensity	304	0.44	<0.0001	Moderate
% positive epithelial cells stained	304	0.34	<0.0001	Fair
Predominant cellular location	304	0.31	<0.0001	Fair
Predominant expression cell type	304	0.98	<0.0001	Almost perfect

Inter-observer agreement between two assessors on immunohistochemistry staining intensity, proportion positive epithelial cells stained, predominant location and predominant expression cell type.

4.6 Discussion

Anaemia parameters were not more severe in the HP+IDA group than the IDA group however the numbers in the study were small (18 vs 40). As expected the anaemia parameters were statistically lower in the HP+IDA and IDA groups when compared to the controls. Gastric inflammation and atrophy was greater in the HP+IDA group than the IDA and control groups. One of the leading causes of gastric atrophy is *H. pylori* infection, and atrophy is associated with anaemia so it is not surprising that the HP+IDA had more severe inflammation and atrophy (Kaye et al., 2008).

Serum hepcidin concentrations were significantly reduced in the HP+IDA and IDA groups compared to the controls (9-fold, P=0.009 and 5-fold, P<0.0001 respectively). In contrast to previously published work (Azab and Esh, 2013). There is a large amount of divergent data in the literature with regards to serum hepcidin and *H. pylori* infection. Azab *et al.* performed a case control study comparing anaemic children with or without *H. pylori* infection and healthy controls. They found there was a six-fold increase in the serum

hepcidin in the *H. pylori* infected anaemic children. Also a decreased serum hepcidin in the non-infected group when compared to controls (Azab and Esh, 2013). Emiralioglu *et al*. also found an increase in serum prohepcidin in *H. pylori* infected children (Emiralioglu et al., 2015). Whilst Cherian measured urinary hepcidin in a cohort of children with *H. pylori* infection or without determined by serology and found there was no difference in hepcidin and IL-6 levels between the groups (Cherian et al., 2008).

The findings of the current study are likely due to gastric atrophy, as there was a significantly higher proportion of gastric atrophy in the HP+IDA and IDA groups when compared to the controls. This is supported by the fact that hepcidin production decreases when iron stores in the body are low, so this would explain why serum hepcidin is low in both infected and uninfected anaemic groups (Nemeth et al., 2004). Also hepcidin is localised in gastric parietal cells (Schwarz et al., 2012). As gastric glands are replaced with fibrous tissue in gastric atrophy (Rugge et al., 2002) parietal cell numbers are reduced; hence serum hepcidin production is reduced. Kim et al also found that serum prohepcidin was lower in adult patients with atrophic gastritis regardless of *H. pylori* status (Kim et al., 2013). In addition Thomas *et al* successfully demonstrated gastric hepcidin expression was reduced in hypergastrinemic INS-GAS mice with *H. pylori* infection which had a knock on effect on certain iron absorption genes. Therefore the reduction in gastric hepcidin expression due to loss of hepcidin secreting parietal cells may act as an iron regulator (Thomson et al., 2012).

Others have failed to show a relationship between *H. pylori* infection and serum prohepcidin (Lee et al., 2010). This could suggest that hepcidin secreted locally from gastric tissue is not sufficient to increase systemic serum hepcidin (Schwarz et al., 2012). Where previous papers have shown an increase in serum hepcidin with *H. pylori* infection or its expression upregulated in response to cytokines, these patients have been very young or children so will have shown little or no gastric atrophy. The *H. pylori* infected anaemic patients in our study probably have negligible *H. pylori* colonisation

so there would be no significant increase in serum hepcidin. Gender, CagA status or drug therapy with PPI or oral therapy did not seem to influence serum hepcidin concentrations. (Arezes and Nemeth, 2015).

The current study was not able to confirm the work of Schwartz *et al.* showing that gastric hepcidin expression is upregulated during *H. pylori* infection (Schwarz et al., 2012). Although not statistically significant, gastric corpus expression was slightly higher in the HP+IDA group when compared to the other groups. There are few published studies investigating local tissue hepcidin expression in *H. pylori* infection. This study also showed that hepcidin was expressed in the duodenal and gastric mucosa but expression scores were higher in the gastric corpus. In summary, *H. pylori* infection does not seem to influence gastric or duodenal mucosal expression in this study. Immunohistochemistry (IHC) scoring only provides semi quantitative data which means data should be interpreted with caution. Schwarz *et al* used RT-PCR to determine the level of hepcidin expression in the gastric mucosa. This is a far more reliable tool than immunohistochemistry evaluation. There is a wide range of variability with IHC scoring of duodenal and gastric mucosa which also leads to difficulty relying on or interpreting the results. With regard to determining the expression scores, the observer agreement was moderate and fair for intensity and percentage of cells stained respectively. This lack of strong agreement would also introduce variability in the results.

Ferroportin was mainly localised in the cytoplasm of enterocytes in the HP+IDA group and cell surface membrane in the controls and IDA group. The localisation of ferroportin in the cytoplasm suggests iron trapping in cells that occurs in cases of anaemia (Nemeth et al., 2004). Although not statistically significant, gastric hepcidin was increased despite reduced systemic levels, suggesting other mechanisms such as local cytokine production could be responsible for ferroportin re-localisation. Ferroportin expression can be influenced by inflammatory cytokines, haemoglobin, hypoxia and transitional metals (Ward and Kaplan, 2012).

In summary, these data suggest that systemic hepcidin may not be a key mediator of IDA in this cohort. Effects of IDA in *H. pylori* infection could therefore be caused by other mechanisms. Serum may not be an appropriate site for hepcidin investigation in this cohort but local expression of hepcidin from tissues could be the mediator of IDA. Mucosal expression could be associated with local production of pro-inflammatory cytokines and recruitment of inflammatory cells. This pilot study helps further the understanding of iron transport in humans. Increasing the understanding will possibly allow for more targeted investigations and adopt new treatment strategies that may be more effective in those with *H. pylori* infection.

Strengths

This pilot study helped characterise local and systemic iron transporters in iron deficiency anaemia patients with or without *H. pylori* infection and dyspeptic controls. The study data help to add to the literature investigating the relationship between hepcidin and *H. pylori* but further studies are needed. This study looked at a variety of serological, haematological and histological parameters and will aid the planning of further mechanism experiments by helping to determine the best parameters to use. Importantly both the histopathologists and scientists were blinded to the clinical and laboratory findings therefore reducing bias. Also recruitment was of unselected prospective patients and numbers were adequate for a pilot study.

Limitations

Intra-observer agreement for hepcidin expression scoring was moderate and fair (k=0.44, 0.34) which may have impacted the final results. A third observer to look over cases where there was discrepancy may have made the scoring more consistent. mRNA gastric hepcidin expression data are also needed to confirm immunohistochemistry expression scores so current results cannot be fully relied on until this is completed. Although patients were investigated extensively for anaemia at presentation they were not followed up for a long period, so it is possible that a cause for anaemia such as undiagnosed

malignancy or chronic GI blood lost could have been later found which may also affect the results. Also due to the pilot nature of this study no power calculations were carried out.

Planned future work

RNA has been extracted and converted from snap frozen gastric and duodenal biopsies and converted to cDNA. In future work we plan to process the duodenal and gastric samples for qRT-PCR looking specifically at iron regulation genes *HAMP, DMT1, TFR1, FERRITIN, Ferroportin* and the pro-inflammatory response examining *TNFa, IFN gamma, IL1, IL10.* This will also help to further evaluate whether local *H. pylori* transcriptionally upregulated hepcidin expression effects iron transport in a way that the immunohistochemistry scores have failed to elicit.

4.7 Conclusions

- Anaemia parameters were similar in the HP+IDA and IDA groups.
- Gastric inflammation and atrophy was greatest in the HP+IDA group.
- Serum hepcidin was lower in the HP+IDA group compared to controls. This is in contrast with previously published data and is likely to be due to the presence of gastric atrophy.
- Corpus hepcidin expression tended to be higher in the HP+IDA group but this difference was not statistically significant.
- Local gastric hepcidin was increased despite reduced systemic levels, suggesting other mechanisms for ferroportin re-localisation e.g. local cytokines production.
- The cellular localisation of ferroportin was different in HP+IDA groups compared to IDA and controls.
- *H. pylori* did not appear to be contributing to iron deficiency anaemia through additional effects on hepcidin.

Chapter 5: Assessing the response to intravenous and oral iron in colorectal cancer patients with iron deficiency anaemia with or without serological evidence of *H. pylori* infection

5.1 Introduction

Colorectal cancer is the second most common cancer in woman and third in men worldwide (Ferlay et al., 2015). In the majority of cases cancer develops from a step wise progression from adenomatous polyps to colorectal carcinoma (Arnold et al., 2005). Age, environmental factors and a strong family history are strong risk factors for the disease (Ballinger and Anggiansah, 2007). In addition to change in bowel habits and rectal bleeding, a common presenting feature is IDA. Up to 10% of patients with IDA have colorectal cancer and are more commonly right sided (Sadahiro et al., 1998, Raje et al., 2007, Goddard et al., 2011) and is likely to be due to chronic occult blood loss. Anaemia associated with cancer is also related to poor iron absorption, nutritional deficiency, and anaemia of chronic disease. The release of inflammatory cytokines also leads to anaemia by a variety of pathophysiological mechanisms. Hepcidin is released from colorectal cancer tumour cells, leading to decrease release of iron into serum from iron transporter ferroportin. The reduced availability of iron leads to lower production of red blood cells. Tumour cells also activate immune system leading to cytokine release including IL-6 which leads to further hepcidin release reducing red cell production via decreased erythropoietin (EPO) production and destruction by macrophages (Munoz et al., 2014).

The primary form of treatment for colorectal cancer is surgical resection and chemotherapy but prognosis is greatly dependent on the stage of disease at diagnosis. The lifetime risk of developing colorectal cancer is around 2% and it is the second most common cause of cancer related death in the UK. The five year survival rates in the UK are around 60% (Poston et al., 2011, Guideline, 2011).

40% of patients with colorectal cancer are also diagnosed with IDA (Ludwig et al., 2004). Cancer associated anaemia impacts negatively on patient's quality of life and affects survival rates, so its management is an essential part of cancer treatment (Caro et al., 2001, Ludwig et al., 2004). The existence of pre-

operative anaemia is an independent risk factor for 30 day mortality with an odds ratio of 1.83 (95% CI 1.05 to 3.19) for moderate anaemia or a prolonged hospital stay (Leichtle et al., 2011). This makes anaemia a potential target for reducing colorectal cancer surgery mortality. Allogenic blood transfusions are a limited resource, expensive and associated with infections, anaphylaxis, fluid overload and haemolytic reactions (Williamson et al., 1999). The use of allogenic blood transfusions in colorectal cancer surgery is associated with increased surgical morbidity and mortality (Acheson et al., 2012). Other options for maintaining haemoglobin levels pre-operatively include intravenous or oral iron supplementation and erythropoietin stimulating agents. The benefits of iron replacement therapy pre-operatively in orthopaedic and gynaecological surgery is established in reducing adverse outcomes but results are inconsistent in colorectal cancer surgery and there are few studies (Cuenca et al., 2006, Munoz et al., 2009). This is mainly due to difference in study designs, iron preparations used, inclusion of non-anaemic individuals and lack of data on surgical outcomes (Borstlap et al., 2015). Randomised controlled trials have not consistently shown that intravenous iron is superior to oral in reducing perioperative blood transfusion rate or fall in haemoglobin prior to colorectal surgery (Edwards et al., 2009).

Oral iron is used because it is reasonably cheap, but its effectiveness is reduced because it is often poorly tolerated due to side effects and poorly absorbed. The intravenous route delivers a supply of iron to the bone marrow at a much accelerated rate but requires administration in a hospital setting. It is rarely associated with infusion related reactions. Intravenous iron can be effectively administered in an outpatient setting and is associated with a reduced blood transfusion requirement and improvement in haemoglobin level (Keeler et al., 2014).

Okuyama *et al* study involving 116 patients showed that giving oral sodium ferrous citrate 200 mg twice a day for two weeks improved haemoglobin and haematocrit levels prior to surgery in iron treatment group. Although this was a small retrospective study it showed iron treatment reduced intraoperative

blood transfusion when compared to no iron treatment 9.4% vs 27.4%, *p*=0.05 (Okuyama et al., 2005). Lidder *et al* investigated similar numbers of randomised prospective patients into oral iron treatment or no treatment. Their findings were similar with regards to improvement in haemoglobin and a 66% cost saving due to reduced transfusion requirement (Lidder et al., 2007). Multiple studies have shown intravenous iron is superior to oral iron therapy in the treatment of IDA in many conditions and prior to surgery. In IDA associated with pregnancy there was a 2g/dL (p=0.005) improvement in the intravenous group and fewer side effects for this randomised controlled trial conducted in India (Kochhar et al., 2013). Iron stores are more effectively restored with intravenous route and subsequently lead to improvement in anaemia parameters when compared with the oral route when given to patients post GI haemorrhage (Bager and Dahlerup, 2014).

Although there was a variation in iron supplements used most studies have shown superiority in using intravenous iron for a variety of medical conditions. The presence of *H. pylori* infection may lead to IDA, influence iron absorption and eradication therapy could improve response to iron therapy (Choe et al., 1999, Huang et al., 2010). No study has investigated the influence of *H. pylori* infection on response to iron therapy in colorectal cancer.

5.2 Aims

- To determine any differences in anaemia parameters between *H. pylori* infected and uninfected groups and the mode of iron therapy.
- To determine whether uninfected individuals respond better to oral iron therapy than *H. pylori* infected individuals.
- To determine if there is a difference in response to intravenous therapy in the *H. pylori* infected and uninfected group.
- To investigate whether the red blood cell transfusion requirement is reduced in the intravenous group compared to oral group up to 12 weeks post operatively.

5.3 Hypotheses

It is hypothesized that intravenous iron will lead to a better improvement in anaemia parameters when compared to oral. Patients with serological evidence of *H. pylori* will be less responsive to oral iron replacement treatment than those individuals without serological evidence of *H. pylori*. *H. pylori* status would help predict responders and non-responders to oral iron therapy. There will be no difference in response to intravenous iron replacement treatment in patients with or without *H. pylori* infection.

5.4 Materials and Methods

5.4.1 Participants

This multicentre, randomised, open label clinical trial was coordinated by the Division of GI Surgery, University of Nottingham, and Nottingham, UK. Other centres involved in patient recruitment and data acquisition included Royal Wolverhampton NHS Trust, University Hospital Birmingham, Derby Hospital NHS Foundation Trust and Yeovil District Hospital NHS Foundation Trust. Prospective patients were recruited 2012 to 2015 after approvals from Nottingham Research Ethical Committee 2 (ref. no. 09/H0408/67), Medicines and Healthcare Products Regulatory Agency, UK (MHRA) and ClinicalTrials.gov identifier: NCT01701310. Inclusion criteria were a diagnosis of non-metastatic colorectal adenocarcinoma in adults with biochemical evidence of IDA (< 12 g/dl for women, < 13 g/dl for men). Main exclusions included anaemia due other causes, previous surgery, current pregnancy or chemotherapy treatment. Mr B Keeler was responsible for the coordination, collection of data and management of the study.

Results from relevant published data were used to estimate sample size and it was calculated that 116 patients were required to achieve a power of 90% with α level of $p < 0{\cdot}05$. A one unit difference in mean transfused volume was

defined as a clinically significant effect (Christodoulakis et al., 2005, Keeler et al., 2017). Power calculations were carried out by Dr Keeler.

Patients with non-metastatic colorectal adenocarcinoma and IDA were randomised to either intravenous iron (ferric carboxymaltose) or control oral iron (ferrous sulphate). 116 patients were recruited but 87 had stored plasma for *H. pylori* and CagA ELISA assays. Enrolled patients were randomised independently to intravenous or group 1:1 by the trial team using a computer generated variable block randomisation programme, stratifying patients by age and sex. The system was run by Clinical Trials unit at the University of Nottingham. Enrolled patients attended an outpatient visit for blood tests and received iron treatment. Those randomised to oral therapy received ferrous sulphate 200mg twice a day for a minimum of two weeks. The intravenous group received one dose of 1000 mg ferric carboxymaltose (Ferinject, Vifor Pharma, Glattbrugg, Switzerland) diluted in 250 ml of 0.9% normal saline which was infused over 15 min under physician supervision 14 days prior to surgery. Blood tests were also taken at the time of surgery and at regular intervals for a total of 12 weeks. Demographic and clinical details were also recorded during study visits.

5.4.2 Laboratory Investigations

Blood samples were also collected for haematological and biochemical analysis by the local pathology department at Nottingham University Hospital. These tests included full blood count, B12, folate, serum ferritin, transferrin saturation, transferrin, serum iron and iron binding capacity.

5.4.3 Histopathology

Colorectal adenocarcinoma samples were examined by the Histopathology department at the University Hospital in Nottingham. Evaluation was carried out by a single blinded GI pathologist (Dr P Kaye).

5.4.4 ELISA assay

6ml of blood was collected in vacutainer non-additive serum tube (Becton Dickinson). The time blood was taken recorded and bottle inverted several times and sample left standing at room temperature for 1 hour then centrifuged at 200 x *g* for 10 minutes. The supernatant (serum) was transferred to three 1.5ml cryotubes and care was taken not to disturb the pellet and stored -80°C. 8mls of blood was collected in EDTA tube and centrifuged at 200 x *g* for 10 minutes at room temperature and 3 aliquots of plasma stored at -80°C

CagA IgG ELISA kit (Genesis) was followed according to manufacturer's instructions to determine *cagA* status from stored plasma. Optical densities were measured with a microplate reader at 450nm with the reference wavelength of 620nm. CagA IgG ELISAs were completed by Dr JR White (87 samples). The concentration of *H. pylori* IgG (Biohit) was determined using an ELISA kit according to manufacturer's instructions. *H. pylori* IgG ELISAs were completed by Dr JR White (49 samples) and Mr B Keeler (38 samples).

5.4.5 Statistics

Statistical analysis was carried out using Prism 6.0 (GraphPad Software 6 USA), unless otherwise stated horizontal lines within bars represented median values. Mann Whitney U tests were used for unpaired data and Wilcoxon signed rank test for paired. A statistical significant difference was considered if p values were ≤ 0.05. Correlations were examined using the Spearman's rank correlation test. All statistical analysis on the 87 patients was carried out by Dr J.R White.

5.5 Results

5.5.1 Demographics

116 were recruited but 87 patients were involved in the final analysis of stored serum. Patient demographic and clinical details are shown in Table 5.1

but were similar between all patient groups. 41 patients received oral ferrous sulphate and 46 received intravenous ferric carboxymaltose (Ferinject, Vifor Pharma, Glattbrugg, Switzerland). There were no immediate adverse events associated with intravenous therapy. The median duration of treatment for oral therapy/*H. pylori* infected and oral therapy/uninfected was 25 days. The median duration of treatment for intravenous therapy/*H. pylori* infected and intravenous therapy/uninfected was 21 days. The intravenous group had received one or two doses of intravenous iron pre-operatively.

Table 5.1 Demographics and anaemia parameters for patients randomised to intravenous or oral iron therapy according to *H. pylori* status

	Oral therapy and *H. pylori* positive	Oral therapy and *H. pylori* negative	Intravenous therapy and *H. pylori* positive	Intravenous therapy and *H. pylori* negative
Number of patients	12	29	15	31
Male: Female ratio	2:1	17:12	2:1	18:13
Previous iron therapy prior recruitment (%)	3 (25%)	17 (59%)	7 (46.7%)	15 (48%)
Median Age (range)	77 (36-85)	78 (46-90)	73.5 (51-86)	73.5 (49-86)
Smoker (%)	1 (8.3%)	5 (17%)	0	5 (16%)
Anticoagulant (%)	3 (25%)	4 (13.8%)	1 (7%)	7 (22.6%)
ASA grade mode (range)	2 (1-4)	2 (1-3)	2 (2-3)	3 (1-3)
Recruitment Hb (g/dL) males	10.65 (9.2-11.7)	9.9 (8.4-12.2)	10.8 (8.5-12.9)	10.85 (7.3-13)
Recruitment Hb (g/dL) females	9.7 (9.1-10.7)	10.9 (9.1-13.2)	9.5 (7.6-11.8)	10.4 (7.5-12.2)
Recruitment MCV	84 (67-94)	83 (70-100)	76 (70-88)	76 (67-109)
Recruitment Ferritin	19 (4-108)	26 (3-613)	13 (3-219)	21 (4-1231)
Recruitment Transferrin	2.95 (1.6-12)	2.9 (1.9-13)	3 (1.9-4.5)	3 (2-369)
Recruitment Serum Iron	4 (2-30)	5.5 (5.5-62)	4 (2-13)	5 (1-24)
Recruitment Transferrin Saturation	7.5 (2-45)	8.5 (3-94)	6 (2-47)	6 (2-27)
CagA Positive	7 (58%)	n/a	8 (53%)	n/a
Median Treatment days (range)	25 (11-56)	25 (12-62)	21 (14-61)	21 (11-57)
Tumour stage T4	2	8	5	4
Tumour stage T3	6	19	7	22
Tumour stage T2	2	0	0	2
Tumour stage T1	0	0	1	2
Tumour stage T0	0	0	2	0
Nodal status N2	0	1	3	3
Nodal status N1	4	8	0	6
Nodal status N0	6	18	12	21
Metastasis status M0	9	26	15	30
Metastasis status M1	1	1	0	0
Operated (%)	10 (83%)	27 (93%)	15 (100%)	30 (97%)
Chemotherapy (%)	4 (33%)	4 (14%)	2 (13%)	9 (29%)

Demographics and anaemia parameters for patients randomised to intravenous or oral iron therapy. Values expressed as median with range or percentages.

No patients required blood cell transfusions prior to surgery. Five patients in the oral therapy group received intraoperative blood transfusions compared to six in the intravenous group. Intravenous group required fewer transfusions after surgery and required less intravenous iron on discharge. Twelve patients in the oral therapy group received blood transfusion up to 7 days post op compared to seven in the intravenous group. Intravenous iron was prescribed post discharge to thirteen of the oral group compared to one in the intravenous group. There was no relation to *H. pylori* status and transfusion or further iron requirement.

5.5.2 Anaemia parameters

There was no statistically significant difference in anaemia parameters between *H. pylori* infected and uninfected at recruitment (Table 5.2). There was no statistically significant difference in anaemia parameters between groups when stratified by therapy and *H. pylori* status (Table 5.3). There was a significant negative correlation between tumour size and recruitment haemoglobin level (r= -0.3 p=0.008) see Figure 5.1. In all cohorts there was a statistically significant increase in haemoglobin post treatment (Figure 5.2). There was a significant increase in the percentage change in haemoglobin with intravenous iron therapy when compared to oral therapy (median 13.6 vs 5.3%, p=0.0003), but no statistically significant change when stratified by *H. pylori* status or mode of iron therapy (Figure 5.3). There was a significant increase in serum iron levels (p=0.003 oral therapy, p=0.0001 intravenous therapy) after treatment regardless of *H. pylori* status (Figure 5.4). There was a significant increase in the percentage change in serum iron with intravenous iron therapy when compared to oral (median 100% vs 33%, p< 0.0001), but no statistically significant change in the percentage change in serum iron when stratified by *H. pylori* status or mode of iron therapy (Figure 5.5).

