Determinants of *Helicobacter pylori* density in the human stomach and implications in disease

Sapna Rohitbhai Patel

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

July 2011

ABSTRACT

Introduction and aims:

Helicobacter pylori establishes a chronic infection in the stomach of almost half the world's population. A small proportion of patients go on to develop peptic ulcer disease and in some cases gastric cancer. Numerous factors influence the outcome of infection, including host, bacterial and environmental factors. The impact of bacterial colonisation density on the outcome of disease is poorly understood. The aim of this study was to investigate the determinants of bacterial density and the implications in disease.

Methods:

Gastric biopsies were collected from 50 *H. pylori* infected patients attending the Queen's Medical Centre, Nottingham for a routine upper GI endoscopy. Patient disease status was determined as having gastritis, duodenal ulcer disease (DUD), or gastric ulcer disease (GUD). Histological analysis for inflammation, activity, *H. pylori* density, atrophy and intestinal metaplasia were assessed according to the Sydney scoring system. Bacterial strains were cultured from gastric biopsies. Isolated bacterial DNA was used for genotyping strain virulence for CagA, VacA and DupA status. In order to determine accurate levels of colonisation in gastric tissue samples, a quantitative real-time PCR (qPCR) assay was developed based on the quantification of *H. pylori 16S rRNA* copy number, relative to the number of human *GAPDH* copy numbers. Quantification of *IL8*, *IL10*, *TGFβ*, *FOXP3*, *hβD1* and *LL37* mRNA levels in gastric tissue were assessed by real-time RT-PCR. The impact of host factors on bacterial density was assessed *in vitro* using *H. pylori* infected or uninfected AGS gastric epithelial cells, treated with recombinant cytokine. Colony forming units (CFUs) were enumerated in culture suspensions. The *in vitro* model was also used to examine the impact of *cagA*, *cage*, and *vacA* isogenic deletion mutant strains on bacterial density. Also, *hβD1* expression levels were measured in culture suspensions, and IL-8 secretion levels in were assessed by ELISA.

Results and conclusions:

The pattern of colonisation in the gastric mucosa was found to be associated with the disease profile that develops, with an antral-predominant colonisation was associated with DUD, whereas corpus-predominant colonisation was associated with GUD. Bacterial factors that may drive the pattern of bacterial density in the stomach were then investigated, and strains expressing the *dupA* virulence factor were coincident with antral-predominant colonisation in the gastric mucosa. *cagA* and *vacA* were not found to be associated with bacterial density *in vivo* or *in vitro*. To assess the relationship between colonisation density and the host response, firstly *IL8* mRNA expression levels were measured in gastric biopsies. An inverse relationship between bacterial load and expression of this pro-inflammatory cytokine was observed, but only at high *IL8* expression levels and with higher *H. pylori* density. The inflammatory cytokines IL-1β, TNFα, IL-17 and IL-22 did not impact CFU counts *in* vitro. No association was observed between bacterial load and expression levels of the immunosuppressive genes *IL10, TGFB1* and *FOXP3*. However, increased CFU counts were found on treatment of *H. pylori* infected AGS cells with recombinant IL-10 and TGFβ *in vitro*. Also, reduced expression of these immunosuppressive genes was associated with the presence of atrophy and intestinal metaplasia. In addition, *IL10* and *FOXP3* expression levels were negatively associated with mononuclear cell (MNC) infiltration.

Reduced expression levels of the antimicrobial peptide (AMP) defensin *hβD1* were found with *H. pylori* infection *in vivo* and *in vitro*, and a negative association between *hβD1* expression and bacterial load was observed *in vivo*. A small increase in *hβD1* expression was observed in AGS cells infected with the *cag*PAI defective mutant strain in comparison to the wild type strain, suggesting a possible role for the CagPAI in modulating *hβD1* expression. In contrast, expression levels of the antimicrobial peptide *LL37* were upregulated in the *H. pylori* infected gastric mucosa.