Table 5.2 Total anaemia parameters of the *H. pylori* infected and uninfected with colorectal cancer pre treatment

Total pre-treatment Haemoglobin, MCV, Ferritin, serum iron, transferrin and transferrin saturation. Values expressed as median with ranges. Mann Whitney U test was carried out for statistical significance. No significant difference in anaemia parameters prior to iron therapy in infected and uninfected patients with colorectal cancer. NS=non-significant.

	***H. pylori* positive**	***H. pylori* negative**	**P value**
Haemglobin g/dl	10.6 (7.6-12.9)	10.45 (7.3-13.2)	ns
MCV fl	82 (67-94)	81.5 (67-109)	ns
Ferritin µg/L	15 (3-219)	22.5 (3-1231)	ns
Serum iron µmol/L	4 (2-30)	5 (1-62)	ns
Transferrin g/L	3 (1.9-12)	2.9 (369)	ns
Transferrin saturations %	3 (1.6-12)	2.9 (1.9-13)	ns

Table 5.3 Anaemia parameters of the oral and intravenous therapy groups stratified by *H. pylori* status

Haemoglobin, MCV, Ferritin, serum iron and transferrin pre-treatment. Values expressed as median with ranges. Mann Whitney U test was carried out for statistical significance. There was no significant difference in haemoglobin, MCV, ferritin, serum iron and transferrin levels in *H. pylori* infected when compared to uninfected. NS=non-significant.

	Oral therapy and *H. pylori* positive	**Oral therapy and *H. pylori* negative**	**P value**	**Intravenous therapy and *H. pylori* positive**	**Intravenous therapy and *H. pylori* negative**	**P value**
Haemglobin g/dl	10.3 (9.1-11.7)	10.5 (8.4-11.9)	ns	10.7 (7.6-12.9)	10.4 (7.3-12.2)	ns
MCV fl	84 (67-94)	83 (70-100)	ns	76 (70-88)	76 (67-109)	ns
Ferritin µg/L	19 (4-108)	26 (3-613)	ns	13 (3-219)	21 (4-1231)	ns
Serum iron µmol/L	4 (2-30)	5.5 (2-62)	ns	4 (2-12)	5 (1-24)	ns
Transferrin g/L	2.95 (1.9-12)	2.9 (1.9-13)	ns	3 (1.9-4.5)	3 (2-369)	ns

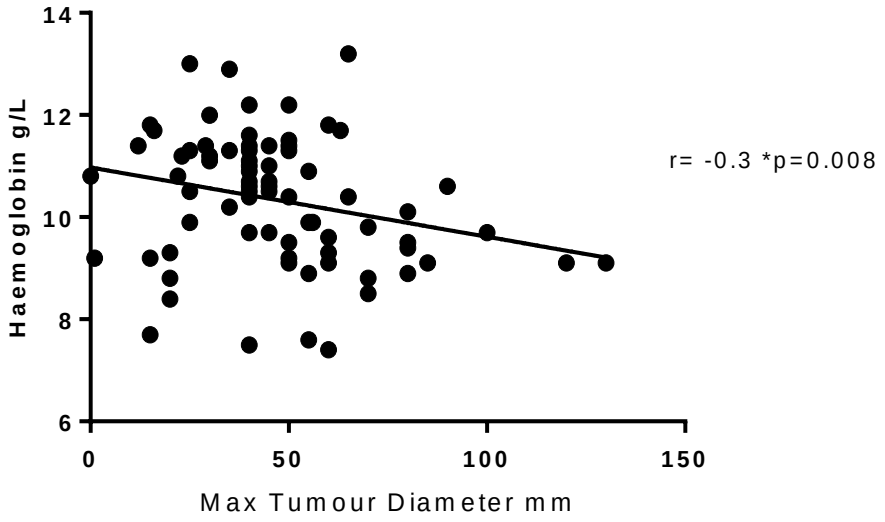


Figure 5.1 Correlation between tumour size and initial haemoglobin levels

Tumour size in mm was plotted against recruitment haemoglobin levels and a scattered graph plotted showing a weak inverse correlation. Correlations were examined using the Spearman's rank correlation test.

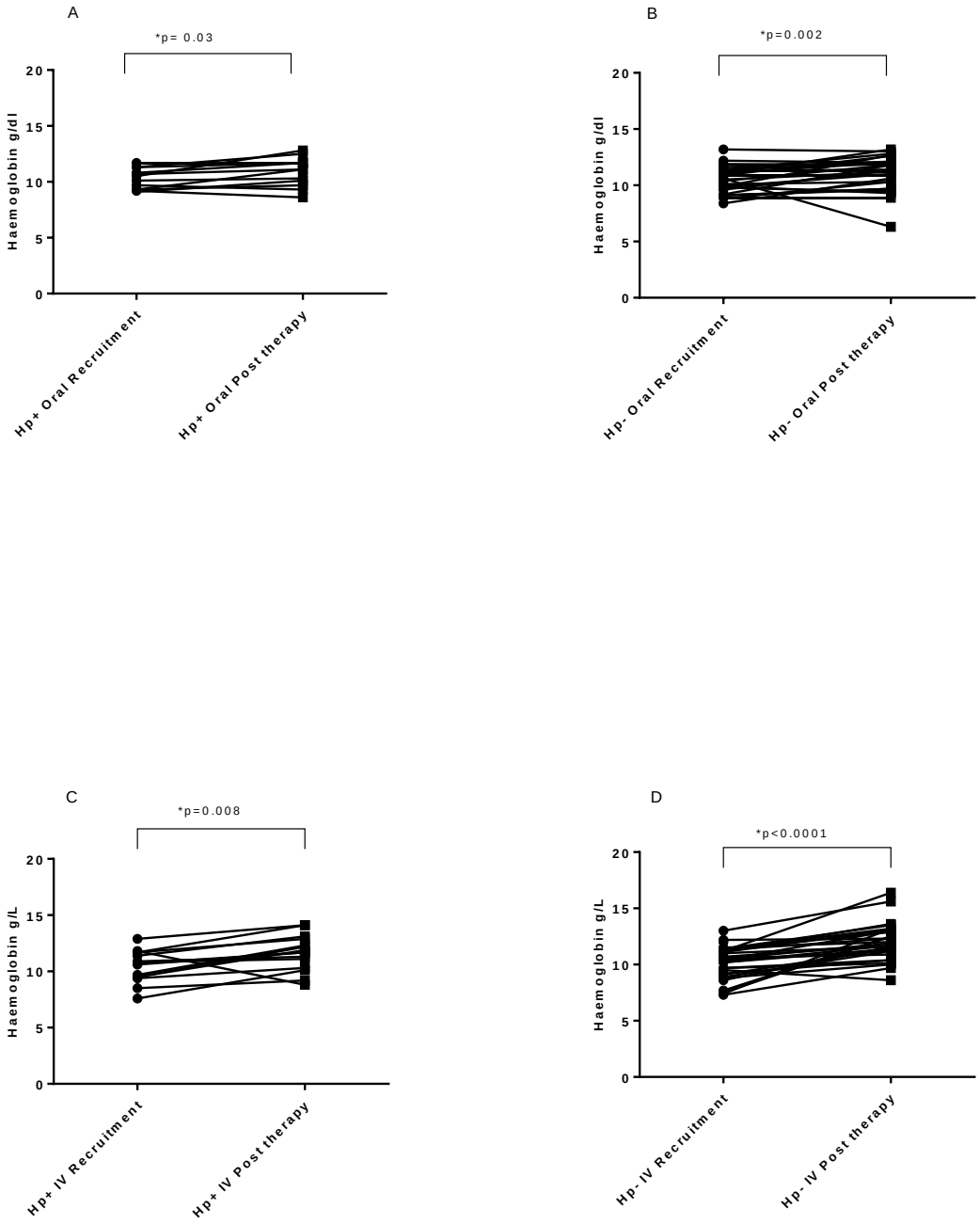


Figure 5.2 Haemoglobin levels before and after iron therapy

Haemoglobin levels before and after therapy in *H. pylori* infected oral group (A), *H. pylori* uninfected oral group (B), *H. pylori* infected intravenous group (C) and *H. pylori* uninfected intravenous group (D). Wilcoxon test was used to test for statistical significance.

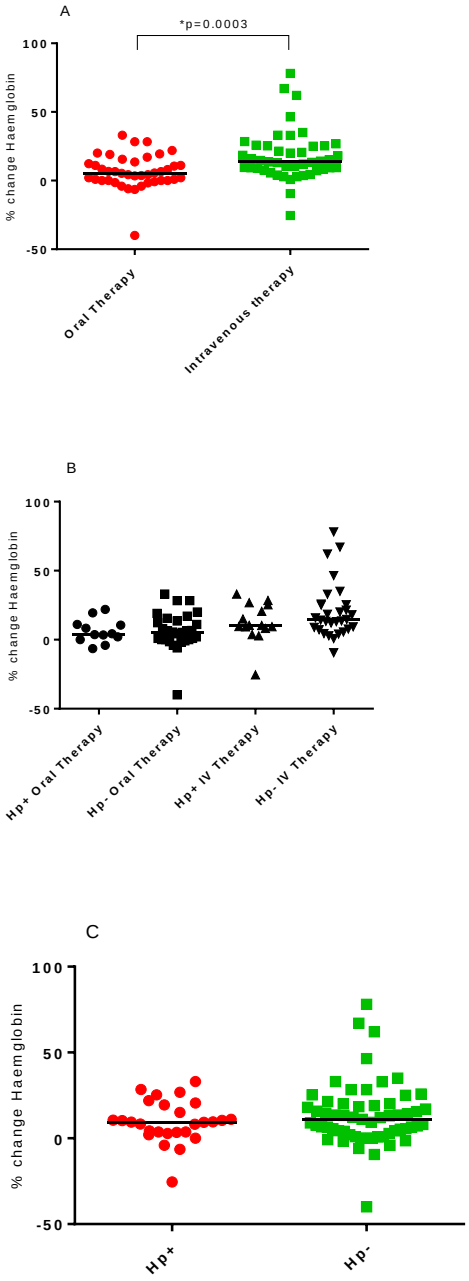


Figure 5.3 Percentage change in haemoglobin of the oral and intravenous therapy groups

Haemoglobin levels at recruitment were subtracted from levels prior to surgery and multiplied by 100 to calculate % change in haemoglobin. Percentage change in haemoglobin when stratified by oral and intravenous therapy (A). Percentage change in haemoglobin when stratified by oral and intravenous therapy and *H. pylori* status (B). Percentage change haemoglobin when compared *H. pylori* infected patients given a combination of oral and intravenous therapy with *H. pylori* uninfected given the same combination. Horizontal line represents medians, Mann Whitney U test was carried out for statistical significance.

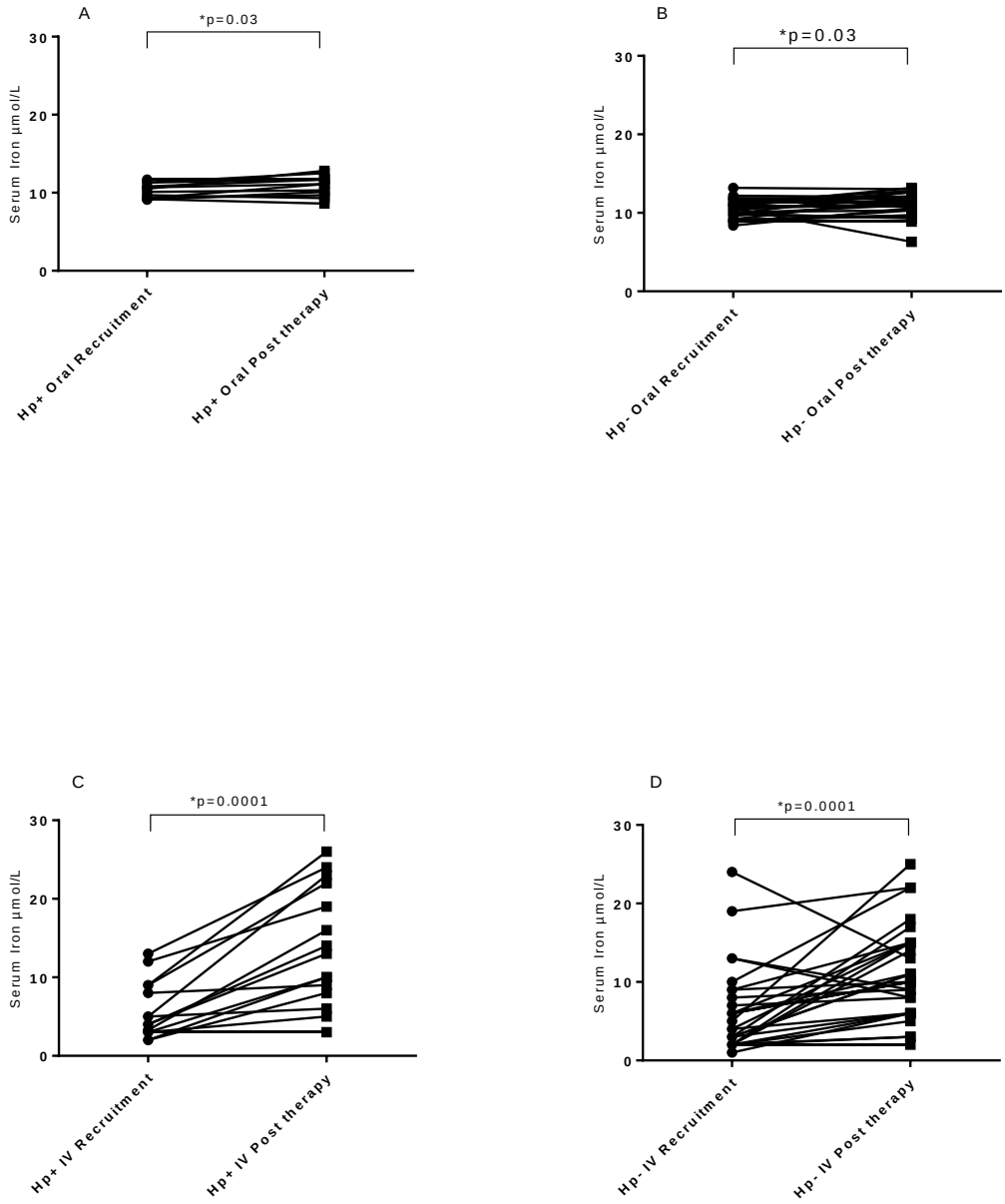


Figure 5.4 Serum iron before and after treatment of the oral and intravenous iron therapy groups

Serum iron levels before and after therapy in *H. pylori* infected oral group (A), *H. pylori* uninfected oral group (B), *H. pylori* infected intravenous group (C) and *H. pylori* uninfected intravenous group (D). Wilcoxon test was used to test for statistical significance.

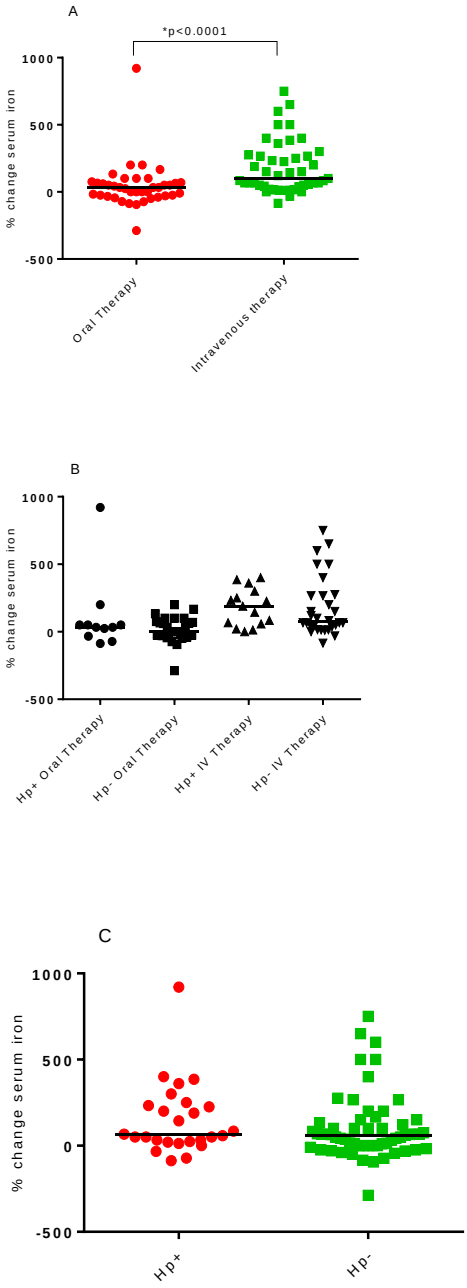


Figure 5.5 Percentage change in serum iron of the oral and intravenous therapy groups

Serum iron levels at recruitment were subtracted from levels prior to surgery and multiplied by 100 to calculate % change in iron. Percentage change in serum iron for the oral and intravenous therapy group (A). Percentage change serum iron when stratified oral and intravenous therapy by *H. pylori* infection status (B). Percentage change serum iron when compared *H. pylori* infected patients given a combination of oral and intravenous therapy with *H. pylori* uninfected given the same combination (C). Horizontal line represents medians, Mann Whitney U test was carried out for statistical significance.

There was a significant increase in MCV (p=0.0008) and decrease in transferrin (p=0.0007) but no change in ferritin and transferrin saturation in oral group including both *H. pylori* infected and uninfected patients (Figure 5.6). In the intravenous group including both *H. pylori* infected and uninfected there was a significant increase in MCV (p<0.0001), ferritin (p<0.001), transferrin saturation (p<0.0001) and decrease in transferrin (p<0.0001) (Figure 5.7). There was a significant greater percentage change in MCV (5.7% vs 2.3% p=0.0007), ferritin (2900% vs 18% p<0.0001) and transferrin saturation (220% vs 25% p<0.0001) and a lower percentage change in transferrin (-30% vs -9.2% p<0.0001) after treatment in the intravenous group. There was no significant change in these anaemia parameters when stratified by *H. pylori* status (Figure 5.8).

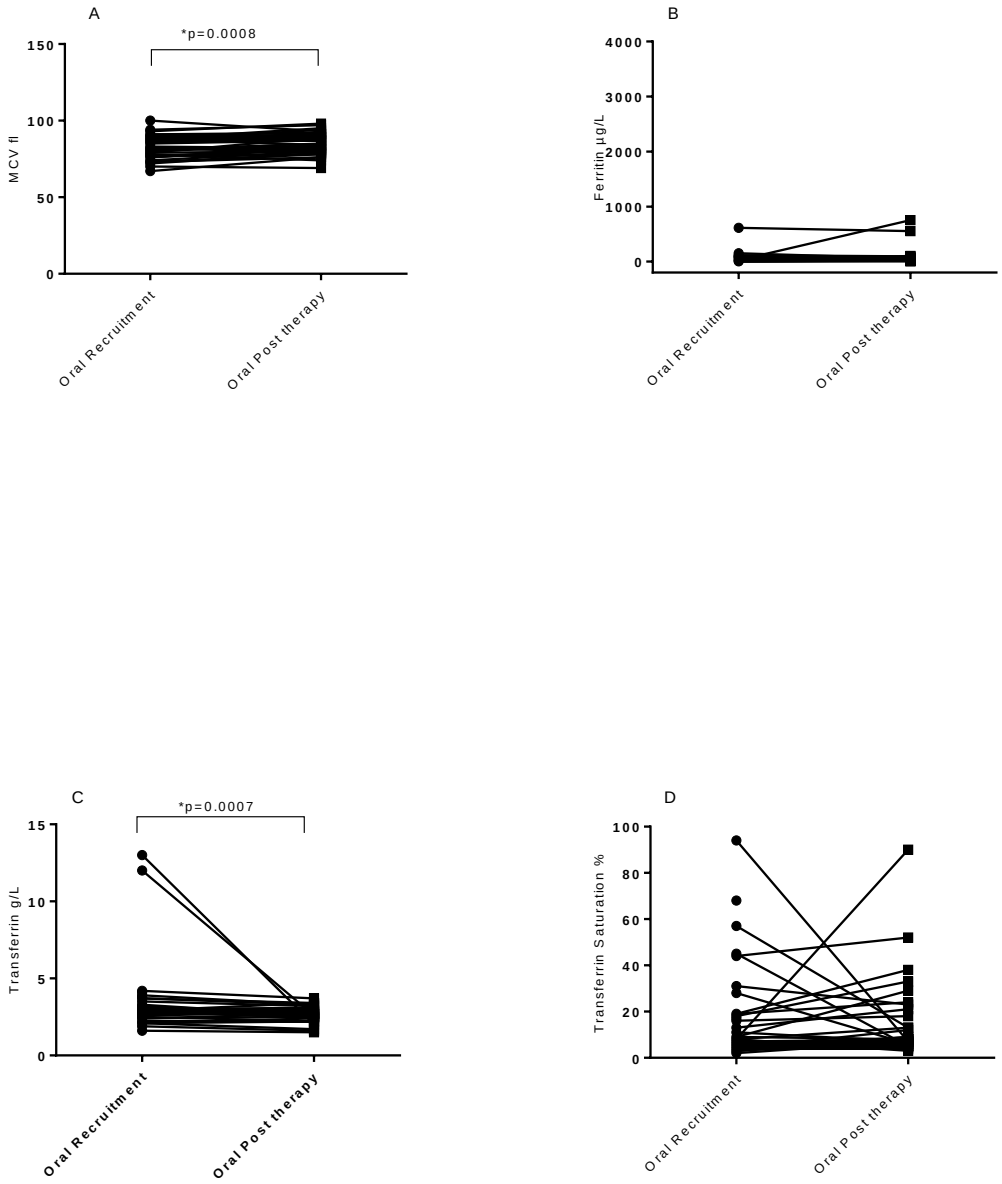


Figure 5.6 MCV, ferritin, transferrin and transferrin saturation before and after oral iron treatment

Anaemia parameters before and after therapy in *H. pylori* infected and uninfected oral iron therapy group. MCV before and after oral treatment (A), Ferritin before and after treatment (B) transferrin before and after oral treatment and transferrin saturation before and after oral treatment (D). Wilcoxon test was used to test for statistical significance.