In conclusion, the localisation of *H. pylori* in the gastric mucosa is associated with the disease profile that develops in the stomach. The patterns of colonisation observed may be influenced by the *dupA* virulence determinant. Host immune responses, were not associated with colonisation density. However, increased Treg responses may be associated with protecting the host from the deleterious

effects of inflammation. The direct modulation of bacterial load may be regulated by AMPs. In the *H. pylori* infected mucosa, reduced *hβD1* expression was observed, which may assist persistent colonisation.

LIST OF PUBLICATIONS

Hussein, N. R., Argent, R. H., Marx, C. K., Patel, S. R., Robinson, K. & Atherton, J. C. ***Helicobacter pylori dupA* is polymorphic, and its active form induces proinflammatory cytokine secretion by mononuclear cells.** *J Infect Dis* 2010, **202**, 261-269

Robinson, K., Kenefeck, R., Pidgeon, E. L., Shakib, S., Patel, S., Polson, R. J., Zaitoun, A. M. & Atherton, J. C. ***Helicobacter pylori*-induced peptic ulcer disease is associated with inadequate regulatory T cell responses.** *Gut* 2008, **57**, 1375-1385.

LIST OF ORAL PRESENTATIONS

Patel, S., Smith, K., Letley, D.P., Atherton, J.C., Robinson, K. ***Helicobacter pylori* infection is associated with a down-regulation in gastric mucosal expression of human beta-defensin 1.** 9th International Workshop on Pathogenesis and Host Response in *Helicobacter* Infections. Helsingor, Denmark 2010.

Patel, S., Kenefeck, R., Letley, D., Atherton, J.C., Robinson, K. **Low-level *Helicobacter pylori*-induced regulatory T-cell responses in the human gastric mucosa are associated with the presence of pre-malignant pathology.** International congress of mucosal immunology, 2009, Boston, USA.

Patel, S., Letley, D.P., Hussein, N., Robinson, K., Atherton, J.C. **Determinants of *Helicobacter pylori* localisation and *IL8* expression in the human gastric mucosa.** British Society of Gastroenterology annual meeting 2009, Glasgow, UK.

LIST OF POSTER PRESENTATIONS

Patel, S. **New advances in cancer research.** Research Showcase 2010, Nottingham, UK.

Patel, S., Kenefeck, R., Hussain, K., Zaitoun, A., Letley, D.P. , Atherton, J.C., Robinson, K. **Low-level *Helicobacter pylori*-induced regulatory t-cell responses in the human gastric mucosa are associated with the presence of peptic ulcer disease and pre-malignant pathology.** British Society for Immunology Annual Congress, Harrogate, UK 2010.

Patel, S., Letley, D.P., Atherton, J.C., Robinson, K. ***Helicobacter pylori* infection is associated with a down-regulation in gastric mucosal hBD1 defensin expression.** Society for general microbiology, 2010, Edinburgh, UK. and European Helicobacter Study Group XXIInd International Workshop on *Helicobacter* and related bacteria in chronic digestive inflammation and gastric cancer, 2009, Porto, Portugal.

Patel, S., Letley, D.P., Robinson, K., Atherton, J.C. **Accurate quantification of *H. pylori* density in human gastric biopsies using real-time PCR.** British Society of Gastroenterology annual meeting 2008, Birmingham, UK.

ACKNOWLEDGEMENTS

First, I would like to thank my supervisors Prof. John Atherton, Dr Karen Robinson, and Mr Darren Letley for giving me the opportunity to work with them. Most of all, I would like to thank them for their support and guidance over the years. They have been an inspiration and truly incredible mentors. It has been a pleasure to work for them.

I thank my friends and colleagues that I have been fortunate to meet during my PhD for creating such an enjoyable atmosphere to work in. I could not have asked for a better work environment.

I would also like to thank Dr Abed Zaitoun for providing support and consultation with histological analysis.

I am also grateful to the Medical Research Council for providing funding for my project.