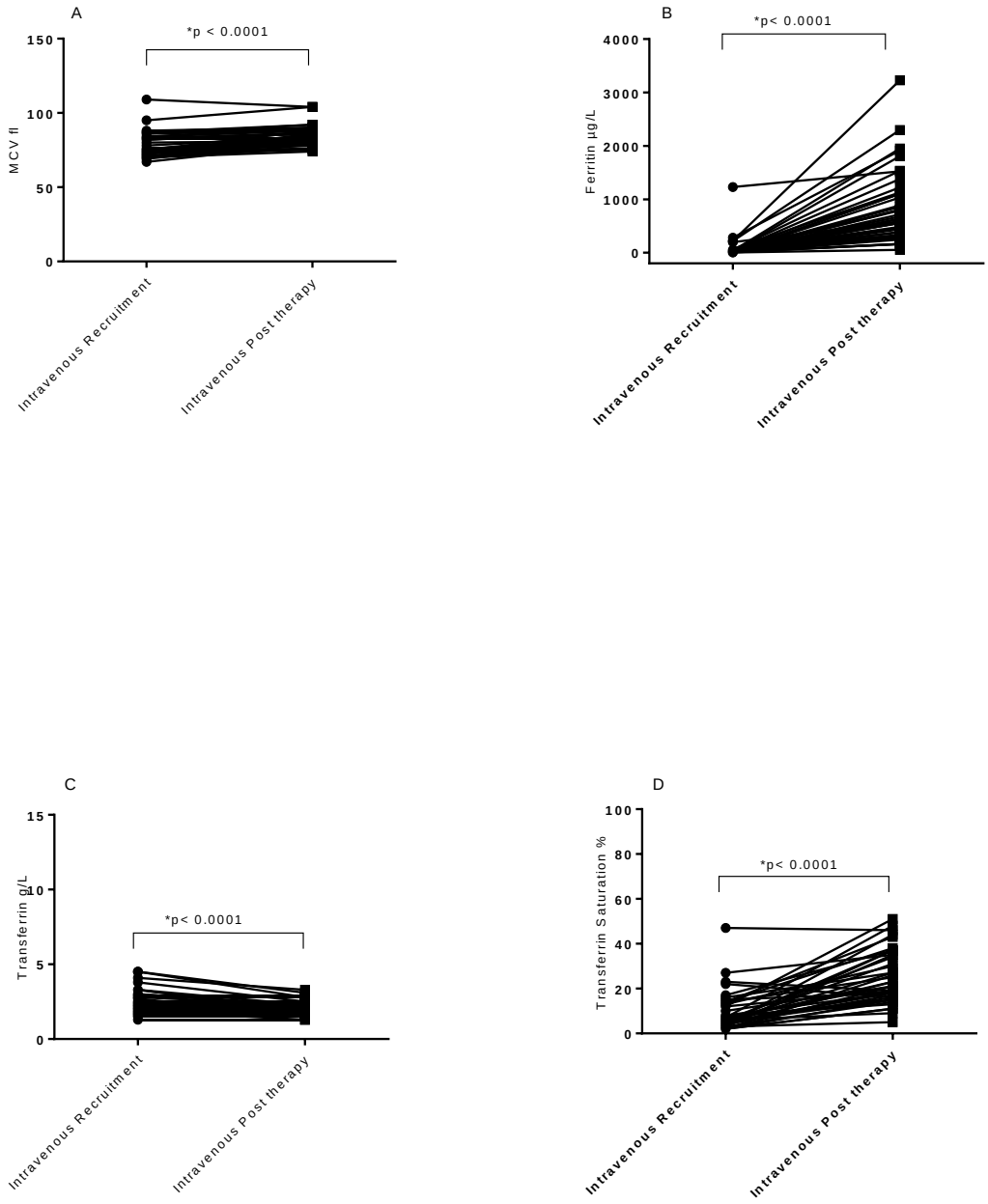


Figure 5.7 MCV, ferritin, transferrin and transferrin saturation before and after intravenous treatment

Anaemia parameters before and after therapy in *H. pylori* infected and uninfected intravenous iron therapy group. MCV before and after intravenous treatment (A), Ferritin before and after intravenous treatment (B) transferrin before and after intravenous treatment and transferrin saturation before and after intravenous treatment (D). Wilcoxon test was used to test for statistical significance.

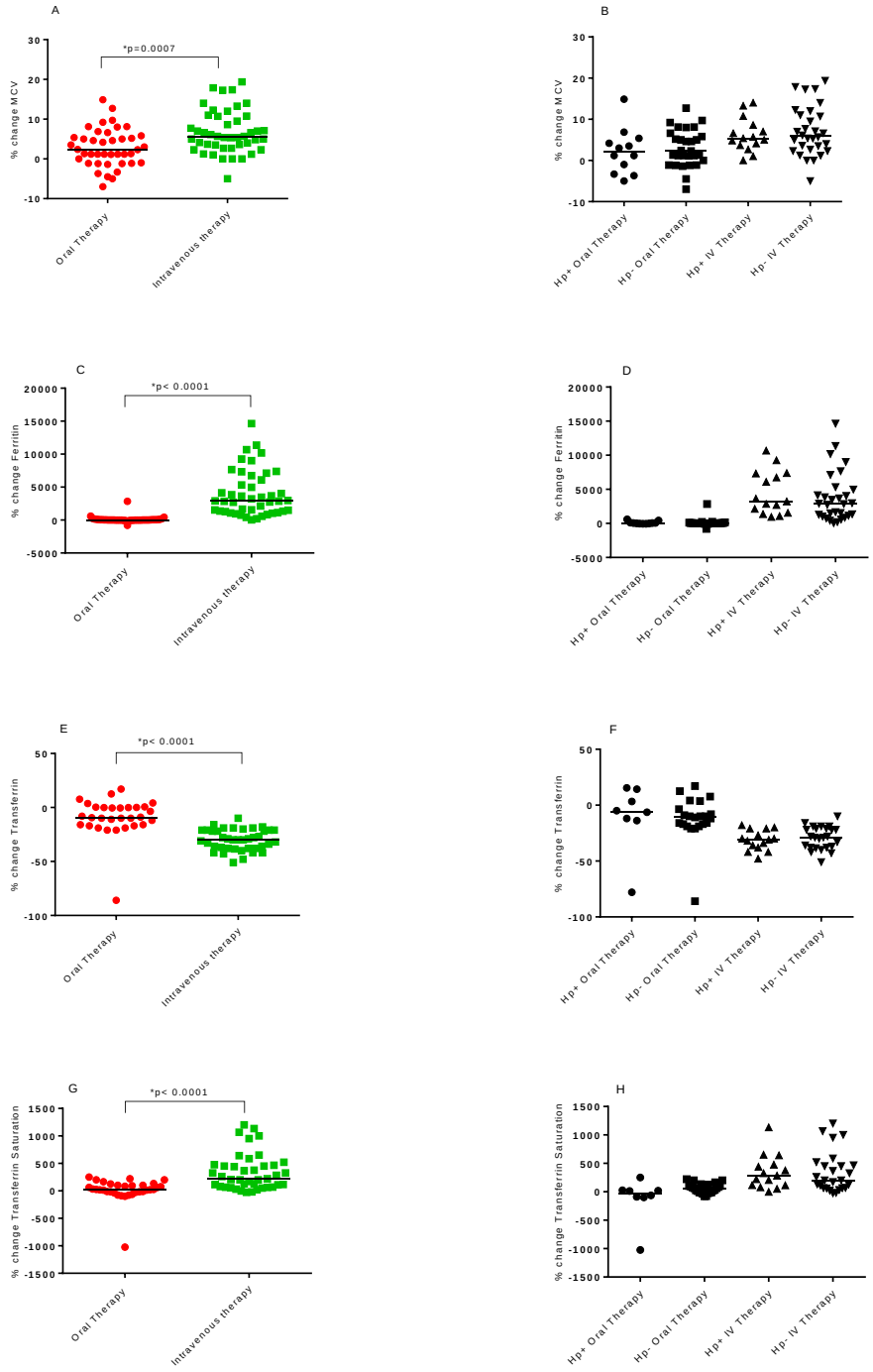


Figure 5.8 Percentage change in MCV, ferritin, transferrin and transferrin saturation

Anaemia parameter levels at recruitment were subtracted from levels prior to surgery and multiplied by 100 to calculate % change in MCV (A), ferritin (C), transferrin (E) and transferrin saturation (G). Anaemia parameters when stratified by *H. pylori* status (B), (D), (F) and (H). Horizontal lines represent medians, Mann Whitney U test was carried out for statistical significance.

H. pylori infection did not influence response to iron therapy in this cohort of colorectal patients. Intravenous therapy did achieve a greater increase in anaemia parameters but any effects of *H. pylori* are likely to be negligible in cases of colorectal cancer.

5.5 Discussion

Blood transfusion requirements were reduced in the intravenous group but were not statistically significant. Intravenous therapy led a greater improvement in iron indices. *H. pylori* and *cagA* status did not influence response to therapy.

Intravenous therapy did not reduce allogenic blood transfusion requirement during surgery (Nielsen, 1995). Blood transfusions during major surgery increase the risk of infectious complications, recurrence of malignancy and death (Chung et al., 1993). During major surgery, there is alteration in immune function leading to immunosuppression; the use of blood transfusion can also lead to post-operative immune impairment and increasing the infection risk further. Other independent factors that contribute to post-operative complications such as infection by immunosuppression include environmental factors such as alcohol consumption or malignant disease (Nielsen, 1995). Although non-metastatic tumours were included in the study, the TNM tumour stage was variable between groups, so the non-reduction seen in this small study could be due to other factors such as tumour which is independent to blood transfusion.

The results from this small study show that both oral and intravenous therapy improves anaemia parameters which are consistent with previous studies (Lidder et al., 2007, Quinn et al., 2010, Okonko et al., 2008). Intravenous iron is more effective than oral therapy in improving iron indices in iron deficiency associated with non-metastatic colorectal cancer which is similar to the previous studies although pregnant and not colorectal cancer patients were investigated (Kochhar et al., 2013, Kim et al., 2009). Our intravenous group required fewer blood transfusions and iron replacement therapy during the

follow up period post-surgery but the evidence in the literature is still inconclusive. Ng *et al* summarised the evidence in their Cochrane review of randomised controlled trials for the use of both oral and intravenous iron in pre-operative anaemia. The three trials studied did not show a statistically significant reduction in the use of blood transfusions. The sample size was small so a reliable conclusion cannot be drawn. Intravenous iron was more effective than oral in increasing haemoglobin level which is similar to this study (Ng et al., 2015). Although this study adds to the literature, larger well designed studies are still needed.

The larger the tumour the more pronounced the anaemia which highlights the importance of measuring anaemia parameters. These data shows a negative correlation between tumour size and recruitment haemoglobin level (r= -0.3 p=0.008) which is similar to a recently published study (Keeler et al., 2016).

H. pylori infection as predicted did not influence outcome in intravenous group. There was a slight improvement in the change in haemoglobin in the *H. pylori* negative group but this was statistically insignificant. There was no correlation between *H. pylori* status and response to iron replacement therapy. It was expected that *H. pylori* infected group would have had a poorer response to oral iron therapy because the presence of the infection would lead to reduced absorption. As the median age ranged from 73.5 to 78 years we would expect a higher incidence of gastric atrophy in this older age group. This is due to hypochlorhydria that is sometimes associated with gastric atrophy which leads to reduced iron absorption (Schade et al., 1968). However, a potential explanation for our results was that *H pylori* status was determined by serology but a positive test does not distinguish between current or previous eradicated infection. Using histology, rapid urea test or urea breath test to diagnose current infection would have helped to stratify patients better. Obtaining a more reliable diagnosis would have led to delay in definitive treatment for colorectal cancer because exposure to antibiotics or stopping PPI therapy would be required for minimum of two weeks. So the use of serology from stored plasma was the most ethical way to proceed.

Gastric atrophy is also associated with IDA (Kaye et al., 2008) but we could not perform serology testing on the stored plasma because patients were not fasted prior to donating the blood sample. In retrospect *H. pylori* infection diagnosis by serology is insufficient to predict those individuals who might respond less well to oral therapy.

Strengths

There are only a few randomised controlled trials investigating response iron therapy in colorectal cancer patients so this study will add to the literature. A large number of patients were recruited with 94% completing the study but unfortunately only 75% of stored serum was available. This study helps to determine the short term benefits of iron replacement therapy.

Limitations

The addition of more reliable tests for current *H. pylori* infection or atrophy would help better stratification of patients and allow for more significant conclusions to be made. The maximum follow up was 12 weeks but having a 12 month follow would help determine long-term benefits of iron treatment. Oral therapy data could be biased because compliance due to intolerance with oral treatment is often higher but this was not recorded. Lower anaemia parameters could be due to poor compliance and not necessary the route of iron delivery. Although colorectal cancer was the likely cause of the profound anaemia not all patients had complete examinations for alternative causes. These alternative causes could influence response rate. Costs associated with treatment and quality of life outcomes would help determine other important results associated with mode of therapy. Transfusion rates were lower than expected thus increasing the chances of type II error. The duration of pre-operative iron therapy was also short and may not have allowed for adequate benefits of oral therapy (Keeler et al., 2017).

Planned future work

To seek ethical approval to assess archived gastric histology samples to allow for assessment of *H. pylori* infection and atrophy grades. This would allow for a better stratification and assess for associations. With stored plasma Mr Acheson's team are planning on testing serum hepcidin before and after treatment as colorectal cancer induces hepcidin production and levels are related to malignancy stage (Ward et al., 2008).

5.6 Conclusions

- Blood cell transfusion requirements are reduced in the intravenous group but the results were not statistically significant.
- Both oral and intravenous therapy improves anaemia indices regardless of *H. pylori* infection status.
- Intravenous therapy led to a larger improvement in iron indices when compared oral therapy.
- *H. pylori* infection does not influence the mode of iron treatment.
- *H. pylori* infection and cagA status do not influence response rates to iron therapy.
- *H. pylori* status as assessed by serology cannot be used as a serological predictor of response to iron therapy.

Chapter 6: Investigating how *H. pylori* eradication treatment affects the host immune response

6.1 Introduction

6.1.1 The immune response to *H. pylori*

Inflammation caused by *H. pylori* promotes an environment for disease and the inadequate host's immune response allows the bacteria to persist throughout life. There are several mechanisms by which *H. pylori* could interact with the immune system of their host. The close interaction between host cell surface and bacteria could initiate immune response, in addition to virulence factors allowing passage through the cell surface and immune induction via Payer's patches (Kim and Blanke, 2012, Nagai et al., 2007). *H. pylori* has evolved to cause both local and systemic effects on the immune response and these are important in the bacteria's pathogenesis. Anti-inflammatory cells and anti-bacterial defences are stimulated with *H. pylori* colonisation via bacterial recognising molecules. The innate immune system is engaged by pattern recognition receptors (PRRs), such as NOD-1. This is stimulated by *cag+* strains and recognises soluble components of the bacterial cell wall (Nurnberger et al., 2004, Robinson et al., 2007). However, the different research approaches used in studies to investigate the role of PRRs in gastritis have produced contradictory results (Robinson et al., 2007). The expression of cytokines and chemokines due to *H. pylori* attracts neutrophils, dendritic cells, macrophages, natural killer cells and lymphocytes which release reactive oxygen and nitrogen species (Robinson et al., 2007, Sundquist and Quiding-Jarbrink, 2010). The eventual development of gastric adenocarcinoma is associated with these oxygen and nitrogen reactive species producing DNA damage or inhibiting tumour suppresser genes by a variety of pathways. The immune response is variable and this in turn would determine the risk of disease that subsequently develops (Na and Woo, 2014, Macarthur et al., 2004).

Another line of innate defence against bacterial colonisation is the production of peptides which possess anti-microbial properties. Human β-defensin-2 (hBD2), neutrophil derived alpha defensins (HNP 1-3) and other related

peptides are found in abundance in infected individuals' gastric fluid compared with that from uninfected people and levels decrease after *H. pylori* eradication therapy (Isomoto et al., 2004, George et al., 2003, Robinson et al., 2007).

A protective mucus layer consisting of a cell associated layer (MUC1) and the secreted mucin (MUCFAC) covers the gastric epithelial surface which is important for the interaction with *H. pylori (*Dunne *et al., 2014)*. *H. pylori* adapts the mucin layer to allow its movement, in addition to acting as the host 'natural antibiotic' with the binding of *H. pylori* to MUC1 affecting the host inflammatory response (Bansil et al., 2013, Fukuda et al., 2006, Park et al., 2012).

H. pylori interactions with gastric epithelial cells results in the secretion of pro-inflammatory cytokines and chemokines. These factors induce the migration of immune cells such as granulocytes to the inflamed mucosa. The level of inflammation is increased and further pro-inflammatory molecules are released because of this. IgA and IgG antibody production is also stimulated by *H. pylori* but the exact role this plays is unknown since immunization of antibody deficient mice resulted in a reduced colonization density. Importantly, *H. pylori* has mechanisms (although exactly how is not entirely clear) to evade being engulfed and destroyed by phagocytes. This is the mechanism in clearing other bacterial infections (Robinson et al., 2007).

6.1.2 Innate immunity

CagPAI interaction with the gastric mucosa enables the transfer of peptidoglycans into cytoplasm, NOD1 activation and the expression of pro-inflammatory genes such as IL-1β, IL-18 tumour necrosis factor alpha (TNFα), IL-6, IL-12, CCL2-5, CCL20, and CXCL1-3 (Viala et al., 2004, Vanaja et al., 2015, Peek et al., 1995, Cook et al., 2014). Polymorphisms which leads to increased pro-inflammatory IL-6, IL-8, TNFα and IL-1β expression or reduced IL-10 (anti-inflammatory) are usually associated with high disease risk (Ramis et al., 2015, El-Omar et al., 2003). Chemokine abundance leads to a collection of immune

cells. The neutrophils associated with these immune cells cause gastritis by releasing cytokines and tissue damaging factors. Further DNA damage occurs when the bacteria undergoes phagocytosis (Allen et al., 2005). *H. pylori* infected mucosa is abundant with macrophages, M1 which secrete pro-inflammatory cytokines and have bactericidal properties and M2 which aid cell growth and repair. *H. pylori* is able to evade phagocytosis inducing macrophages to form megasomes, which are fusions of phagosomes. These phagosomes provide a protective intracellular niche and allow the infection to persist (Italiani and Boraschi, 2014, Schwartz and Allen, 2006, Allen, 2001). *H. pylori* is able to counteract the effects of reactive oxidative species and nitric oxide but the prolonged exposure of host to these aid cancer development. The pathway for breaking down damaged cells aids regulation of inflammatory signalling. Any abnormality in the autophagy regulation leads to DNA damage and the production of reactive oxygen species which can also eventually lead to cancer formation (Joven et al., 2014).

Mast cells are likely to be involved in inflammation, tissue repair and clearance of *H. pylori* and these have been shown to be present in higher numbers in the gastric mucosa of infected individuals (Bamba et al., 2002). Dendritic cells are important for the adaptive immunity, in *H. pylori* infection dendritic cells produce less retinoic acid (inflammation regulator) which in turn leads to more inflammation increasing gastric cancer and peptic ulcer risk (Bimczok et al., 2015). Although not fully investigated in the context of *H. pylori* infection, natural killer cells may lead to damage to hosts cells; increased numbers are found in blood and gastric mucosa of infected individuals (O'Keeffe et al., 2008, Rudnicka et al., 2013).

6.1.3 Adaptive immunity

6.1.3.1 Pro-inflammatory response

H. pylori provoke an antibody response (IgA and IgG) which react with parietal cell antigens of the gastric mucosa which could stimulate an autoimmune response leading to increased inflammation. Evidence for this comes from the

presence of autoreactive antibodies in the serum of *H. pylori* infected patients (Wilson and Crabtree, 2007, Smyk et al., 2014, D'Elios et al., 2004). Inflammation and disease seen in *H. pylori* infected individuals are induced by a strong CD4+/CD8+ T lymphocyte response (Figueiredo Soares et al., 2007). *H. pylori* induces main two forms of T helper responses, pro-inflammatory Th1 and Th17 and regulatory T lymphocyte (Tregs) which are anti-inflammatory. The cytokines produced by these responses influence disease outcomes (Robinson et al., 2008, Serrano et al., 2013). Both Th-1 and Th-17 responses have been demonstrated to be important in mediating host immunity to *H. pylori*. The literature is conflicting on exactly which is dominant (Muller and Solnick, 2011). The release of these inflammatory cytokines leads to both acute and chronic inflammation. Th1 and Th17 secrete cytokines which lead to inflammation and in turn leads to further enrolment of neutrophil, lymphocytes, macrophages and other cells involved in pro-inflammatory cytokines release. IFNγ and TNFα are secreted by Th1 lymphocytes for bactericidal properties and production of pro-inflammatory factors. Th-17 can lead to reactive species, chemokines and antimicrobial peptides by IL-17A, IL-17F, IL-21, and IL-22 production (Popova et al., 2011, Wilke et al., 2011). Th2 and Th22 have also been implicated in the pro-inflammatory response (Zhuang et al., 2015, Robinson et al., 2008). The main function of Th-2 cells is to promote an antibody dependent response. These produce cytokines of a less pro-inflammatory nature therefore a biased Th2 response leads to increased *H. pylori* bacterial density. This response controls the pathology but is less effective in clearing the bacteria (Robinson et al., 2007).

Th1 lymphocyte response could lead to cancer formation but conflicting evidence shows a high Th1 response leads to stronger anti-tumour immunity and better gastric cancer prognosis but high Th17 and Th22 could be involved in tumour angiogenesis and invasiveness leading to a poorer cancer prognosis (Chang et al., 2014, Liu et al., 2012). The Th-1 cell mediated response to *H. pylori* has many implications in leading to disease states and is normally the dominant response. But once gastric malignancy has developed the host

immune response can be directed towards a Th-2 response which can in turn create a reduction in anti-tumour response of certain T cells (Gallimore and Godkin, 2008). IFNγ levels are higher in *H. pylori* gastritis which causes a greater degree of inflammation but lower colonisation (Mohammadi et al., 1997) but IFNγ deficient mice have a reduction in inflammation (Obonyo et al., 2002).

6.1.3.2 Regulatory T lymphocyte (Tregs) response

Tregs are an immunosuppressive type of T lymphocyte which steered immune the system away from a pro-inflammatory response (Kao et al., 2010). There are many different subtypes (Table 2) and some are auto-reactive and essential to prevent autoimmune diseases. Others play a central role to dampen down inflammation. Some are $CD8^+$ but the vast majority (90%) are $CD4^+$. Natural Tregs include $CD4^+$, $CD25^+$, FoxP3, FoxP3 promoter Treg-specific demethylated region (TSDR). These are created by the thymus and are specific for self-antigens. They mediate their actions via CTLA-4 or non-specific antigens. $CD25^{hi}$ $CD8^+$, $CD25^-CD4^+$, $CD4^-CD8^-$ Tregs express an elevated level, low level and zero level respectively of FoxP3 (Khattri et al., 2003). Treg responses are induced by some pathogens as a means to evade immunity and to avoid clearance. Inducible Tregs include the Tr1 subset which secretes IL-10, Th3 which secretes TGFβ and possibly Th9 which secretes IL-9, IL-10 and TGFβ. These inducible Tregs are generated in the periphery and act on non-self-antigens. They are important for preventing inflammatory diseases. Persistence of the colonisation with *H. pylori* is believed to be possible by the organism heightening Treg responses (Robinson et al., 2007). Tregs can be considered beneficial or detrimental depending on the clinical situation. There is no one reliable marker for Treg function: the majority can be expressed by other subsets of T lymphocytes, and this all complicates matters and makes studies difficult to design and interpret.