I save my final and greatest thanks for my family – Team Patel. Thank you for always believing that there is nothing I can't achieve. For always supporting me and giving me everything and more, I am eternally grateful.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

1. INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative, motile bacterium that persistently colonises the human stomach. All infections are associated with the development of gastritis (Blaser & Parsonnet, 1994; Marshall & Warren, 1984). Although most infections remain asymptomatic, a small proportion of the infected population develop gastric and duodenal ulcer disease (Honda *et al.*, 1998; Kuipers *et al.*, 1995a), and persistence of infection has been linked with gastric cancer (Huang *et al.*, 1998; Uemura *et al.*, 2001).

The aim of this study was to determine the implications of bacterial density in disease, and to determine factors that influence bacterial load in the human host. This introduction gives a general background to *H. pylori* infection and the host immune system.

The presence of a curved bacillus in the human stomach has been documented for over a century. As early as 1875, spiral bacteria were found colonising the mucus lining of the human stomach, and later spiral bacteria were discovered in the stomach of dogs (Bizzozero, 1893), but the bacteria could not be cultured (Krienitz, 1906; Luger, 1917). It was not until 1983 that Robin Warren and Barry Marshall successfully isolated and cultured the organism from human stomachs, and suggested a causal association between this bacterium and peptic ulcer disease, which had originally been linked to environmental factors such as stress and spicy food (Marshall & Warren, 1984). In one of their most significant experiments, Barry Marshall fulfilled Koch's postulates by infecting himself with *H. pylori* and subsequently developed gastritis, and then went on to show the successful treatment of gastritis with antibiotics, work for which they were awarded the Nobel Prize in 2005.

The bacterium was initially named *Campylobacter pyloridis*, later corrected to *Campylobacter pylori*, and in 1989 DNA sequencing, as well as other data, showed that the bacterium did not belong in the *Campylobacter* genus and was placed in its own, named *Helicobacter* (Goodwin *et al.*, 1989). There are now over 30 species recognised within this genus that infect the gastrointestinal tract of

different animals including cats, dogs (Hanninen *et al.*, 1996; Paster *et al.*, 1991), rodents (Fox *et al.*, 1995) and other mammals (Fox, 2002).

1.1 *H. PYLORI* EPIDEMIOLOGY AND TRANSMISSION

It is estimated that almost half the world's population are infected with *H. pylori* (Torres *et al.*, 2000). Infection rates differ between developing and western countries, where rates of infection have been declining for years (Group, 1993; Torres *et al.*, 2000). Estimated prevalence in developed countries such as western Europe and the US varies between 30-50%, whereas prevalence can range between 50-90% in developing countries such as in Africa and Asia (Perez-Perez *et al.*, 2004). The high prevalence rate recorded in developing countries is largely thought to be due to low socio-economic status, crowded living conditions, bed sharing between several siblings, and unclean water sources that are common to these geographical regions (Dominici *et al.*, 1999; Ertem *et al.*, 2003; Lindkvist *et al.*, 1998; Webb *et al.*, 1994). The incidence of *H. pylori* infection appears to be declining worldwide, however this is more rapid in developed countries, which is thought to be caused by improved sanitation reducing the chances of childhood infection (Cullen *et al.*, 1993; Parsonnet *et al.*, 1992; Sipponen *et al.*, 1996).

Another marked difference between *H. pylori* infection in western and developing countries is the age at which infection is acquired. Individuals are often infected at much younger ages in developing countries compared to western countries. Previous studies have reported *H. pylori* infection rates of 23% in children younger than 3 in Vietnam (Rowland *et al.*, 1999), 33% in Egypt (Okuda *et al.*, 2007), and 40% in children younger than 6 in Brazil (Frenck *et al.*, 2006). In Bangladesh, prevalence was reported to reach as high as 80% in children younger than 5 (Nguyen *et al.*, 2006). Comparative studies in Western countries show much lower incidence of infection, with rates of 13.6% reported in Sweden (Rothenbacher & Brenner, 2003), 6% in Finland (Rehnberg-Laiho *et al.*, 1998), and 2.4% in Germany (Rowland *et al.*, 2006) for children younger than 3.