Table 6.1: Treg populations

Subset	Phenotype	Origin	Action
Regulatory T cells	$CD4^+$, $CD25^+$, $FOXP3^+$, T helper 1 & 2 cytokines	Thymus/Possibly periphery	Cell to cell contact, secretion TGF-β, IL10
IL10 secreting regulatory T cells	$CD4^+$, secrete IL10 but not T helper 1 & 2 cytokines	Periphery	IL10 secretion
T helper 1	$CD4^+$, IFN-γ	Periphery	Secretion IFN-γ, IL10
T helper 2	$CD4^+$,IL4, IL5, IL13	Periphery	Secretion IL4/IL10
T helper 3	$CD4^+$, TGF-β	Periphery	Secretion TGF-β

Table shows Treg population, phenotype, origin and action. Table adapted from *(Hawrylowicz and O'Garra, 2005).*

Table 6.1 shows the different Treg populations and their distinguishing markers. Others not included in Table 6.2 include neuropilin (Nrp1-), toll like receptor 4 (TLR4), LAG3, and Helios. Other subsets include Th-17 regulatory cells, and $CD4^+$ cells which secrete both IL-17 and TGFβ (Hawrylowicz and O'Garra, 2005). Another subset is Th-9 cells with phenotype $CD4^+$, secreting IL-9 and IL-10. Th-9 originates in the periphery and acts on IL-10 (Robinson, 2012).

CD25 is a transmembrane protein present in T cells, B cells and other lymphocyte cells. This has being used as a marker to identify CD4+ *FOXP3*+ Tregs. Most mice Tregs are $CD4^+CD25^+$, but in humans Tregs are $CD25^{hi}$, approximately 30% of $CD4^+$ T cells are $CD25^+$. Most are $CD25^{int}$ and under 3% $CD25^{hi}$. As not all Tregs are $CD25^{hi}$ and CD25 is present on other cells it cannot be used as an accurate marker (Robinson, 2012). $CD25^{hi}$ represents a high level of expression (Figure 6.1).

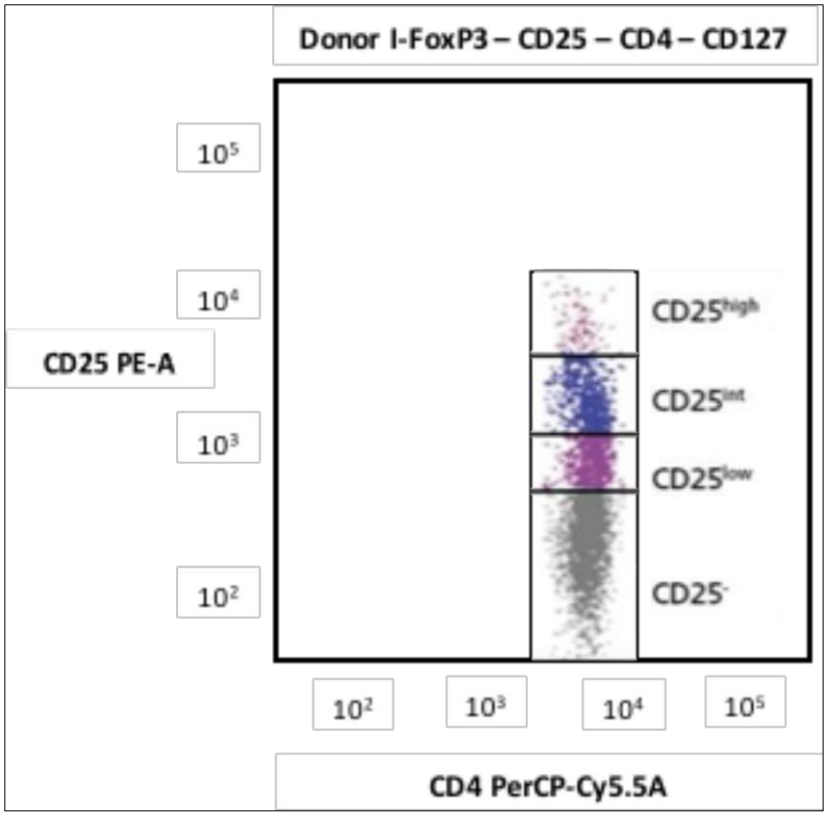


Figure 6.1 Enrichment of Tregs with CD4, CD25, and CD127

PBMCs were stained with anti-human CD4, CD25, CD127 and FoxP3. Lymphocytes were analysed using flow cytometry by forward and side scatter and then gated with CD4. Figure adapted from (Bdbiosciences.com, 2017).

6.1.4 Forkhead box P3 (*FoxP3)*

Mice lacking FoxP3 due to deletion have fatal autoimmune diseases and their $CD4^+$ response is greater (Fontenot et al., 2003). Not all human Tregs express FoxP3, in contrast to mice. This therefore cannot be used as a reliable marker in humans for Treg activity. Humans with defective FoxP3 genes suffer from fatal autoimmune disorders such as immune dysregulation, polyendocrinopathy, enteropathy X linked syndrome (IPEX syndrome) (Gambineri et al., 2003). The properties of FoxP3 are not yet fully established, but it is known to down regulate the expression of Treg genes by binding to specific promoter regions. Its activity is influenced by oestrogens and TGFβ (Robinson, 2012). $CD4^+$ and $CD25^{hi}$ expressing FoxP3 cells, IL-10 and TGFβ are higher in subjects infected with *H. pylori* when compared to those not infected (Lundgren et al., 2005, Lindholm et al., 1998, Lundgren et al., 2003).

There is conflicting evidence on whether Tregs reduce inflammation scores (Robinson et al., 2007).

Tregs which express FoxP3 transcription factor are mostly $CD4^+$ T cells that also express CD25 and affect the function of $CD4^+$, CD8 T cells, NK cells, B cells and antigen presenting cells (Sakaguchi et al., 2008). They prevent tumour development but can also suppress anti-tumour responses by the innate and adaptive immune system. Other cells after specific stimulation can possess Treg regulatory function. These include Tr1 secreting IL-10 cells, T helper 3 cells, TGFβ, $CD4^-CD8^-$ T cells and $CD8^+CD28^-$ T cells. These cells are different to $CD4^+FoxP3^+$ Tregs whose development is determined whilst still in the thymus. There has being some conflicting evidence with regard to the development and function of FoxP3 expressing cells (Sakaguchi et al., 2010). Helios is another transcription factor that may be a marker of Treg activity, most FoxP3+ cells are also helios positive (Getnet et al., 2010, Akimova et al., 2011).

The exact mechanism of Treg suppression is unknown but many mechanisms have being speculated from down regulation APC co-stimulatory functions to induction of programmed cell death (see Table 6.2).

Table 6.2: T cell suppression mechanisms in humans and mouse

Molecule	Function
Cytotoxic T lymphocyte antigen 4 (CTLA 4)	Antigen presenting cell (APC) down-regulation
CD95	Induction apoptosis
TGF-β	Induction Foxp3
Granzyme A	T cell lysis
IL10	Conversion T cells to regulatory T cells,
CD25	Absorption IL2

Table of T cell suppression mechanisms, adapted from (Sakaguchi et al., 2010).

6.1.5 Regulatory T cell response in *H. pylori* infected individuals

Robinson *et al* investigated both T helper and T regulatory immune responses in the gastric mucosa of subjects infected with *H. pylori*. Th-1, Th-2 and Tregs are all present in gastric mucosa of *H. pylori* infected individuals. Mice models have shown that Tregs suppression leads to reduced inflammation and persistence of bacteria. Higher Tregs are reported in *H. pylori* infected mucosa and the majority are $CD4^+$ expressing $CD25^{hi}$ marker. $CD25^{hi}CD4^+$ Tregs express high FoxP3 and function by suppressing cytokines such as IL-10 and TGFβ. Flow cytometry and real time polymerase chain reaction assay (PCR) were utilized to measure these responses. The results successfully found a response to *H. pylori* infection consisted of both Th-1 and Th-2 with high IL-10 producing Tregs. Peptic ulcer disease subjects had a 3.2 ($p=0.038$) and 6.1 ($p=0.029$) fold increase in Th-1 and Th-2 responses respectively, and a 2.4 fold decreased ($p=0.05$) $CD4^+CD25^{hi}IL\text{-}10^+$ response when a comparison was made with individuals without ulcers. They successfully showed *H. pylori* induce higher Tregs numbers and peptic ulcers occur when this response is inadequate, i.e. reduced Treg numbers (Robinson et al., 2008).

H. pylori lead to the release of pro-inflammatory cells from the gastric mucosa which attract neutrophils and other cells leading to ulceration and damage of the mucosa (Robinson et al., 2007). When Th-1 or Th17 responses predominate, with the action of defensins this leads to effective bacterial destruction and tissue damage. Treg responses suppress Th-1and Th17 responses and other inflammatory cells leading to less ulceration of the mucosa. This Treg suppression of these inflammatory cells leads to *H. pylori* colonisation and persistence (Figure 6.2) (Robinson et al., 2008).

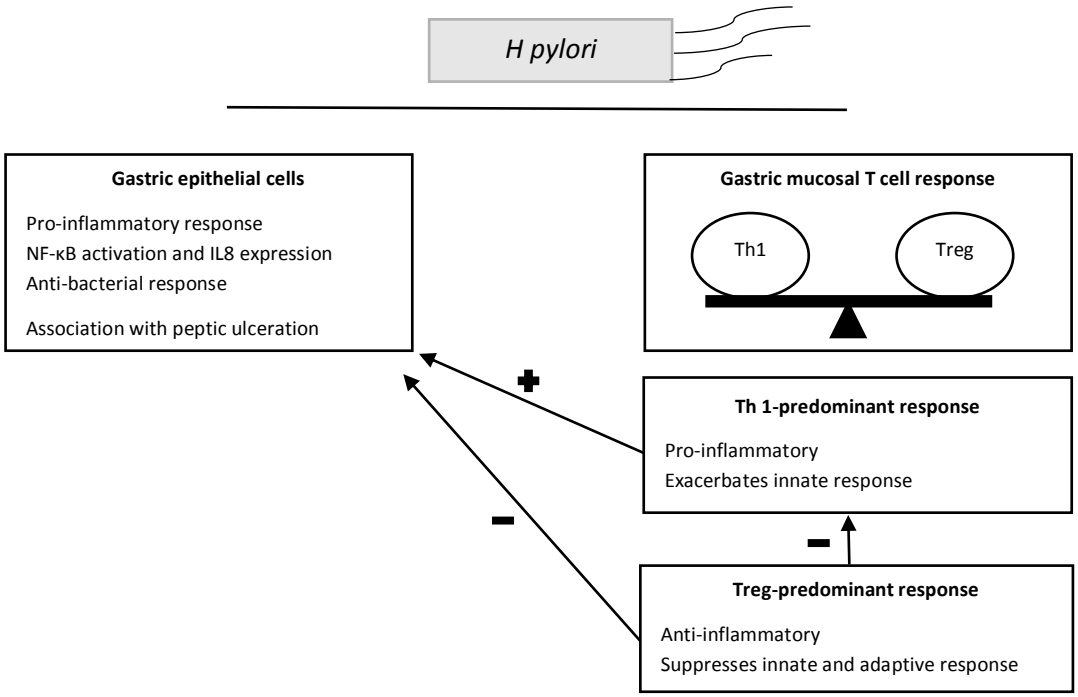


Figure 6.2: How T cell response influences colonisation, inflammation and *H. pylori* related diseases

Figure adapted from (Robinson et al., 2008).

However, little is known about the Treg response after *H. pylori* eradication.

6.1.6 Asthma, Allergy and Atopic conditions

Due to many factors including better hygiene and increasing use of antibiotics in the community, the prevalence of *H. pylori* has reduced but at the same time the prevalence of atopic conditions has risen (McCaig et al., 2002, Gupta et al., 2007). Atopy occurs when there is an exaggerated immune response to particular allergens. Atopy development usually occurs due to early childhood environmental exposure but genetic predeposition also contributes (Tattersfield et al., 2002, Dodge and Burrows, 1980, Ege et al., 2011). Asthma is more common in urban areas of developing countries (Yemaneberhan et al., 1997). Atopy protection may be mediated by a number of microorganisms but as *H. pylori* prevalence is on the decrease, atopy is on the increase leading

some to suggest *H. pylori* is linked to disease development in atopic conditions (Robinson, 2015, Austin et al., 1999). Kosunen et al described a 3-fold increase in allergy incidence and a 30% decrease in *H. pylori* prevalence between 1970 and 1990's (Kosunen et al., 2002). In a systematic review and meta-analysis carried out by Taye *et al H. pylori* colonisation was associated with an 18% reduction in odds for atopy (Taye et al., 2015), whilst others have only found a weak association (Wang et al., 2013).

It has also been hypothesised that being colonised with *H. pylori* is associated with a reduced risk of allergic disorders such as allergic rhinitis, eczema and asthma. These conditions are complex and have multi-factorial components, but one explanation could relate to the hygiene hypothesis originally proposed by Strachan. This states the development of atopic conditions could be due to lack of exposure to bacteria and viruses during childhood development (Holster et al., 2012, Bloomfield et al., 2006, Strachan, 1989) potentially due to eradication of microbes that may have had a protective effect. Work done by Blaser has shown that these conditions have an inverse correlation with *H. pylori* infection (Chen and Blaser, 2007). Some experts have rejected the hygiene hypothesis and believe infections like *H. pylori* act as a trigger rather than providing protection. GI infection could alter the mucosal permeability allowing for allergen entry and reduction of tolerance to these allergens (Lionetti et al., 2014).

Blaser's 2008 review on *H. pylori,* asthma and allergy identified twelve cross-sectional studies that showed a significant inverse relationship that smaller case-control studies had not shown before (Blaser et al., 2008). However, one case-control study by Reibman *et al* (318 cases and 208 controls) did show an inverse relationship between CagA+ *H. pylori* strains and asthma (Reibman et al., 2008). Further work by Chen and Blaser using a large cohort confirmed the association that adults colonised with *cagA+ H. pylori* strains were less likely to have childhood asthma but found it to be restricted to younger subjects with disease onset earlier in life. They concluded that it is plausible for *H. pylori* strains to play a biologically protective role in atopic disease via

pathways that are complex and poorly understood at present (Chen and Blaser, 2007).

A great number of other studies have found this inverse association of *H. pylori* and atopy but a smaller number have shown conflicting data with no association (Bodner et al., 2000). This protective association is greatest against childhood asthma (Chen and Blaser, 2008). As both *H. pylori* acquisition and the onset of asthma develop at a young age this could explain some of the contradictive results. An alternative explanation for differences in study results could be the complex nature of asthma with multiple triggers, GORD is known to worsen asthma in individuals by aspiration of gastric fluid into lungs therefore confounding some studies (Robinson, 2015).

H. pylori are thought to elicit a peripheral and local Th1 response, which offsets or suppresses Th2 response. A reduction in Th1 stimulation results in overstimulation of Th2 which could lead to allergy (Sommer et al., 1998, Wang et al., 2007, Trujillo and Erb, 2003). CagA+ strains lead to an increase Th1 response and decreased Th2 (Hida et al., 1999, Orsini et al., 2003). Th2 responses are modulated in both mice and humans by virulence factor *H. pylori* neutrophil activating protein (HP-NAP). Mice undergoing ovalbumin allergen sensitization and treated with HP-NAP have reduced eosinophilia in lungs, reduced serum IgE and lower Th2 bronchoalveolar concentrations (Codolo et al., 2008, D'Elios et al., 2009, Amedei et al., 2006). Increase in gastrin and decrease somatisation hormone leads to Th1 release and hinders Th2 release further influencing this interaction (Zavros and Merchant, 2005).

The T lymphocyte regulatory mechanism helps to inhibit the immune response in allergy and is currently the most studied mechanism. Microorganisms are detected by immune system which later matures to Tregs that mount responses to these organisms rather than leading to pro-inflammatory responses. This leads to a constant bystander suppression of other responses by release of cytokines. Increasing numbers of regulatory

dendritic cells leads to an increased Treg response which may mediate protection from allergy and autoimmunity (Rook, 2007). Tregs suppress Th2 inflammation in asthma via IL-10. It also inhibits mast cells and eosinophil cytokine release, IgE production is inhibited and IgG4 production is promoted which is protective against allergy. Transforming growth factor β (TGF-β), a suppressive cytokine is also involved in airway tissue remodelling and fibrosis (Shi and Qin, 2005, Lloyd and Hawrylowicz, 2009, Urry et al., 2006). *H. pylori* infected individuals and mice have higher Treg numbers both locally and peripheral (Robinson et al., 2008, Cook et al., 2014, Kaparakis et al., 2006). Both Treg and IL-10 response are more pronounced in CagA+ strains which could explain the protection seen with CagA+ and asthma (Hida et al., 1999).

Arnold *et al* provided the first experimental evidence for asthma protection in a mouse model. CagA+ *H. pylori* infected mice had reduced airway hyper responsiveness, reduced asthma markers, pulmonary inflammatory markers and reduced goblet metaplasia (Arnold et al., 2011). Delivery of purified VacA or *H. pylori* factor recombinant gamma-glutamyl transpeptidase (GGT) to mice produced protection against asthma similar to those seen in *H. pylori* infected mice. Once fully investigated these could be future potential therapeutic targets (Oertli et al., 2013, Engler et al., 2014, Robinson, 2015). Mutations in the *FOXP3* gene lead to severe autoimmune diseases in mice and humans. The role of defective regulatory immune pathways has been shown to be important in allergic and autoimmune disorders (Akdis et al., 2004, Fontenot et al., 2003). This was also demonstrated in animal models of inflammatory bowel disease, but the data from human studies have been less convincing (Rook, 2007).

In other settings, bee keepers exposed to bee sting venom have increased levels of allergen specific IL-10 T cells (Bellinghausen et al., 1997) and individuals exposed to extreme levels of pet specific allergens are protected from certain allergic conditions (Simpson and Custovic, 2005). Asthma patients with the severe form of the disease also have reduced number of IL-

10+ CD4+ T cells (McGuirk et al., 2010). Also in cancer patients elevated levels of peripheral Tregs have been found. These higher Tregs levels correspond with a poorer prognosis. Tumours have also been found to develop more slowly in $CD25^+$ Treg depleted mice. Thus manipulating the Treg response could be a potential target for future cancer treatment (Ghiringhelli et al., 2006, Gallimore and Godkin, 2008).

Other inflammatory and autoimmune disorders associated with *H. pylori* protection include inflammatory bowel disease(Papamichael et al., 2014), systemic lupus erythematous (Sawalha et al., 2004) and multiple sclerosis (Li et al., 2007) but there is also conflicting evidence in the literature. *H. pylori* is also linked with the development of the autoimmune disorder ITP.

6.2 Aims

The aims of the study were as follows:

- Characterise frequencies of Tregs and associated markers from peripheral blood from uninfected and infected individuals using RT-qPCR.
- Compare CD25, CD4, FoxP3 and IL-10 frequencies in PBMCs before and after treatment using flow cytometry.
- Investigate differences in these Treg markers before and after eradication treatment in a subset of patients followed over a two year period using RT-qPCR and flow cytometry.
- Compare total plasma IgE levels between uninfected and infected individuals by ELISA and correlate this with IL-10 data.
- Investigate differences in total plasma IgE levels before and after eradication treatment in a subset of patients followed over a two year period.
- Investigate for any potential influences of *CagA* status, gender, smoking, seasonal variation and disease outcome on Treg markers and total IgE levels.

6.3 Hypotheses

Regulatory T lymphocytes (Tregs) are vital for control and prevention of inflammation and diseases that are immune mediated. At present, there is no single reliable marker for Treg cells. However, gastric tissue from *H. pylori* infected patients contains higher levels of *H. pylori* responsive Tregs, which express the anti-inflammatory cytokine IL-10. This factor acts to reduce the degree of inflammation. The suppressive action of Treg cells could potentially explain the chronic infectious state of *H. pylori* in the absence of peptic ulcer disease, and thus why only a small proportion of *H. pylori* untreated people have ulcers (Robinson et al., 2008). It is unclear whether similar findings exist in peripheral blood. This study aims to confirm these previous findings using peripheral blood.

In addition, there is little known about what happens to this increased Treg population after successful eradication treatment. There have been no reported studies to our knowledge that have investigated this. This study hypothesises that after *H. pylori* eradication treatment, the Treg response in peripheral blood will be markedly reduced as the infectious stimulus is removed. By understanding the immune response post eradication, this could help develop a simple blood test to evaluate treatment success.

It has also been suggested that as *H. pylori* has evolved with humans it may have a protective role. *H. pylori* has been the subject of intense research but some important questions still remain unanswered. In particular, there is evidence that *H. pylori* may be a major contributor to the hygiene hypothesis. By stimulating the production of Tregs which control immune and inflammatory cells, it may suppress allergic associated conditions, including asthma. The evidence comes from observational and animal experimental data in the literature but further human studies are needed (Chen and Blaser, 2008). This study will provide data on the long term effects of *H. pylori* eradication on Tregs. Although it has being suggested that eradication

treatment may adversely affect atopy and allergy (Holster et al., 2012), there has been no firm biological evidence for this.

The study hypotheses are that eradication treatment reduces Th1 and Treg responses, therefore no longer suppressing allergy and thus leading to an increase in plasma IgE concentration over time, more specifically:

A

- Levels of IL10 mRNA levels will be greater in *H. pylori* infected than those uninfected

B

- IL10 mRNA response will be reduced after eradication
- IL10 mRNA will remain the same in individuals who remain positive for *H. pylori* infection despite multiple courses of antibiotics

C

- There will be a reduction in peripheral blood Tregs after eradication treatment
- $CD4^+$ $IL10^+$ cells that express markers of Tregs will reduce after *H. pylori* eradication treatment
- $CD4^+$ $IL10^+$ lymphocytes frequency will be higher in CagA+ strains
- Treg markers will remain the same in individuals who remain positive for *H. pylori* infection despite multiple courses of antibiotics

D

- IgE levels will be similar between *H. pylori* infected and uninfected individuals
- IgE levels will increase after successful eradication therapy
- IgE levels will remain the same in individuals who remain positive for *H. pylori* infection despite multiple courses of antibiotics

6.4 Materials and Methods

6.4.1 Participants and clinical materials

6.4.1.1 Uninfected and *H. pylori* infected

The work was carried out after approval from Nottingham Research Ethical Committee 2 (REC Ref: 08/H0408/195) and (REC Ref: 12/EM/0446). Written informed consent from adult patients attending Nottingham University Hospitals NHS Trust for a diagnostic gastroscopy was obtained. Peripheral blood samples were taken from 124 otherwise healthy patients who attended routine endoscopy appointments. The most common indication for a gastroscopy was dyspepsia. This unselected group of patients were prospectively collected. In additional to clinical samples to determine *H. pylori* status, endoscopic features, age, sex and smoking status was recorded at the time of endoscopy. Patient exclusions for the study included using certain medications in the preceding 4 weeks; non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics and proton pump inhibitors (PPIs), regular anticoagulant use, immunosuppressive therapy or where biopsy was contraindicated. The male to female ratio of uninfected and *H. pylori* infected group was 0.46 and 0.42 respectively, with median ages of 53 and 55 years. 32% of *H. pylori* infected patients had evidence of peptic ulcer disease (Table 6.3). In addition to research blood sample, clinical biopsies were collected from gastric antrum and corpus and immediately placed in formalin for later histopathological scoring, one from the antrum placed in urease medium for rapid urease detection, and final sample in iso-sensitest broth (Oxoid, Cambridge, UK)/10% glycerol for *H. pylori* isolation and culture. Uninfected patients had all three negative tests. Patient recruitment and sample collection was done by Prof JC Atherton and Dr JR White for 74 patients. Blood sample processing and analysis for 74 patients was done by Mr AB Greenaway. The remaining 50 patient blood samples were collected, processed and analysed by JR White.