The acquisition of *H. pylori* usually occurs during childhood and persists for life unless treated with antibiotics. Reinfection of children older than 5 years is rare

regardless of socio-economic status, and infections occurring in adults for the first time are similarly rare (Malaty, 2007; Rothenbacher & Brenner, 2003). Epidemiological studies have reported several possible modes of transmission; however, the exact mechanisms are not yet fully understood. It is widely thought that person-person contact is the most likely mode of transmission. Many studies have reported the role of familial clustering as key to *H. pylori* transmission, with mother-to-child transmission regarded as a more predominant route of infection (Farrell *et al.*, 2005; Kivi *et al.*, 2005; Tindberg *et al.*, 2001). Furthermore, the rarity of reinfection in children older than 5, after which they spend less time at home, also highlights the importance of close family contact in the transmission of *H. pylori* (Malaty, 2007). However, a difference in strains between family members is common in developing countries, and suggests other modes of transmission in such areas (Schwarz *et al.*, 2008). An oral-oral route, probably through oesophageal reflux into the mouth or from vomit (Allaker *et al.*, 2002; Kabir, 2004) is supported by the detection of *H. pylori* DNA in vomitus, saliva, and dental plaque (Brown, 2000). Waterbourne transmission, possibly through faecal contamination, is another potential source of transmission. Infection rates in children were found to be much higher in households using municipal water supplies (Klein *et al.*, 1991), whereas families using containers that separated their drinking water were less likely to be infected (Glynn *et al.*, 2002).

1.2 *H. PYLORI* RELATED CLINICAL MANIFESTATIONS

H. pylori colonisation of the gastric mucosa induces gastritis in all infected individuals, but only a minority of infections develop clinical signs. It is estimated that infected patients have a 10-20% risk of developing peptic ulcer disease and 1-2% risk of developing gastric cancer, (Ernst & Gold, 2000; Kuipers, 1999). The risk of developing disease is dependent on a variety of host, bacterial and environmental factors (Kuipers, 1999).

In some circumstances, clearance of the infection can occur, although this is rare with seroreversion rates ranging between 5-10%. It is not known if this clearance

is spontaneous, or as a result of antibiotic use for treatment of other conditions (Everhart, 2000).

Once colonisation is established in the gastric mucosa, *H. pylori* persistently colonises resulting in chronic inflammation of the gastric mucosa, which can progress to atrophic gastritis and occasionally gastric carcinoma. A close correlation exists between the level of acid secretion and the pattern of gastritis that develops, and differences in the distribution of gastritis are crucial in determining *H. pylori* related disease outcome (discussed further in section 1.2.1).

1.2.1 Peptic ulcer disease (PUD)

Peptic ulcer disease is a major health concern to the world population, with high rates of morbidity and substantial mortality (Sonnenberg, 2006). The disease encompasses both gastric and duodenal ulcers, and symptoms include epigastric pain, often accompanied by other dyspeptic symptoms including nausea, heartburn, and bloating. The most common and severe complication of the disease is bleeding, occurring in 50-170 per 100,000 cases, with the highest risk to those older than 60 years of age (Longstreth, 1995; Rockall *et al.*, 1995).

Epidemiological studies have revealed a strong association between peptic ulcer disease (PUD) and *H. pylori* infection. Colonisation of the gastric mucosa with this bacterium is reportedly the cause of approximately 95% of duodenal ulcers and 85% of gastric ulcers worldwide (Kuipers *et al.*, 1995a). However, recent reports have revealed a lower incidence of 81% for *H. pylori* associated duodenal ulceration, suggesting other factors are driving the development of the disease (Gisbert & Calvet, 2009). This causal relationship is strengthened by studies which demonstrated that eradication of the bacterium greatly reduced the risk of recurrent ulceration (Rauws & Tytgat, 1990).

The underlying mechanism by which *H. pylori* contributes to peptic ulcer disease is thought to involve a combination of host genetic predisposition, bacterial virulence factors, and damage of the mucosa (Cohen, 2000).

1.2.1.1 Duodenal ulcer disease (DUD)

Acid production and the distribution of inflammation in the gastric mucosa are key factors in determining the development of PUD. In antral-predominant *H. pylori*-associated gastritis, both basal and stimulated acid output are increased,

and even more so in patients with duodenal ulcers (Schultze *et al.*, 1998) which appear in the duodenal bulb (Kusters *et al.*, 2006). Stomach acidity is regulated by a number of hormonal responses. Under normal conditions low pH in the antrum is detected and stimulates the release of somatostatin from D cells, which exhibits inhibitory control over gastrin secretion from adjacent G cells, and hence acid production from parietal cells in the corpus. *H. pylori* infection impairs the inhibitory effects of somatostatin through the activity of its urease enzyme, which neutralises local acidity and prevents D cells from detecting the true level of acidity in the antrum. This leads to reduced levels of somatostatin secretion (Moss *et al.*, 1992), resulting in hypergastrinemia, and consequently excessive acid secretion (El-Omar *et al.*, 1995; Graham *et al.*, 1993; Olbe *et al.*, 1996) (Figure 1.1). Gastrin was previously suggested to act as a specific growth factor for *H. pylori*, effectively creating a positive feedback-loop (Chowers *et al.*, 2002). However, such effects on bacterial growth were later disputed (Doherty & Atherton, 2009).

As a protective response to the resulting gastric acid hypersecretion and acid overload in the duodenum, gastric metaplasia develops in the duodenum. Such changes are required to support the growth of *H. pylori* which specifically and exclusively colonises gastric epithelial cells (Khulusi *et al.*, 1996; Ohkusa *et al.*, 2003).

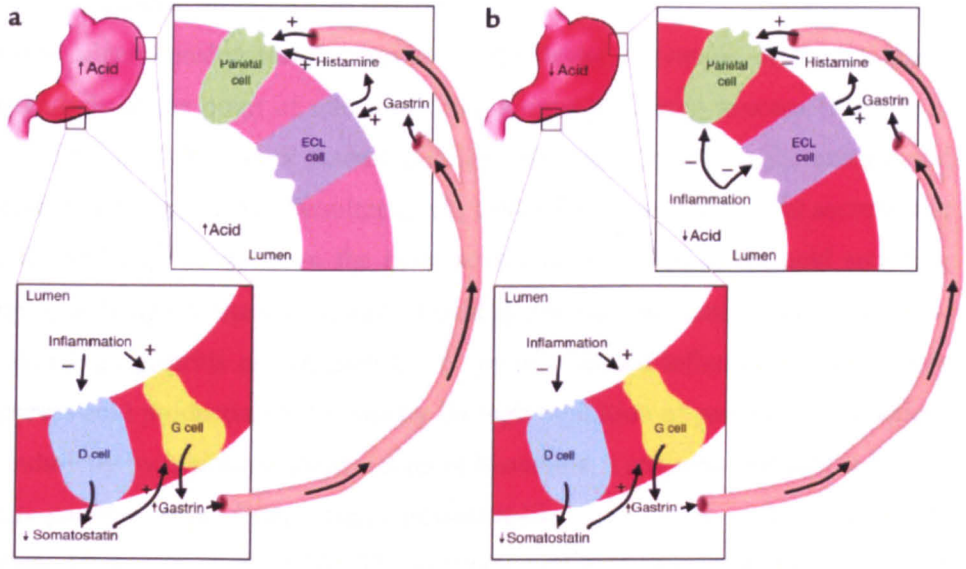


Figure 1.1: Patterns of gastritis involved in the development of PUD.