Table 6.3: Patient demographics of Uninfected and *H. pylori* infected patients

	Uninfected	*H. pylori* Infected
Number of patients	26	98
Male: Female	12:14	41:57
Age	53 (21-78)	55 (21-78)
Non-smoker	54% (14/26)	52%
Ex-smoker	27% (7/26)	21%
Current smoker	19% (5/26)	27%
1x Biopsy based test	0	38%
2x Biopsy based test	100% (26/26)	62%
Peptic ulcer disease	0	32%

Proportion of males to females represented as a ratio, age represented as median with range.

6.4.1.2 *H. pylori* eradication study

The work was carried out after approval from Nottingham Research Ethical Committee 1 (REC Ref: 12/EM/0446) and informed consent from adult patients attending Nottingham University Hospitals NHS Trust for a diagnostic gastroscopy. Patient recruitment, sample collection, blood sample processing and analysis for 50 patient's initial and multiple subsequent follow up visits were done by Dr JR White. Adults aged between 18 and 70 years were included in this study. Patients below 18 years were excluded because they rarely suffer from *H. pylori* related diseases and those over 70 years because they are unlikely to show any reversibility in their immune response. Patients with one or more positive *H. pylori* tests (histology, rapid urease, bacteria culture or Urea Breath Test) were prospectively recruited. Patient exclusions for the study included using certain medications in the preceding 4 weeks; recent antibiotic use or immunosuppressive therapy. After written informed consent and positive *H. pylori* status was confirmed, a blood sample was obtained at the initial visit. Patients were usually treated according to NUH antibiotic policy. Alternative regimes were used for those patients who have previously failed recommended regimes and the choice of antibiotic depended on antibiotic culture and sensitivity testing. Participants attended the research unit before and on multiple times after treatment over a two

year period (Figure 6.3). Over 160 blood samples were processed at different time points by Dr JR White (84%) and Miss D Reddiar (16%). Table 6.4 details patient demographics.

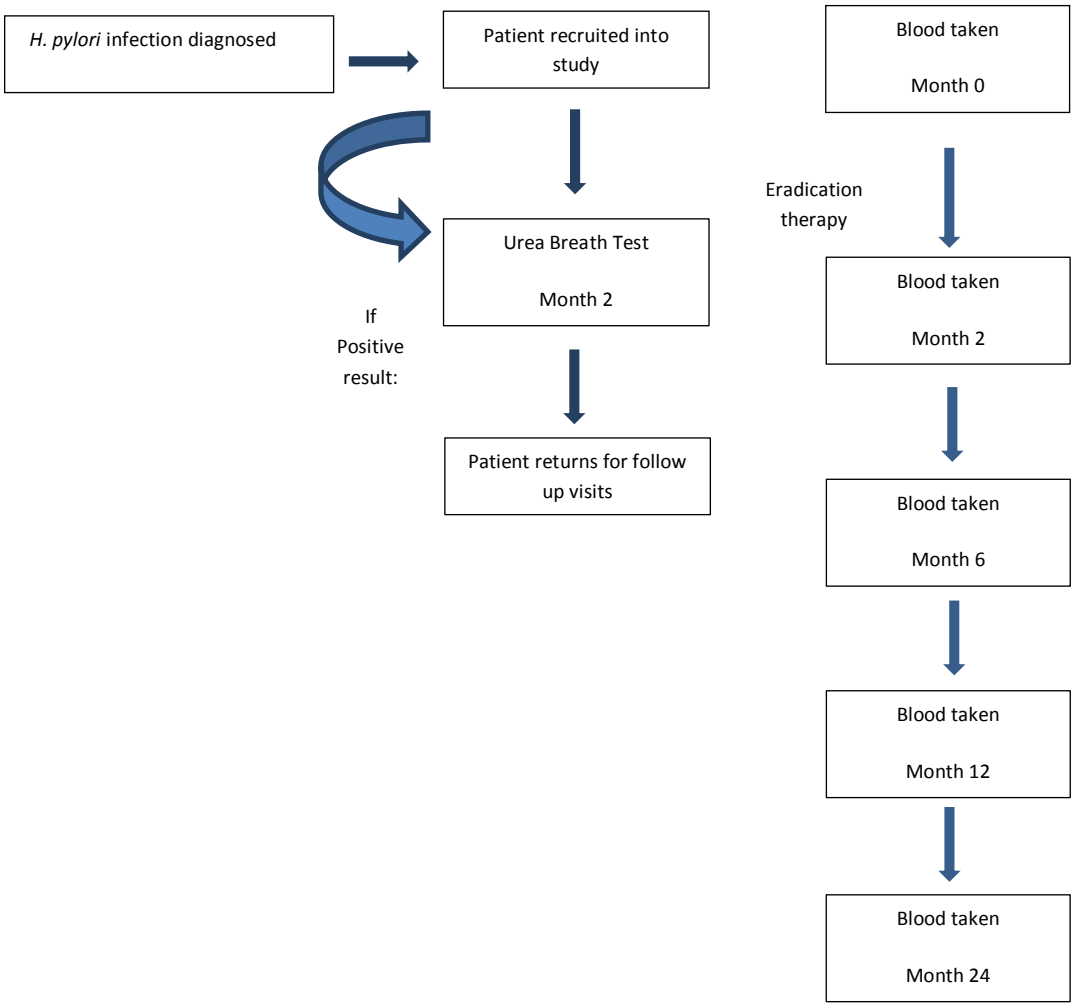


Figure 6.3: Patient flow chart with follow up visits time points

H. pylori infected patients donated a blood sample and received eradication treatment at initial visit at month 0. Two months later a Urea Breath Test was carried out to confirm whether eradication was successful. If eradication was successful each patient returns at 6, 12 and 24 months for further blood samples. Those who fail treatment received further antibiotics and a repeat Urea Breath Test. Patients could return for a maximum of 7 visits or involved in the study for a maximum of 24 months.

Table 6.4 Demographics of patients in the *H. pylori* eradication study

	Infected (*H. pylori* Eradication study)
Number of patients	50
Male: Female	24:26
Age	55.5 (21-70)
Non-smoker	52% (26/50)
Ex-smoker	20% (10/50)
Current smoker	28% (14/50)
Allergy/atopy	18% (9/50)
Adult Asthma	6% (3/50)
1x Biopsy based test	74% (37/50)
2x Biopsy based test	26% (13/50)
Peptic ulcer disease	20% (10/50)
Multiple eradication courses	14% (7/50)

The male to female ratio in the *H. pylori* eradication study group was 0.48, median ages of 55.5 years. 18% had a history of atopy and 20% had evidence of peptic ulcer disease.

6.4.1.3 *H. pylori* lysate preparation

H. pylori strains were isolated from patients with gastroduodenal disease to make the *H. pylori* mixed strain lysate used in section 6.4.1.5. Table 6.5 shows details of *H. pylori* strains used collectively in a lysate to stimulate PBMCs *in vitro*. *H. pylori* strains were partly thawed then were cultured on 5% horse blood agar plates (Oxoid) at temperature of 37°C (10 % O_2 and 5 % CO_2) for 48 hours in a microaerophilic incubator. Growth was carefully extracted using 10 µl loops and passed onto fresh agar plates for a further 48 hours. Each strain was grown until confluent and growth was then collected and re-suspended in 1ml ice cold phosphate buffered saline (PBS). Bacterial suspension were measured at an O.D of 1 at a wavelength of 600nm, and then collected together into a single tube for sonication. The suspension was centrifuged and the pellet was then re-suspended in 1ml PBS. Soniprep 150 sonicator (SANYO,

Watford, UK) was used to disrupt the solution and create the final *H. pylori* lysate with appropriate ear protection and in a Class 1 cabinet to prevent the spread of aerosolised bacteria. Cells were sonicated in 10 second bursts at 10 microns, each burst usually being followed by 30 seconds of cooling.

Total protein estimation was calculated using Pierce BCA Protein Assay kit (ThermoScientific) according to manufacturer's instructions to obtain a working concentration of 250µg/ml. Aliquots were stored at -20°C until needed then diluted with 5ml culture media (which consisted of RPMI 1640 medium, 10% heat in-activated fetal calf serum (FCS) and 100 µg/ml penicillin and 0.1 /ml streptomycin) to make a final concentration of 25µg/ml. This was completed by D. Letley and JR White.

Table 6.5 Strain details for *H. pylori* lysate

Strain	Strain details	Disease
Q86B	*vacA* s1/m1, *CagA+*	Duodenal Ulcer
Q109A	*vacA* s2/m2, *CagA-*	None
Q110A	*vacA* s2/m2, *CagA-*	Oesophagitis
Q122B	*vacA* s1/m1, *CagA+*	Hiatus hernia
Q126B	*vacA* s1/m2, *CagA+*	Gastric Cancer
Q131B	*vacA* s1/m2, *CagA+*	Duodenal Ulcer

Details of *H. pylori* strains used collectively in a lysate to stimulate PBMCs *in vitro*.

6.4.1.4 Plasma and PBMC isolation

20ml to 50ml of blood was collected in EDTA-vacutainer tubes (Greiner Bio-One) which contained spray dried K2EDTA which acted as an anticoagulant (Robinson et al., 2002). This blood sample was then centrifuged at 200 x *g* for 10 minutes and the plasma layer separated from blood cells and placed in three cryogenic tubes each containing 1ml of plasma and immediately stored at -80°C. The remaining blood cells were re-suspended in washing media

which consists of RPMI 1640 medium (Sigma), 2% fetal calf serum (FCS) (Sigma), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma).

The purification and enrichment of peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation was then carried out. This consisted of layering 10-15mls of the blood cell suspension onto 5ml Histopaque1077 (Sigma-Aldrich) separation solution. A 20 minute centrifugation at 300 x *g* followed and the enriched interphase of PBMCs was collected from the layer between Histopaque and washing media. Three subsequent washes with washing media followed at a speed of 200 x *g* for 5 minutes. The final pellet was re-suspended in 5ml culture medium which consisted of RPMI 1640, 10% heat in-activated fetal calf serum (FCS) and 100 µg/ml penicillin and 0.1 /ml streptomycin. 25µl cells were stained with 75µl 0.4% Trypan Blue solution (Thermo Scientific) and 10µl placed on a haemocytometer slide with a coverslip. The number of dye-excluding (white) cells was counted within two set of 16 squares on the grid. Dead cells take up the dye and appear blue. The number of cells per ml equals the mean count multiplied by the dilution factor (4). This count was then multiplied by 5 to give the total number of cells in 5mls. An appropriate amount of culture medium was added to make final concentration $1x10^6$cells/ml. 74 samples were processed by Mr AB Greenaway and 131 patient samples by Dr JR White and 26 by Miss D Reddiar.

6.4.1.5 In vitro stimulation

1×10^6 PBMCs in 1 ml culture media were then aliquoted into six sterile culture tubes (Elkay). 100µl of *H. pylori* lysate (25µg/ml) was added to four tubes for antigenic stimulation and nothing added to two unstimulated tubes (Robinson et al., 2005). PBMC incubated at 37°C with 5% CO_2 for one hour before 10 µl brefeldin A (10µg/ml) added. For the antigenic stimulation a total of 16 hours at 37°C with 5% CO_2 was then completed. Cells were then stored at 4°C before cellular staining.

6.4.1.6 Cells for RNA extraction or repeated cellular staining

Three aliquots of $5x10^6$ pelleted PBMC were re-suspended in 250µl of 10µl 2-mercaptoethanol (ME)/1ml lysis solution mixture to lyse cells and inactivate RNase. This was then stored at -80°C until required for RNA purification. Any remaining cells were then added to 250µl-500µl freezing medium (10ml FCS, 2ml dimethyl sulfoxide DMSO, 8ml culture medium) and stored at -80°C for repeat cellular staining at a later date.

6.4.1.7 Fluorochromes

Fluorochromes are chemical compounds which are able to re-emit light upon light excitation. Fluorochrome-conjugated antibodies were excited at different wavelengths of light in the flow cytometer to produce fluorescence. This allowed cell events to be sorted into distinct populations by their expression of different markers. Fluorochrome dyes properties used for the FC 500 MCL flow cytometer (Beckman Coulter) are detailed in Table 6.6 and Table 6.7.

Table 6.6 Fluorochrome-conjugated antibodies

Marker/ Isotype	Fluorochrome	Species/ Subtype	Company	Catalogue no.	Clone	Concentration	Volume
CD4	ECD	Mouse IgG1	Beckman Coulter	6604727	SFCI12T 4D11	0.5 mg/ml	2.5µl
CD25	PE/Cy7	Mouse IgG1, κ	BioLegend	302612	BC96	0.1 mg/ml	10µl
Foxp3	AF488	Mouse IgG1, κ	BioLegend	320212	259D	0.1 mg/ml	5µl
Iso-Foxp3	AF488	Mouse IgG1, κ	BioLegend	400134	MOPC-21	0.1 mg/ml	5µl
IL-10	PE	Rat IgG2a	ABD Serotec	MCA2250	JES3-12G8	0.5 mg/ml	2.5µl
Iso-IL-10	PE	Rat IgG1,κ	BioLegend	400408	RTK207 1	0.2 mg/ml	1µl

Fluorochrome-conjugated antibodies detecting markers which are expressed by human regulatory T lymphocytes. Antibodies were used according to manufacturer's instructions. Isotype controls are described as 'iso'.

Table 6.7 Fluorophore properties

Fluorophore	Abbreviation	Excitation laser (nm)	Emission bandwidth (nm)
Alexa Fluor 488	AF488	Argon Ion-488	519-525
Phycoerythrin-Texas Red	ECD	Argon Ion-488	610-635
Phycoerythrin	PE	Argon Ion-488	568-590
Phycoerythrin-Cyanin 7	PE/Cy7	Argon Ion-488	750-810

Table detailing fluorophore properties

6.4.1.8 Extracellular staining

$1x10^6$ PBMC were centrifuged for 5 minutes at 250 x g and human extracellular markers; anti-CD4-phycoerythrin-Texas Red (ECD; Beckman Coulter UK Ltd., High Wycombe, UK) (2.5 µl/ test) and phycoerythrin-Cyanin 7 anti-human CD25 (Biolegend) (10 µl per/ test) added to the pellet and then incubated for 30 minutes at 4°C. The cells were then washed three times with 1ml of buffer of PBA/2% FCS buffer (2% FCS, 0.1% bovine serum albumin (BSA) and 0.1% sodium azide). Once washes were complete, cells were fixed in 0.5ml of 0.5% formaldehyde at 4°C in the dark overnight (Robinson et al., 2002, Robinson et al., 2008, Cook et al., 2014)

6.4.1.9 Intracellular staining

0.5ml of PBMC in 0.5% cell fix were pelleted at 250 x *g* for 5 minutes then washed with 1ml cell staining buffer (BioLegend). The cells were permeabilised with 1ml FoxP3 perm buffer solution which consists of 900µl PBS, 100µl FoxP3 perm buffer x10 (BioLegend). PBMCs were later re-suspended in 1ml of perm buffer for 15 minutes at room temperature then pelleted and intracellular antibodies 2.5 µl/test of IL-10 PE (Coulter) and 1µl Rat IgGI-PE Isotype (BioLegend) were added. PBMCs were then incubated in the dark at 4°C for 90 minutes. After which, 100µl FoxP3 perm buffer, Alexa Fluor®488 anti-human FoxP3 (BioLegend) (5µl/test) and Alexa Fluor®488 Mouse IgG1 isotype control (BioLegend) (5µl/test) were added. PBMCs were

then incubated in the dark at room temperature for 30 minutes, and then washed three times with cell staining buffer (Biolegend). Once washes were complete, cells were fixed with 0.5% formaldehyde at 4°C in the dark until flow cytometry analysis (Robinson et al., 2002, Robinson et al., 2008, Cook et al., 2014). Experiment tube list Table 6.8.

Table 6.8: Extracellular and Intracellular antibodies tube list

Tube Number	Stimulus	Extracellular	Intracellular
1	None	CD4-ECD, CD25-PC7	FoxP3-A488, IL-10PE
2	None	CD4-ECD, CD25-PC7	Isotype A488, Isotype PE
3	*H. pylori* lysate	CD4-ECD, CD25-PC7	FoxP3-A488, IL-10PE
4	*H. pylori* lysate	CD4-ECD, CD25-PC7	Isotype A488, Isotype PE
5	*H. pylori* lysate	CD4-ECD, CD25-PC7	FoxP3-A488 only
6	*H. pylori* lysate	CD4-ECD, CD25-PC7	IL-10PE only

Peripheral blood was collected from patients and PBMC isolated. $1x10^6$ PBMC in 1ml culture media placed in six FACS tubes. 100µl of *H. pylori* lysate (25µg/ml) added to tubes 3-6 for antigenic stimulation and nothing added to tubes 1-2 (unstimulated). After in vitro stimulation extracellular and intracellular staining was carried with the appropriate antibodies.

6.4.1.10 Flow cytometry analysis

A multi-colour flow cytometry data acquisition was carried out using a FC500 MCL cell sorter (Beckman Coulter). With a predetermined lymphocyte gate, data on 250,000 events was acquired and analysed using Kaluza software (Beckman Coulter), using the appropriate isotype controls. Each sample were gated on forward and side scatter under the 488nm laser, this was to exclude debris and dead cells. An example flow cytometry plot and analysis is shown in Figure 6.4. Samples were excluded from analysis if there were fewer than 50,000 events, inconsistent staining, or if there were positive isotype control

stained events. Flow cytometry analysis was done by Dr JR White (79 samples) and Miss D Reddiar (78 samples).

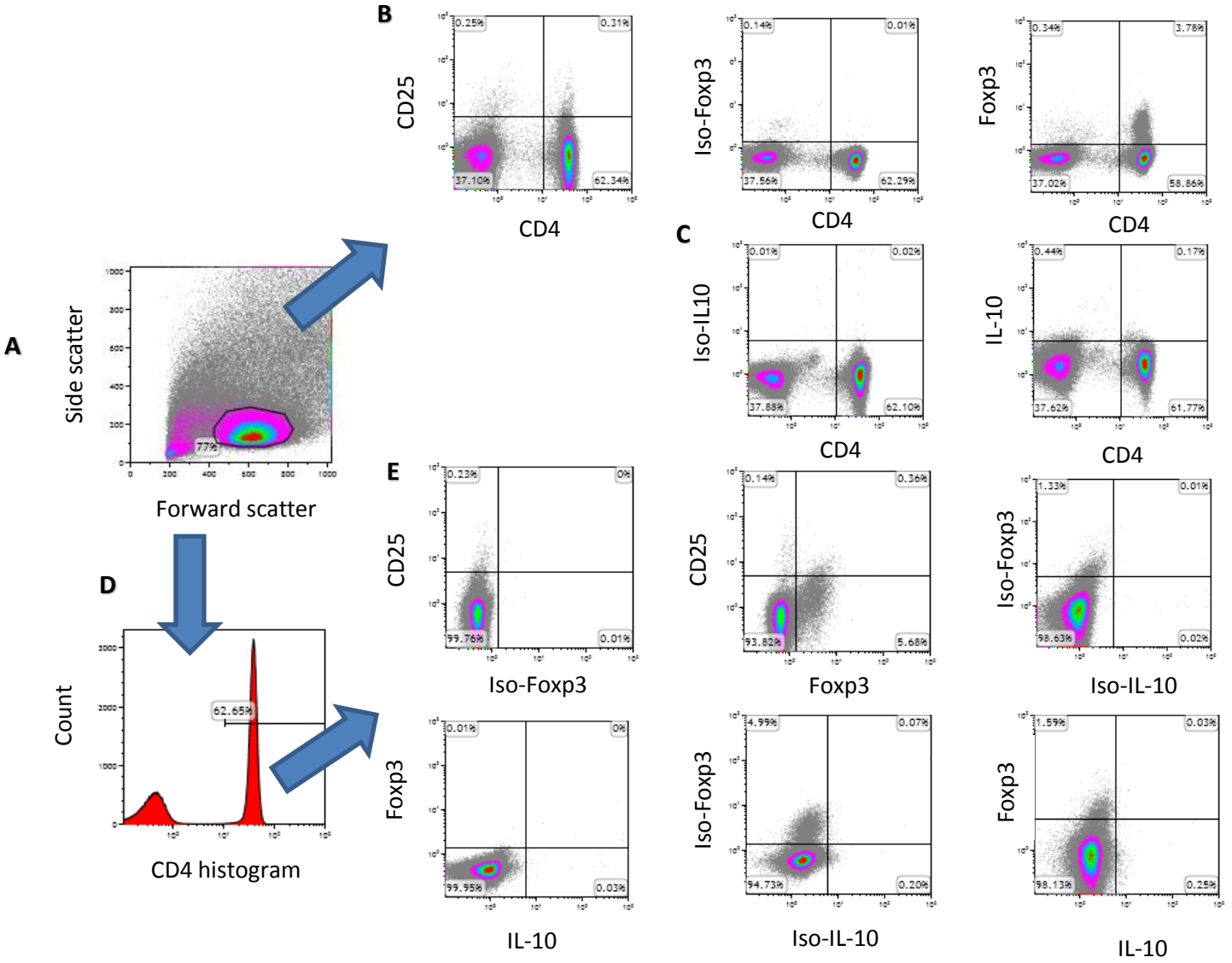


Figure 6.4 Gating strategy for flow cytometry analysis

Flow cytometry gating detailing the analysis strategy for Tregs. PBMCs were isolated from recruited patients and stained with fluorochrome conjugated antibodies to identify $CD4^{+}CD25^{hi}Foxp3^{+}$ Tregs, and their IL10 production. The lymphocyte population was gated by forward and side scatter under the 488nm laser (A). (B) Left to right represents CD4-CD25 and CD4-Foxp3 plots and its corresponding isotype control. (C) CD4-IL10 plots gated from the lymphocyte gate, left to right: isotype control and anti-IL10 stained cells stimulated with *H. pylori* lysate. (D) Histogram showing $CD4^{+}$ events from the gated lymphocytes. Flow cytometry plots with their corresponding isotype controls gated from CD4+ events gated in D (E).

6.4.1.11 IgE ELISA assay

Human IgE ELISA Quantification kit (Bethyl, USA) was followed as manufacturer's instructions to quantify plasma IgE concentrations. Optical densities were measured with a microplate reader at 450nm with the reference wavelength of 595nm. Standard curves on each plate were used to determine the final IgE concentrations. IgE concentrations were expressed in ng/ml. 2.4ng/ml is equal to 1 Ku/L with normal levels below 75Ku/L (i.e. <180 ng/ml) (Seagroatt and Anderson, 1981, Amarasekera, 2011). All IgE ELISA were completed by Y. Falcone and M. Lingaya.

6.4.1.12 *CagA* ELISA assay

CagA IgG ELISA kit (Genesis) was followed according to manufacturer's instructions to determine *cagA* status from stored plasma. Optical densities were measured with a microplate reader at 450nm with the reference wavelength of 620nm. CagA IgG ELISAs were completed by Y. Falcone, M. Lingaya and J.R White.

6.4.1.13 RNA isolation

Total RNA was extracted using GenElute^{TM} Mammalian Total RNA Miniprep kit (Sigma-Aldrich, USA) according to manufacturer's instructions. $5x10^6$ pelleted PBMC were placed in 250µl of lysis solution/2- mercaptoethanol (ME) mixture to lyse cells and inactivate RNase and stored at -80°C until later RNA extraction. After the extraction process the final RNA was eluted in 50µl of elution solution and the nucleic acid concentration was quantified using a NanoDrop ND-1000 spectrophometer (NanoDrop Technologies, USA). To determine the absence of protein contamination, the ratio of absorbance at 260 to 280 nm (A260/A280) was assessed. Values between the 1.8 and 2.1 indicate absence of contamination. Appendix 3 details nucleic acid concentration and 260/280nm absorbance values. The purified RNA was later diluted to 20ng/l using DNase/RNase-free water. RNA extractions for 74

patient samples were done by Mr AB Greenaway and 131 patient samples done by Dr JR White.

6.4.1.14 Complementary DNA (cDNA) synthesis

cDNA was synthesised from purified diluted 20ng/µl RNA using oligo(dT) primer (Invitrogen, Paisley, Scotland), deoxynucleotide (dNTP) (Roche), DNase/RNase-free water, 5x first strand buffer (Invitrogen, Paisley, Scotland), 0.1M DTT (Invitrogen, Paisley, Scotland) and RNase out (Invitrogen, Paisley, Scotland) according to the manufacturer's instructions. For each sample 3 volumes of reverse transcriptase (+RT) and 1 volume –RT control was prepared. The –RT control ensures that signals in RT-PCR do not originate from contaminating genomic DNA. cDNA for 74 patient samples were done by Mr AB Greenaway and over 131 patient samples done by Dr JR White.

6.4.1.15 RT qPCR

This was performed on a Rotor-Gene 3000 (Corbett Research, Cambridge, UK) using primers described in Table 6.9 (Robinson et al., 2008) and DyNAmo HS SYBR green kit (Thermo scientific). Cycling conditions are listed below in Table 6.10. Each run included a no template control; a melting curve analysis was completed to test the primer specificity. Duplicate samples were run in addition to one –RT control samples; these results were analysed according to Pfaffl method (Pfaffl, 2001). The efficiencies of each PCR were 1.72 (*GAPDH*) and 2.00 (*IL-10*) and were calculated from slopes of cDNA input verses CT plots (data not shown). The *IL-10* expression levels were created by normalising against the reference gene *GAPDH*, and later comparing this value to the normalised levels from five pooled *H. pylori* negative donors (negative comparator) to enable a fold difference to be calculated. A positive control, commercial pooled human cDNA (Clontech) was included in each run. cDNA for 74 patient samples were done by Mr AB Greenaway and 131 patient samples done by Dr JR White.

Table 6.9: Primer sequences for RT-qPCR analysis

Primer Name	Primer sequence	Tm (°C)	Product Size (bp)
GAPDH Forward	5'-CCACATCGCTCAGACACCAT-3'	66.3°C	114 bp product
GAPDH Reverse	5'-GGCAACAATATCCACTTTACCA-3'	65.8°C	114 bp product
IL10 Forward	5'- GCTGGAGGACTTTAAGGGTTACCT -3'	65.3°C	109 bp product
IL10 Reverse	5'- CTTGATGTCTGGGTCTTGGCT -3'	65.3°C	109 bp product

Tm, Melting temperature; GADPH, glyceraldehyde-3-phosphate dehydrogenase; IL-10, interleukin 10; (Robinson et al., 2008).

Table 6.10: Cycling conditions for RT-qPCR analysis

Step 1	95°C 15 mins
Step 2	95°C 15 sec, 62°C 30 sec, 72°C 30 sec (acquiring) (40 cycles)
Step 3	72°C 10 min
Step 4	melt 72-95°C
Step 5	72°C 5 mins

Amplification carried out over 45 cycles (Robinson et al., 2008).

6.4.1.16 Statistical Analysis

Statistical analysis was carried out using Prism 6.0 (GraphPad Software 6 USA), unless otherwise stated horizontal lines within bars represented median values. Mann Whitney U tests were used for unpaired data and Wilcoxon signed rank test for paired data. Correlations were examined using the Spearman's rank correlation test. Statistical significant difference was considered if p values were ≤ 0.05. All statistical analysis was carried out by J.R White.

6.5 Results

6.5A: *IL10* mRNA levels in PBMCs from *H. pylori* infected and uninfected individuals

The Nottingham *H. pylori* group have previously shown that IL10 secreting Tregs are significantly higher in *H. pylori* infection (Robinson et al., 2008). We investigated variation among the infected group and the possible underlying factors-age, sex, gender, smoking status and peptic ulcer disease.

PBMCs from 98 *H. pylori* infected and 26 uninfected patients was collected and processed as previously described in section 6.4.1.1. *IL10* mRNA levels were elevated 0.8-fold (Figure 6.5) in PBMCs from infected patients when compared with uninfected patients expressed as median and range 1.08 (0.09-11.35) in uninfected and 1.9 (0.04-90.9) in infected patients p=0.0258). There was a wide range of variation within each group.

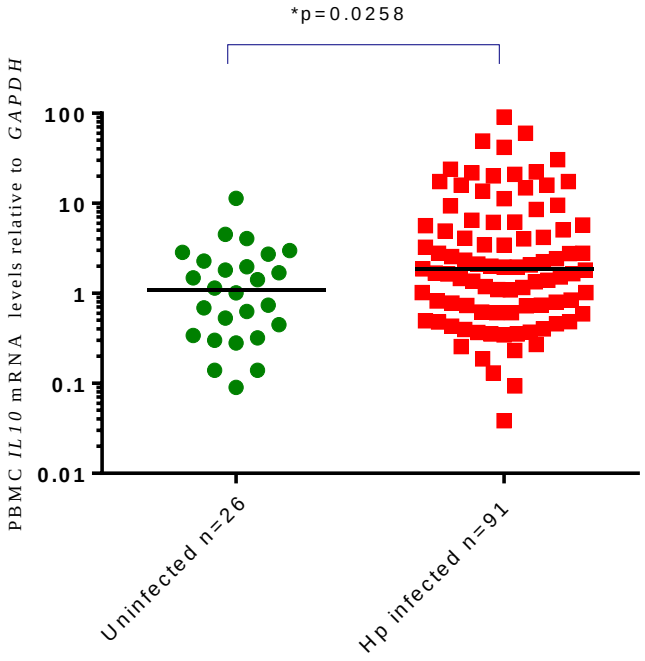


Figure 6.5 *IL10* mRNA expression levels in PBMCs from uninfected and infected *H. pylori* patients

Peripheral blood samples were collected from uninfected and *H. pylori* infected patients. mRNA was extracted from five million purified PBMCs. Using RT-qPCR *IL10* expression levels were determined and data normalised relative to *GAPDH* expression. A pooled cDNA control prepared from the PBMCs of 8 uninfected patients was used as the internal comparator to generate the fold difference. Horizontal bar represent median and Mann Whitney U test was used to calculate statistical significance

With regards to *IL10* mRNA expression levels the possible confounders of sex, smoking status, age, *cagA* status and the presence of peptic ulcer disease were closely scrutinised amongst the *H. pylori* infected group. No significant difference in *IL10* mRNA was observed between the genders (Figure 6.6A). There was no observed correlation between age and *IL-10* expression levels (Figure 6.6B). Neither smoking status, nor *cagA* genotype of the colonising strain seemed to influence *IL10* expression in the studied individuals (Figure 6.6C-D). Findings were similar in uninfected patients (data not shown). Patients were also stratified according to the presence or absence of peptic ulcer disease (PUD) (Figure 6.6E). PUD is defined as mucosal breaks in the

stomach and duodenum with depth and is usually greater than 5mm (White, 2016b). Erosions were not included as PUD but healing ulcer scars were. There was no difference between duodenal and gastric ulcers (data not shown). Those with evidence of PUD (median 1.7) had a 1-fold decrease in *IL10* expression level when compared with those without PUD (median 3.4, p=0.0357).

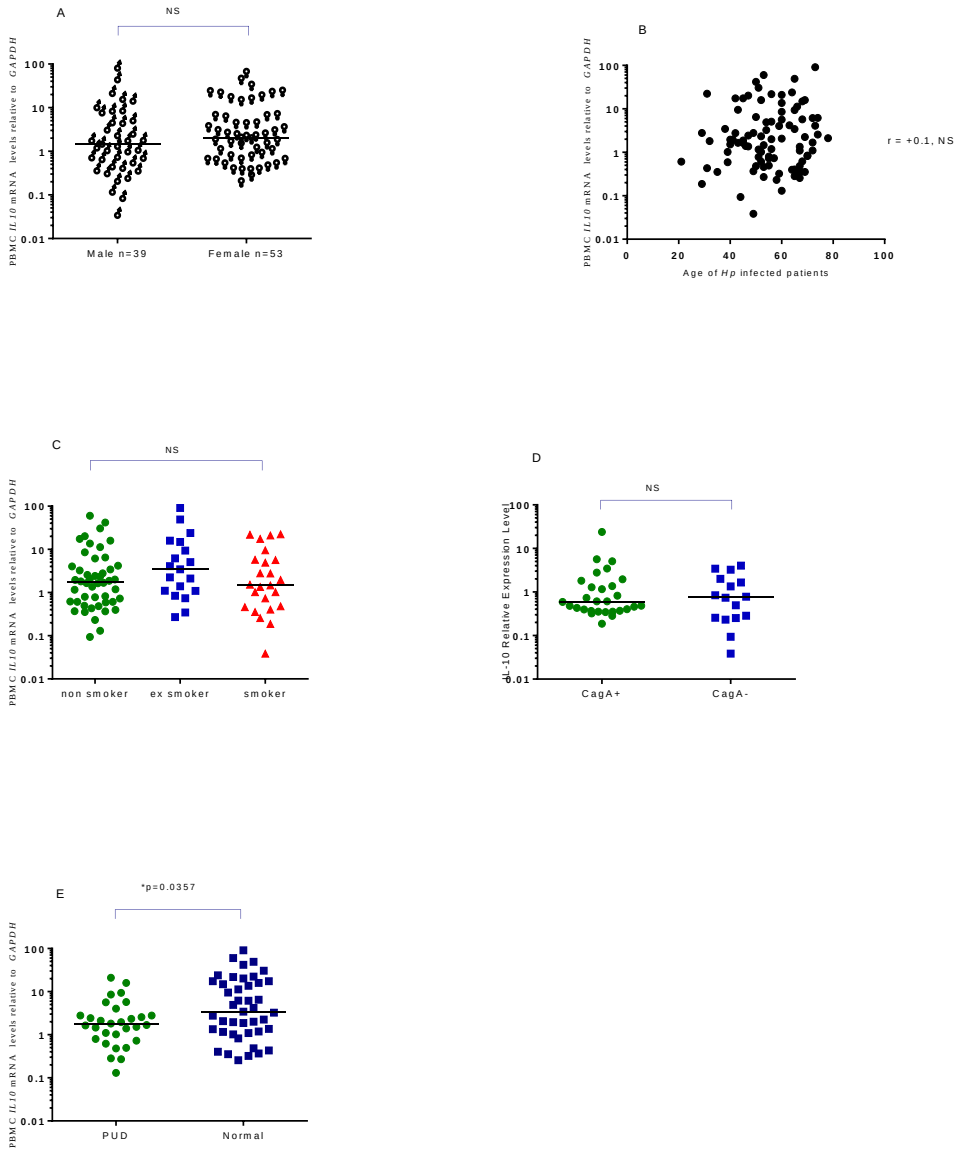


Figure 6.6 Relationship of sex, age, smoking, *cagA* status and peptic ulcer disease with PBMC *mRNA* expression levels

IL10 mRNA expression levels were correlated with gender (A), age (B), smoking status (C), *CagA* status (D) and Peptic ulcer disease status (E). Horizontal bar represent medians. The Mann Whitney U test was used to calculate statistical significance. Correlations were examined using the Spearman's rank correlation test. NS represent non-significant p values.

6.5B: *IL10* mRNA levels before and after eradication therapy

No study has investigated T lymphocyte response and total IgE levels post eradication. Blood samples were taken from 50 *H. pylori* positive patients to determine their response to eradication treatment. Figure 6.7A did not show a difference in mRNA *IL-10* expression levels after eradication therapy in the six individuals who completed the two year follow up, but the general trend was a reduction. Similarly there was no difference in mRNA *IL-10* expression levels in patients currently still followed up in the study and have provided blood samples at specified time points (Figure 6.7B). There was no statistical significant difference in mRNA *IL10* expression at 2, 6, 12 and 24 months after *H. pylori* eradication treatment.

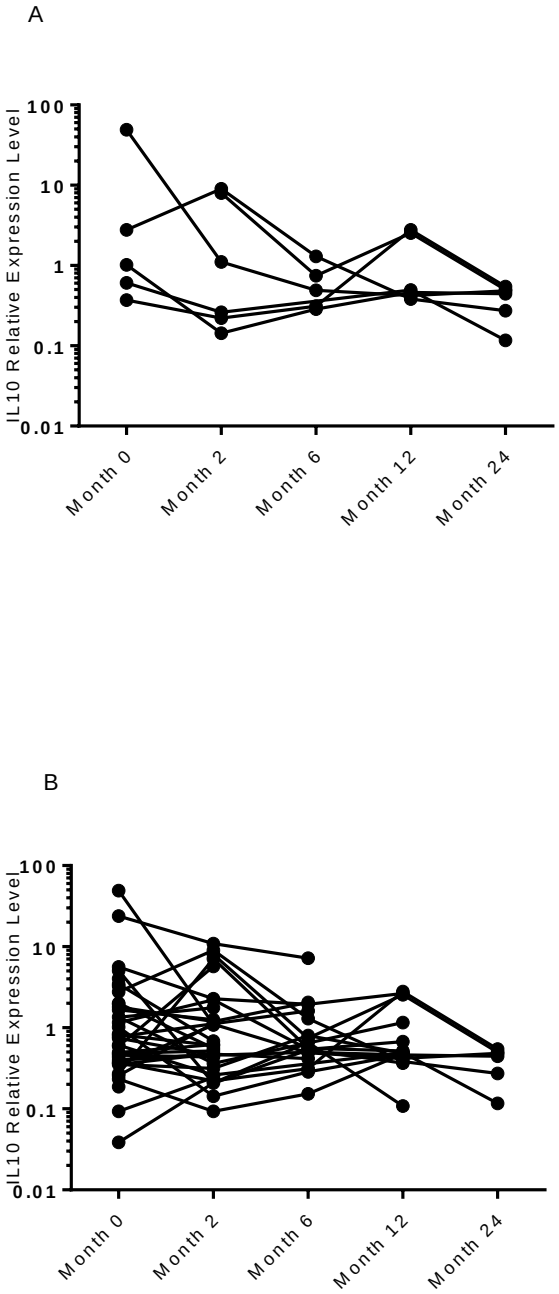


Figure 6.7 mRNA *IL10* levels before and after eradication treatment

Data from 6 patients who completed the study and provided blood samples over a 2 year period (A) and all patients who have completed or currently still in the study before and after treatment (B). The Wilcoxon test was used to calculate statistical significance.

IL10 mRNA levels remained unchanged in individuals exposed to antibiotics but remained *H. pylori* positive (Figure 6.8).

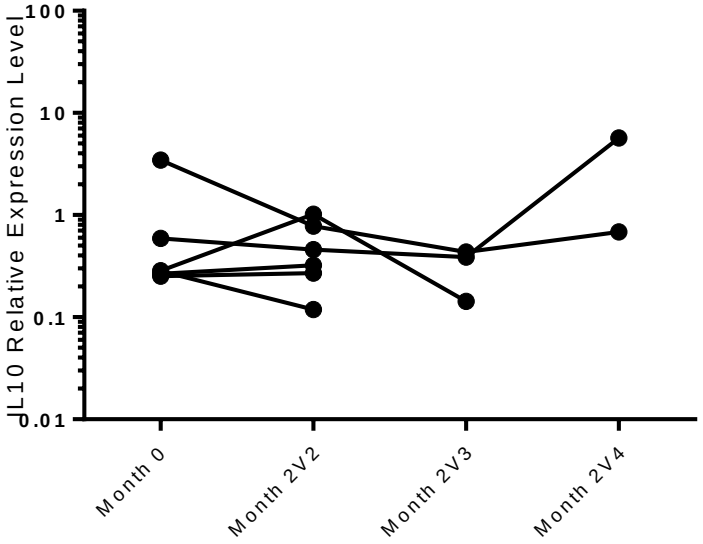


Figure 6.8: *IL10* mRNA levels in 6 individuals requiring multiple courses of *H. pylori* eradication treatment

6 patients received multiple courses and each remained positive until last time point.

6.5C: Quantifying Treg response before and after treatment using flow cytometry

There was no difference in total peripheral blood Tregs (Figure 6.9) during *H. pylori* infection and after successful eradication therapy. There was a significant difference in the percentage of $CD4^{+}IL10^{+}$ events (IL10 expressing CD4+ lymphocytes) among gated lymphocytes before and at 12 months post eradication treatment (p=0.0015). The median value of $CD4^{+}IL10^{+}$ events among gated lymphocytes at month 0 was 0.19 and 0.11 at month 12, a 0.42-fold decrease. There was a significant difference in the percentage of $IL10^{+}$ events amongst $CD4^{+}$ gated lymphocytes (markers of Tregs) before and after treatment (p=0.0029). The median values of $IL10^{+}$ events amongst $CD4^{+}$ gated lymphocytes at month 0 and 12 were 0.42 and 0.25 respectively, representing a 0.4-fold decrease, (Figure 6.10).

There was a significant difference among the percentage of $CD4^{+}CD25^{hi}IL10^{+}$ events among gated lymphocytes before treatment and 12 months post-treatment (p=0.0029). The median values of $CD4^{+}CD25^{hi}IL10^{+}$ events among gated lymphocytes at month 0 and month 12 were 0.08 and 0.03 respectively, representing a 0.63-fold decrease, (Figure 6.11). There was not a significant difference in the percentage of Foxp3 events in $CD4^{+}$ gated lymphocytes, or percentage of Foxp3 that are $CD4^{+}CD25^{hi}$ (data not shown). The proportion of $CD25^{hi}IL10^{+}$ and $Foxp3IL10^{+}$ expressed from $CD4^{+}$ gated lymphocytes did not significantly change before and after treatment (Figure 6.12). There was no significant difference in $CD4^{+}IL10^{+}$ events when CagA status, gender, peptic ulcer disease status and smoking status was investigated (Figure 6.13).

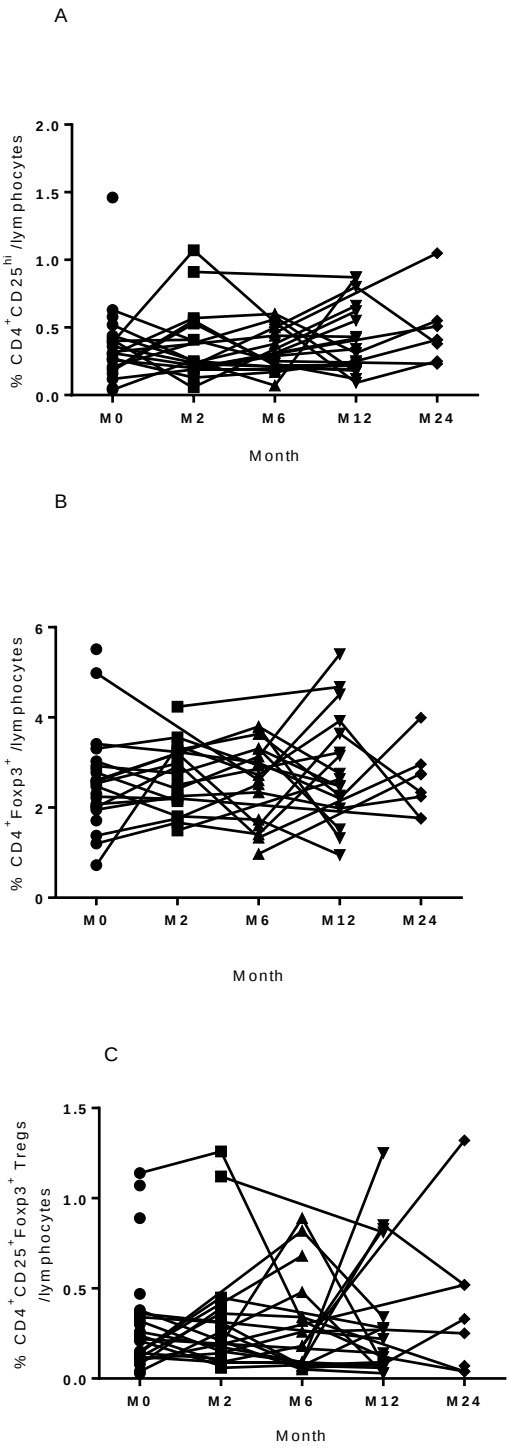


Figure 6.9: Regulatory T cells in peripheral blood of 34 patients before and after treatment

The percentage of $CD4^{+}CD25^{hi}$ events among gated lymphocytes (A), the percentage of $CD4^{+}Foxp3^{+}$ events among gated lymphocytes (B), the number of $IL\text{-}10^{+}CD4^{+}CD25^{hi}$ Tregs (C). The Wilcoxon test was used to calculate statistical significance.

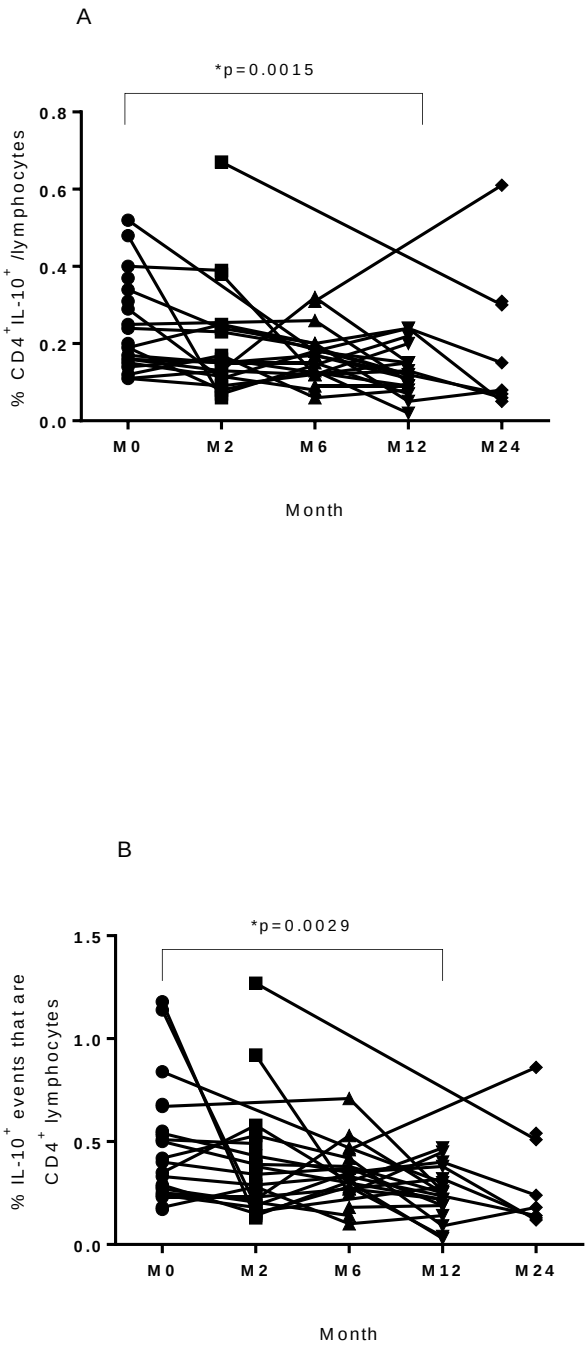


Figure 6.10 IL10 specific regulatory T cells response before and after treatment in 34 patients

The percentage of $CD4^+IL10^+$ events among gated lymphocytes (A). The percentage of $IL10^+$ events amongst $CD4^+$ gated lymphocytes (B). The Wilcoxon test was used to calculate statistical significance.

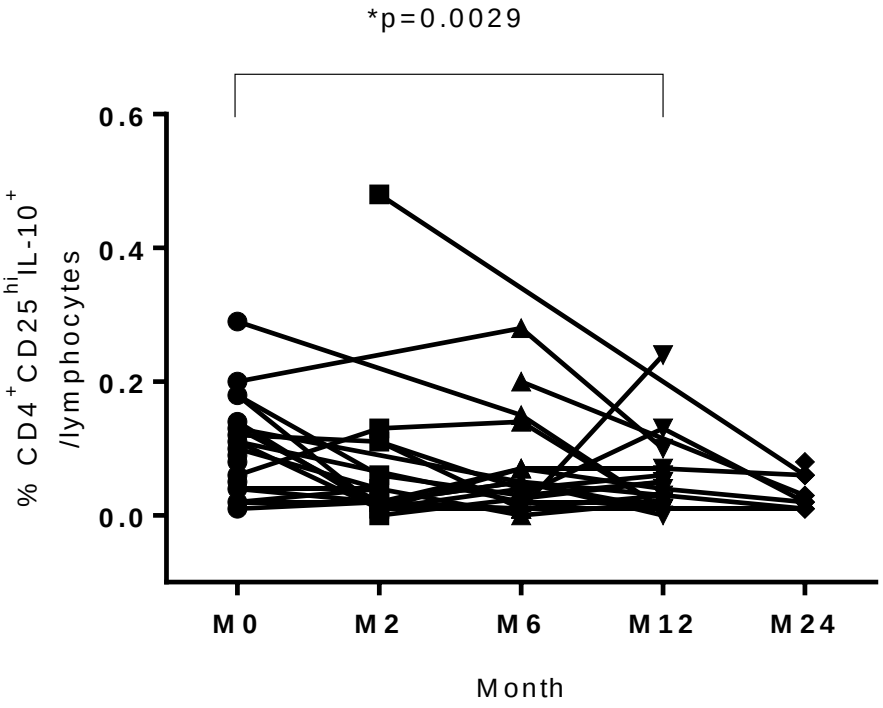


Figure 6.11 $CD4^{+}CD25^{hi}IL10^{+}$ events among gated lymphocytes before treatment and 12 months in 34 patients

The percentage of $CD4^{+}CD25^{hi}IL10^{+}$ events among gated lymphocytes. The Wilcoxon test was used to calculate statistical significance.

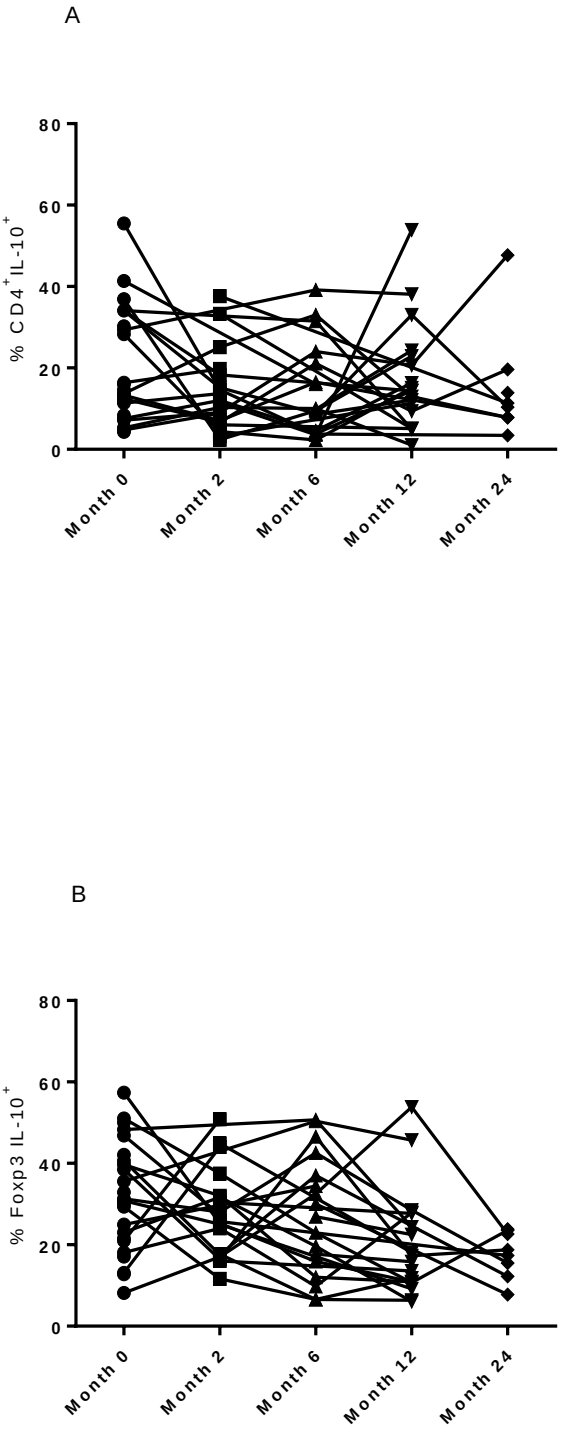


Figure 6.12: Percentage of $CD25^{hi}IL10^{+}$ and $Foxp3IL10^{+}$ expressed from $CD4^{+}$ gated lymphocytes for 34 patients

The percentage of $CD25^{hi}IL10^{+}$ (A) and $Foxp3IL10^{+}$ (B) expressed from $CD4^{+}$ gated lymphocytes. The Wilcoxon test was used to calculate statistical significance.

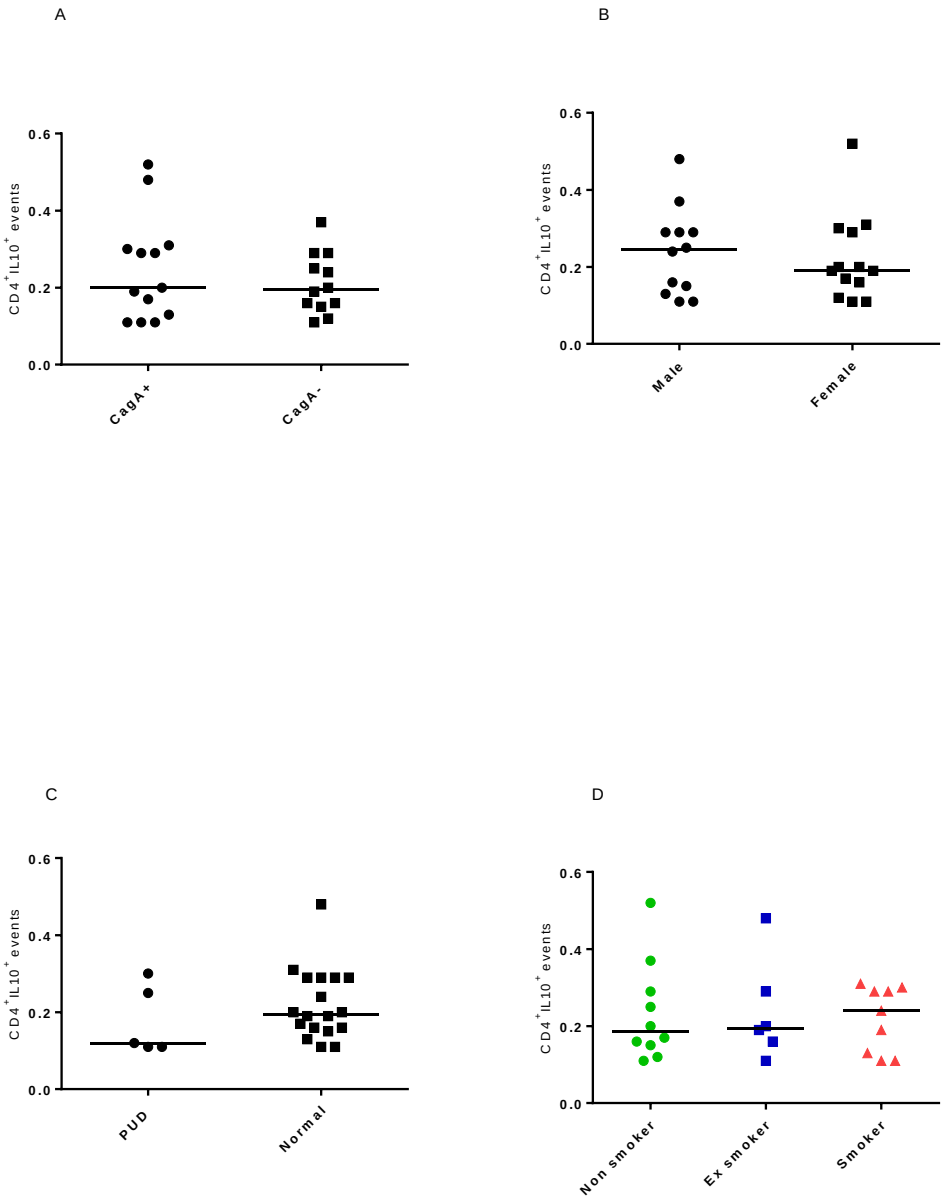


Figure 6.13 $CD4^+IL10^+$ events according to CagA status, gender, disease and smoking status for 25 patients

$CD4^+IL10^+$ events were grouped by CagA status (A) gender (B), peptic ulcer disease status (C) and smoking status (D). Horizontal bar represent medians. The Mann Whitney U test was used to calculate statistical significance.

Treg response levels remained similar in individuals exposed to antibiotics but who remained *H. pylori* positive, (Figure 6.14).

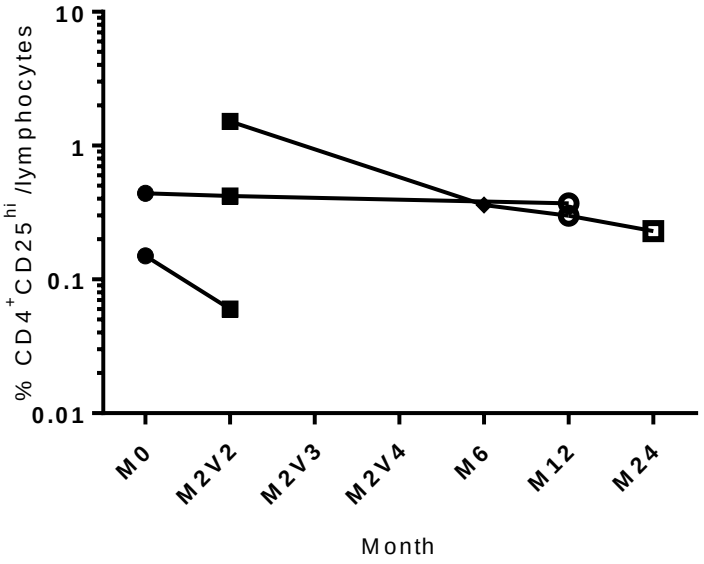


Figure 6.14: Percentage of CD4+CD25hi events amongst lymphocytes in 3 individuals requiring multiple courses of *H. pylori* eradication treatment

Three patients received multiple courses but two were successfully eradicated and returned for month 6, 12 and 24 visits.

6.5D: IgE levels in uninfected and *H. pylori* infected individuals and after eradication treatment

We measured IgE levels in uninfected and *H. pylori* infected individuals to try establish whether there was a difference and if this changes post eradication. Data from section 5a confirm previous findings that PBMC *IL10* mRNA levels are higher in *H. pylori* infected patients. Recently published data from the Nottingham *H. pylori* group did not find a significant difference between the plasma IgE concentrations of uninfected and *H. pylori* infected individuals. There was a difference according to *H. pylori* virulence genotype; those colonised with the more virulent *cagA+* and *vacA+* strains had lower total IgE plasma concentrations (Hussain et al., 2016).

To investigate the correlation between plasma IgE concentrations and *H. pylori* infection plasma was collected from peripheral blood as described in section 6.4.14. 31 uninfected and 94 *H. pylori* infected patients IgE concentrations were determined using a commercial ELISA kit.

Figure 6.15A shows plasma IgE concentrations between uninfected and infected patients. Total IgE levels were similar (median 67.1ng/ml uninfected, 67.2ng/ml infected) between the two groups of patients confirming previous published findings. Figure 6.15B shows that *cagA+ H. pylori* infected individuals (median 47.2ng/ml) had a 1.6-fold lower plasma IgE concentration when compared *cagA- H. pylori* infected individuals (p=0.0178). This was similar to the findings from the Nottingham *H. pylori* group but they described a threefold difference (Hussain et al., 2016). To assess any confounding seasonal variability in IgE concentrations, IgE concentration was correlated with the month that the blood sample was collected (Figure 6.15C). When plasma IgE concentrations were compared with mRNA *IL10* expression levels, there was no correlation (Figure 6.15D). There was also no significant correlation when IgE was compared to the percentage of $CD4^{+}CD25^{hi}IL10^{+}$ events amongst lymphocytes before treatment at month 0 (Figure 6.16).

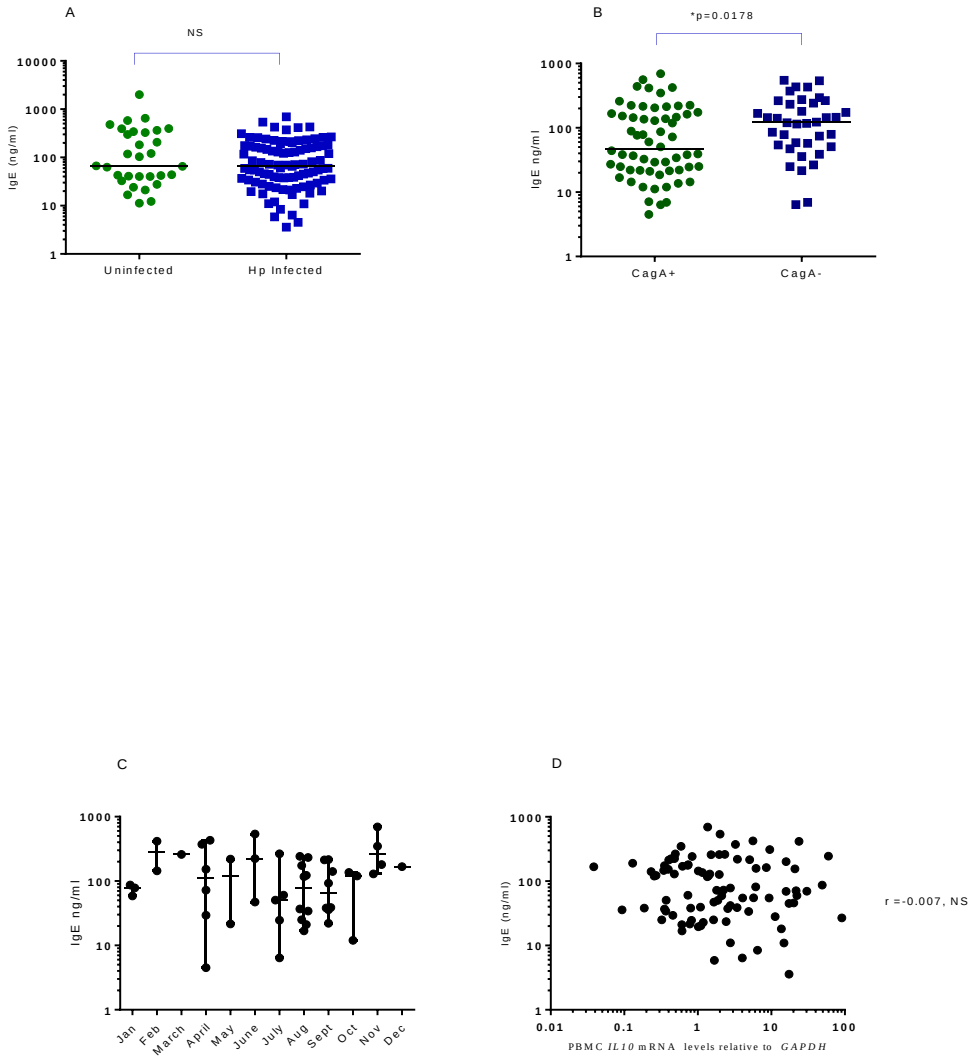


Figure 6.15 Plasma IgE levels of uninfected and infected individuals, association with *cagA* status, and season and PBMC *IL10* expression levels of *H. pylori* infected patients

IgE levels in *H. pylori* infected and uninfected indivduals (A). *H. pylori* infected individuals were stratified according to *cagA* status (B). *H. pylori* infected individuals IgE levels were correlated with the month of the year the blood sample were collected (C). Total IgE concentration and mRNA *IL10* expression levels were plotted against each other (D). Medians are represented by horizontal bars. The Mann Whitney U test was used to calculate statistical significance of differences. Correlations were examined using the Spearman's rank correlation test.

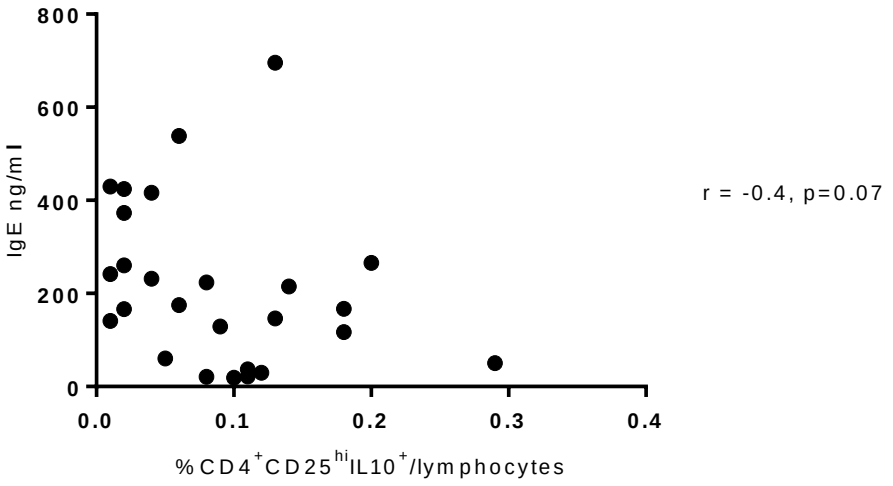


Figure 6.16 *H. pylori* infected individuals IgE levels compared with % $CD4^{+}CD25^{hi}IL10^{+}$ events amongst lymphocytes

Total IgE concentration and the percentage of $CD4^{+}CD25^{hi}IL10^{+}$ events amongst lymphocytes were plotted against each other. Correlations were examined using the Spearman's rank correlation test.

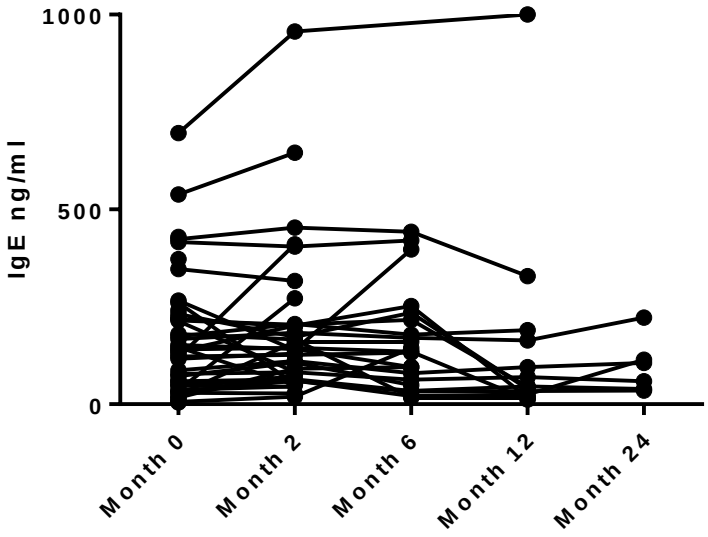


Figure 6.17 Plasma IgE levels before and after eradication treatment

Data on total IgE levels pre and up to 24 months post *H. pylori* eradication treatment was obtained. The Wilcoxon test was used to calculate statistical significance.

IgE levels do not increase after eradication treatment. There was also no significant difference in total IgE levels before and follow up time points (Figure 6.17). There was no statistical significant difference in total plasma IgE levels at 2, 6, 12 and 24 months after *H. pylori* treatment.

IgE levels remained similar in individuals exposed to antibiotics but who remained *H. pylori* positive (Figure 6.18).

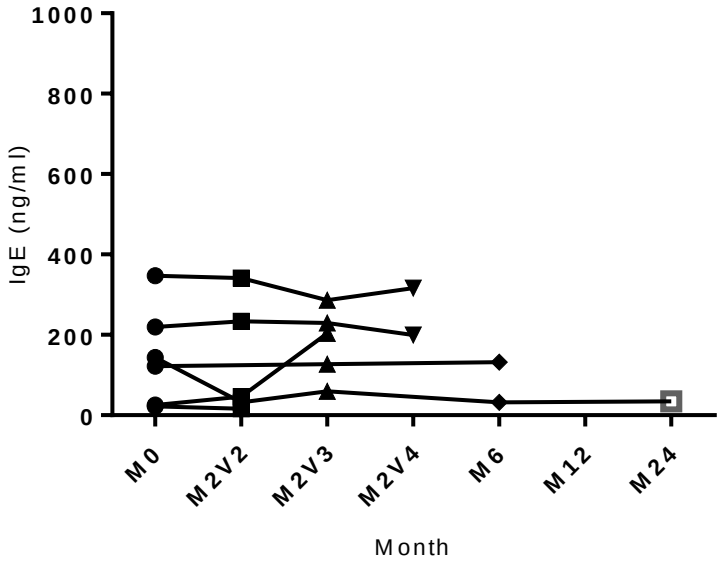


Figure 6.18 IgE levels in 6 individuals requiring multiple courses of *H. pylori* eradication treatment

6 patients received multiple courses and each remained positive until last time point, but two were successfully eradicated and returned for month 6, 12 and 24 visits.

6.6 Discussion

As previously shown, *H. pylori* infection has effects on the peripheral immune system. Those patients with evidence of PUD had a 1-fold decrease (median 1.7) in *IL10* mRNA expression level when compared to those without PUD (median 3.4, p=0.0357). This decreased *IL10* response maybe associated with an increased likelihood of PUD as the equilibrium is disrupted and the pro inflammatory response dominates (Robinson et al., 2008). These study data

confirm previous published findings on the relationship between PUD and *IL10* mRNA expression levels seen in the gastric mucosa. This study however did show that *H. pylori* infected individuals had higher *IL10* mRNA levels as previously reported (Robinson et al., 2008, Serrano et al., 2013).

The percentage frequency of $CD4^{+}CD25^{hi}$ Tregs overall also decreased after successful eradication treatment but this was not statistically significant. Although our results did not confirm our hypothesis, this was the first time these observations have been reported. However, $IL10^{+}$ secreting Tregs did significantly decrease after eradication. The fold difference was 0.4 and 0.63 respectively, and although small we cannot ascertain the exact biological effects due to the relatively short follow up period. There was an almost equal gender mix, no correlation between ages or season when these samples were taken so these confounders are unlikely to be responsible for the observed effects. The presence of PUD and cagA status significantly influenced *IL10* mRNA levels and IgE levels but none of these factors influenced Treg frequencies quantified by flow cytometry.

Treg markers $CD4^{+}CD25^{hi}$ have previously been shown to suppress the immune system and are upregulated in those infected with *H. pylori* (Baecher-Allan et al., 2001, Cao et al., 2003, Lundgren et al., 2005, Mitchell et al., 2012). We showed that in *H. pylori* infected individuals there was a general trend in these markers to decrease after eradication. *IL10* mRNA levels were similar in the *H. pylori* infected and uninfected groups but this was in contrast to previous studies which showed an increase in the *H. pylori* infected group (Robinson et al., 2008, Lundin et al., 2007, Windle et al., 2005). With regards to FoxP3 Hussain *et al.* found no difference in mRNA levels between the *H. pylori* infected and uninfected (Hussain et al., 2016) groups. We also found no statistical difference before and after eradication using flow cytometry. However, it is important to note that the investigation of Tregs is limited due to the small number in the peripheral circulation.

$CD4^{+}CD25^{hi}IL10^{+}$ Tregs are known to be inversely associated with antral inflammation severity scores. Therefore, IL10 has some mechanistic control over an excessive immune response influencing disease outcome (Robinson et al., 2008). Unfortunately, as our patients only donated blood samples it was not possible to check for this association and to see if this altered after eradication therapy.

In view of our results and relationship with antral inflammation, quantifying Treg responses could be a novel way to predict those individuals who are at risk at developing disease. Patients with a high Treg response may be less likely to develop disease so if they are colonised with *H. pylori* and if in future beneficial effects are determined, eradication treatment could be avoided.

Our study did not see a decrease in total IgE levels in the *H. pylori* infected group as previously described (Linneberg et al., 2003). This may be because only a limited number of patients recruited into the study reported a history of atopy at screening (18%) and none were clinically significant requiring specific treatment or affecting quality of life. Therefore, our study did not have the power to detect any changes between the *H. pylori* infected and uninfected groups. Also evidence from the literature about the link between *H. pylori* and atopy was obtained in children but our study investigated adults. Furthermore it may be argued that *H. pylori* infection could be a confounder for other infections with similar risk factors (Hussain et al., 2016).

When looking at the effect of eradication therapy on Treg responses, we found that total IgE did not change but IL10 specific Treg responses did reduce. Hussain *et al*. found total and allergen specific IgE were lower in individuals when stratified for those with strong Treg responses (Hussain et al., 2016).

H. pylori can cause the differentiation of naïve T cells to Tregs by acting on dendritic cells. This may lead to protection against extra gastric inflammatory conditions such as asthma (Oertli et al., 2012). Lowering of IL10 Tregs can lead to colitis and hypersensitivity (Rubtsov et al., 2008). We have shown that

frequencies of IL10 producing Tregs are reduced post eradication. Thus if IL10 is providing protection against allergic or autoimmune conditions, this reduces future anti-inflammatory function. Most *H. pylori* related disease is seen in adults and are rarely seen in children. Also epidemiological data have shown potential protection against GORD and allergic conditions, so in the future we may need to consider possible detrimental effects prior to eradication therapy (Hojsak and Kolacek, 2014). Essentially, eradicating *H. pylori* may pose a clinical dilemma in some individuals as we may iatrogenically increase the risk of other disease. However, the next question is how to determine who is at risk of these diseases: considering family history, clinical profiles or measured immune responses. It is difficult to know at present whether the changes seen in our data will have clinical implications without longer term follow up.

Ondex ® founded by Prof Barry Marshall is a company who are developing natural immune modulatory products such as vaccines targeting allergic conditions. The aim is to develop a non-viable form of *H. pylori* that retains its immune regulatory properties but without the disease risk. If successful they could potentially develop new treatments for allergic conditions using *H. pylori* as the target. If successfully developed it would provide insight into longer term immune effects of *H. pylori* infection. It would also provide data on whether the immune system is set at a certain age or can be altered. This research could help determine whether auto-immune protection from *H. pylori* is limited or lifelong. It is possible *H. pylori* is beneficial in childhood and leads to potential disease later in life so treatment could be avoided if possible until a later age (Blaser et al., 2008). Our data suggest this protection is unlikely to continue if *H. pylori* is eradicated, therefore infection may still be needed for Treg response.

Strengths

This was the first interventional study to our knowledge investigating Treg and total IgE responses before and after eradication in humans. Novel data were collected on Treg response post eradication and have confirmed that *H. pylori*

eradication has systemic effects on the immune system. The same patients were followed up over time, demonstrating a more personalised Treg/IL-10 response, rather than studying a general cohort which would be less likely to show an emerging trend due to large variation in human host immune responses.

Two sensitive tools, flow cytometry and RT qPCR, were used for quantifying gene expression to assess Treg markers and they both showed a similar downward trend after eradication. Gating analysis was also done consistently between patients using published gating strategies.

Limitations

Patients were selected from those referred for upper GI endoscopy, therefore are not representative of the general population. We also planned to collect preliminary data on specific asthma measurements before and after treatment but due to the low number of patients with asthma this was not possible.

Exposing patients to antibiotics is likely to alter normal gut microbiota and the changes seen in the Treg response may not exclusively be due to the successful eradication of *H. pylori*. The duration of impact on the gut flora population is unknown. Also potential confounders such as host polymorphisms and other infections were not taken into account. Patients were followed up for a maximum of two years due to the feasibility of the study. However, the duration of immunological memory is unknown so the changes seen at 12 months could be transient and longer follow up data are required.

Another limitation was that there was no uninfected group of patients followed up over the same time frame to act as a control because this was a pilot study. Also we were unable to have an untreated positive group due to ethical considerations as all patients were referred with symptoms or evidence of peptic ulcer disease so remaining untreated for further two years

was not ideal. The failed eradication group was also too small a population to act as controls and only one patient remained positive despite multiple antibiotic exposures.

We found a huge variety in the Treg markers which makes it unclear as to whether differences seen are biological significant. The study was powered on calculations based on infected and uninfected patients so this raises the possibility that the study may have been underpowered. A greater number of patients would have allowed for the stratification of individuals by initial high or low Treg response. These two groups could then have been analysed separately to investigate whether those with an initial high Treg response change significantly over time and whether this has any impact on autoimmune disorders.

Finally, we excluded samples where the isotopes were greater or less than 50 thousand events or showed inconsistent staining. Also the IL10 isotype controls were previously used in our group work but it was obtained from a different company than the IL10 antibody: a potential source of variability. There were no isotype controls used for CD25 and CD4 in contrast to the other antibodies.

Planned future work

This study is still ongoing and many patients will be returning for the month 12 and 24 visits. We plan to continue to process samples for flow cytometry, total IgE and *IL10* mRNA. With stored samples we plan to perform *H. pylori* IgG serology on plasma to assess for changes in titre levels over a two year period, therefore providing some insight into the antibody response. We plan to also measure mRNA levels for *IL17*, *IFN Gamma* and *IL4.* Repeat cellular staining will be carried out using frozen stored PBMC using the same antibody markers to help confirm findings described here and to ensure there is no variations in results due to differing processing technicians. Once the study is completed we will apply to the local research ethics committee for permission to enable us to contact patients for year five follow up bloods.

6.7 Conclusions

- *IL10* mRNA levels were significantly higher in *H. pylori* infected individuals
- *IL10* mRNA levels were significantly lower in PUD, but other factors investigated did not significantly alter IL10 levels
- General Treg frequencies decrease over time after successful eradication treatment but was not statistically significant
- *H. pylori* specific IL10 Tregs ($CD4^{+}CD25^{hi}IL10^{+}$) significantly decrease over time after successful eradication treatment
- Treg frequencies were similar when potential cofounders were investigated
- IgE levels were significantly higher in *cagA*- individuals, but no other factor investigated significantly altered these IgE levels
- IgE levels did not change after successful eradication treatment

Chapter 7: Final Conclusion

H. pylori is a type 1 carcinogen and the morbidity associated with GI haemorrhage from peptic ulcer disease is significant. The current challenge in clinical practice is to detect *H. pylori* associated disease at an early stage so curative intervention can be carried out.

This work has shown that advanced endoscopy imaging techniques and serology can diagnose premalignant disease. A new proposed simplified classification system for intestinal metaplasia is highly accurate and user friendly, and could potentially be used in clinical practice. This classification system can be used by endoscopists at all grades of training, allowing universal description which would aid training and ultimately improve disease pick up rate.

The data from investigating the role of systemic hepcidin suggests this may not be the most important mediator in patients with IDA and *H. pylori* infection. We have shown that mucosal hepcidin expression is likely to be a key area to investigate and we plan to assess mucosal pro-inflammatory cytokine production in the near future.

The studies have demonstrated that in patients with colorectal cancer *H. pylori* and *cagA* status does not influence a patients response to oral or intravenous therapy suggesting that *H. pylori* is likely to cause only a mild anaemia and highlights the importance of excluding other causes during initial investigation.

Finally, the data have provided the first insight into the immunological effects of *H. pylori* eradication, with a reduction in $IL10^{+}$ Tregs suggesting that the immune system is influenced even in adulthood. This has potential consequences for immune mediated disease. The long-term effects of this were not studied but our pilot data provide a starting point for further work.

In summary, we need to better establish ways of identifying individuals at risk of disease without potentially aggravating allergic disorders in the process with eradication. At present, more evidence is needed to allow clinicians to

decide whether alteration of current clinical guidelines on eradication therapy is needed.

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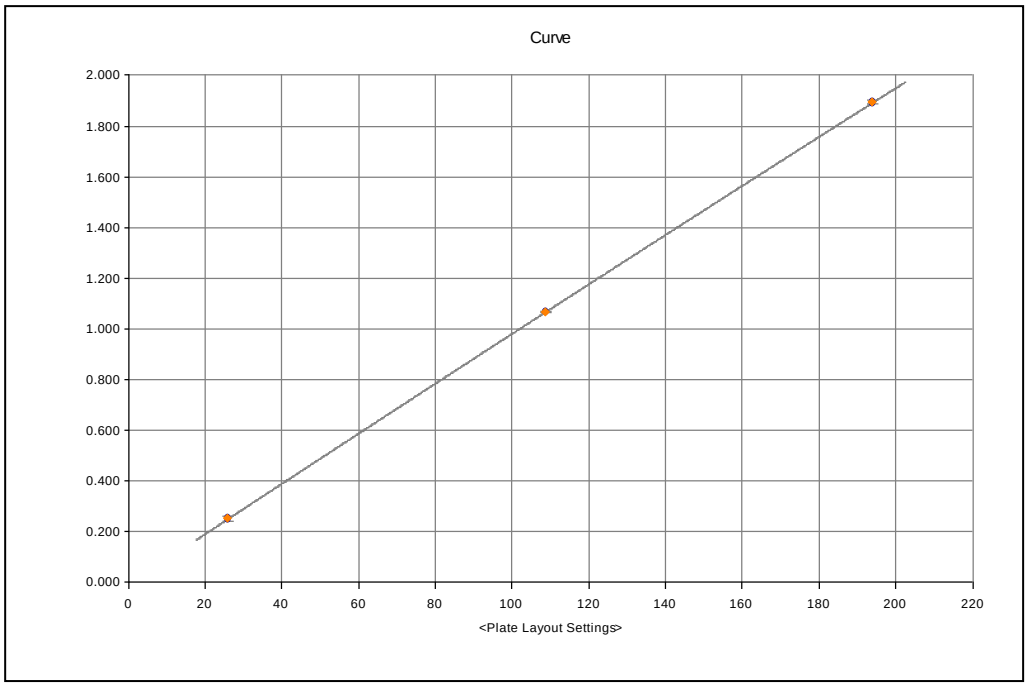
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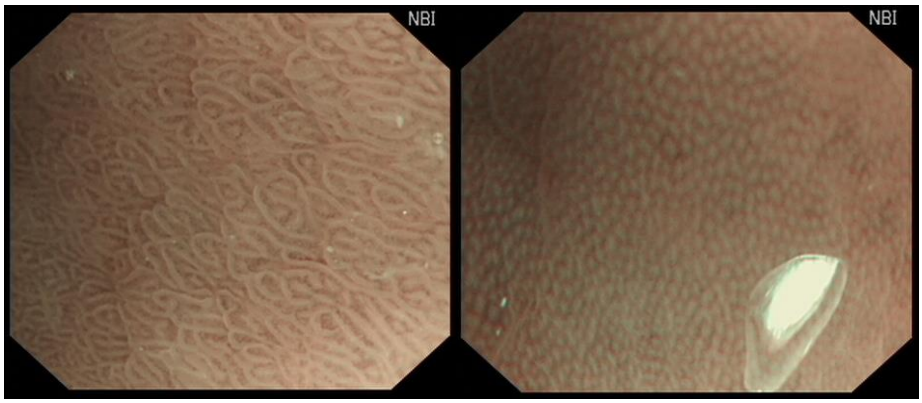
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Appendices

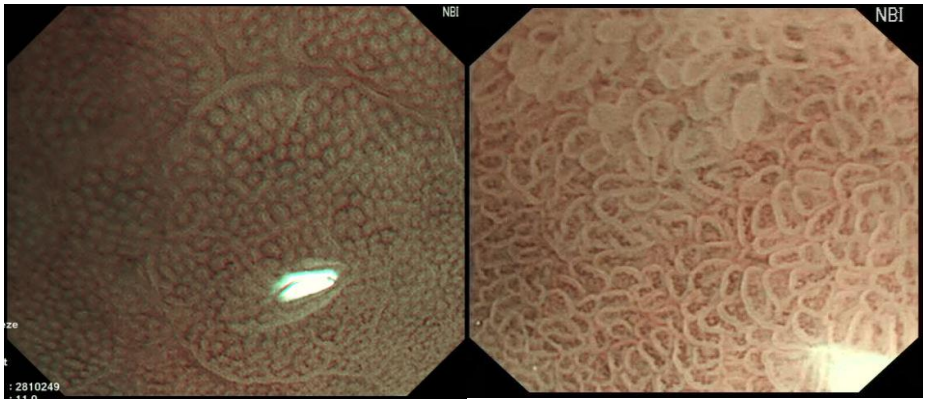


Appendix 1 Example of a typical calibration curve for Pepsinogen I

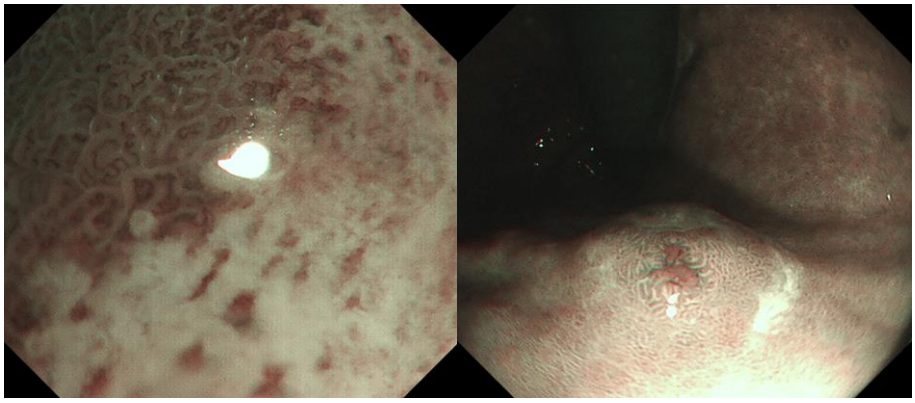
Absorbance 450nm (y axis) and PGI concentration (µg/l) (x axis)



A B



C D



E F

Appendix 2 Endoscopic NBI classification (Pimentel-Nunes et al., 2016)

A- Pattern Aa (normal antrum) regular circular mucosa surrounding regular vessels

B- Pattern Ab (normal gastric corpus) regular circular mucosa surrounding regular vessels

C- Pattern Ab+ (*H. pylori* gastritis and normal corpus mucosa) variable vascular density

D- Pattern Ba (intestinal metaplasia in antrum)

E- Pattern Bb (intestinal metaplasia in corpus) with some light blue crest

F- Pattern C (low grade dysplasia) irregular mucosa and vessels

Appendix 3 Nucleic acid concentration and absorbance at 260 to 280nm

Concentration ng/µl	260/280 ratio	260/230
63.55	2.12	1.7
100.57	2.08	2.28
113.39	2.08	1.8
41.67	2.11	1.14
63.12	2.21	0.48
130.51	2.12	1.73
70.44	2.08	2.02
104.97	2.06	2.14
108.9	2.11	1.14
60.01	2.17	2.01
34.07	2.2	1.22
79.74	2.08	2.21
35.35	2.1	1.95
75.61	2.09	2.05
40.9	2.06	2.92
63.58	2.04	1.66
131.36	2.07	2.19
125.31	2.06	1.82

42.55	2.19	1.29
32.8	2.01	1.12
56.24	2.05	2.27
152.27	2.08	1.89
39.16	2.15	0.66
90.48	2.11	1.87
40.9	2.06	2.92
63.58	2.04	1.66
131.36	2.07	2.19
125.31	2.06	1.82
86.52	2.13	2.04
33.85	2.11	2.94
84.67	2.07	1.25
51.13	2.06	1.95
89.19	2.07	1.76
61.68	2.06	1.29
46.16	2.06	1.4
130.16	2.04	2.2
130.16	2.04	2.2
103.35	2.13	2.11
95.95	2.06	2.17
25.42	2.25	0.86
63.58	2.07	0.97
54.04	2.03	0.6
113.74	2.05	2.28
89.71	2.08	2.15
81.28	2.1	2.38
102.18	2.11	1.42
156.26	2.08	2.09
73.21	2.02	2.4
98.2	2.15	1.48
112.46	2.06	1.01
91.59	2.06	1.65
100.66	2.12	0.73

89.71	2.08	2.15
81.82	2.08	1.53
73.25	2.11	1.9
107.03	2.07	1.18
83.06	2.1	2.25
52.48	2.1	2.66
78.74	2.09	1.35
89.61	2.09	2.09
114.13	2.08	1.63
112.81	2.08	1.7
156.26	2.08	2.09
86.71	2.07	1.46
54.71	2.06	1.52
58.67	2.16	2.13
59.29	2.07	1.58
94.05	2.06	1.21
60.77	2.21	1.45
88.07	2.11	1.62
59.03	2.21	2.3
64.39	2.06	3.2
98.47	2.12	1.71
63.85	2.19	1.42
108.7	2.09	2.16
115.89	2.08	1.73
94.31	2.1	1.68
69.95	2.18	1.46
58.16	2.1	2
53.99	2.11	0.73
36.87	2.22	1.12
91.23	2.11	1.96
87.49	2.07	1.22
44.23	2.04	1.13
60.64	2.15	1.52
78.57	2.17	2.29
87.49	2.07	1.22
65.64	2.16	1.35
111.24	2.11	1.23

67.8	2.07	1.47
98.73	2.14	1.01
94.43	2.11	1.23
40.29	2.03	1.5
91.7	2.07	2.21
65.59	2.06	1.27
78.88	2.2	0.79
68.28	2.19	0.21
52.72	2.09	1.96
45.03	2.04	1.08
34.97	2.21	0.27
81.45	2.08	1.94
84.13	2.05	1.66
68.13	2.04	1.66
37.52	2.01	1.32
70.16	2.05	1.52
74.8	2.22	1.2
64.56	2.05	1.55
54.22	2.03	0.94
86.3	2.04	1.74
56.07	2.02	1.52
105.01	2.14	1.04
64.78	2.04	1.18
56.8	2.19	0.75
72.52	2.1	1.25
54.27	2.05	1.67
41.76	2.25	1.27
69.59	2.09	1.36
82.59	2.07	1.34
44.1	2.24	0.49
79.62	2.06	1.5
60.58	2.17	1.53
62.87	2.07	1.25
79.14	2.16	1.17
97.87	2.15	1.5
136	2.18	0.49
42.11	2.21	1.03

45.9	2.21	1.25
78.54	2.14	1.38
82.1	2.16	1.52
40.54	2.17	0.73
74.43	2.21	0.77