The patterns of gastritis associated with acid secretion and disease outcome. (**a**) *H. pylori* induced antral-predominant inflammation impairs somatostatin inhibitory control of gastrin release from adjacent G cells. Increased gastrin levels signal the release of acid from parietal cells located in the corpus, resulting in hypergastrinaemia. This leads to the development of DUD. (**b**) *H. pylori* induced pan-gastritis impairs acid output through direct inhibition of parietal cells, and indirect inhibition of histamine release from enterochromaffin-like (ECL) cells. This inflammatory profile is associated with the development of GUD. Continual proliferation of parietal cells increases the risk of developing gastric cancer (Taken from Blaser & Atherton, 2004).

1.2.1.1 Gastric ulcer disease (GUD)

Gastric ulcers predominantly appear in the lesser curvature of the stomach, particularly at the point of transition from corpus to antrum mucosa (Kusters *et al.*, 2006). In gastric ulcer disease (GUD), inflammation affects the antrum and corpus to similar extents (Schultze *et al.*, 1998). Unlike in DUD, acid secretion in GUD can be reduced due to the more severe involvement of the acid secreting corpus, although a crucial amount of acid is always maintained. With a corpus-predominant gastritis or pan-gastritis, *H. pylori*-induced inflammatory mediators suppress acid production both directly through inhibition of parietal cell function, or indirectly by inhibiting the secretion of histamine, a stimulant for acid secretion from parietal cells, from enterochromaffin-like cells (ECL cells) (Beales & Calam, 1998; Fox *et al.*, 2000). The resultant reduction in acid production further augments gastrin output, which, although it has little effect on raising acid production from the inflamed corpus, provides a stimulus for the proliferation of gastric epithelial cells (Figure 1.1). Continuing proliferation and inflammation has effects on epithelial cell cycle characteristics (Beales & Calam, 1998; Peek *et al.*, 2000) probably leading to loss of gastric glands and these changes may increase the risk of developing gastric adenocarcinoma (Kuipers *et al.*, 1995c; Peek & Blaser, 2002). However, reduced acid levels do show a protective role against duodenal ulceration (Koike *et al.*, 2001; Yamaji *et al.*, 2001).

1.2.1.2 Other factors contributing to PUD

Most non-*H. pylori* related PUD is thought to be a result of non-steroidal anti-inflammatory drug (NSAID) use. Environmental factors such as smoking and excessive alcohol use have been described to contribute to the development of ulcer disease, but these factors have not been identified as individual ulcerogenic agents (Kato *et al.*, 1992; Rosenstock *et al.*, 2003). Emotional stress has been suggested to contribute to ulcer disease, but there is little evidence to support this role (Medalie *et al.*, 1992; Peters & Richardson, 1983).

1.2.2 Gastric adenocarcinoma

Gastric adenocarcinoma is the 2nd leading cause of cancer-related death worldwide, and is responsible for over 700,000 deaths each year (Peek & Blaser, 2002). Since the discovery of *H. pylori*, numerous studies have reported a strong association between the disease and *H. pylori* infection, supported by geographical associations between the prevalence of *H. pylori* and the incidence of gastric cancer (Forman *et al.*, 1990). These studies demonstrated that *H. pylori* infection increased the risk of developing gastric cancer (GC) by approximately 10-fold, and the bacterium was later designated a class I carcinogen by the WHO. This risk association was later found to be higher in case-control studies (Ekstrom *et al.*, 2001), and was supported in Mongolian gerbil studies, in which *H. pylori* infection induced atrophic gastritis and GC (Honda *et al.*, 1998; Rieder *et al.*, 2005; Watanabe *et al.*, 1998). Some case-control studies failed to show such a strong risk association with developing GC (Huang *et al.*, 1998), and it is now understood that the rate of *H. pylori* infection was underestimated due to loss of the bacterium as the gastric mucosa undergoes pre-malignant transformation, a change that no longer supports growth of the bacterium.

Despite the number of gastric cancer associated deaths, the rate of GC occurrence in developed countries has been declining, and the reason for this is now believed to be associated with the decreasing prevalence of *H. pylori*, particularly in western countries. However, GC is still the 4th most common cancer worldwide as the disease is still prevalent in many developing countries (Figure 1.2).

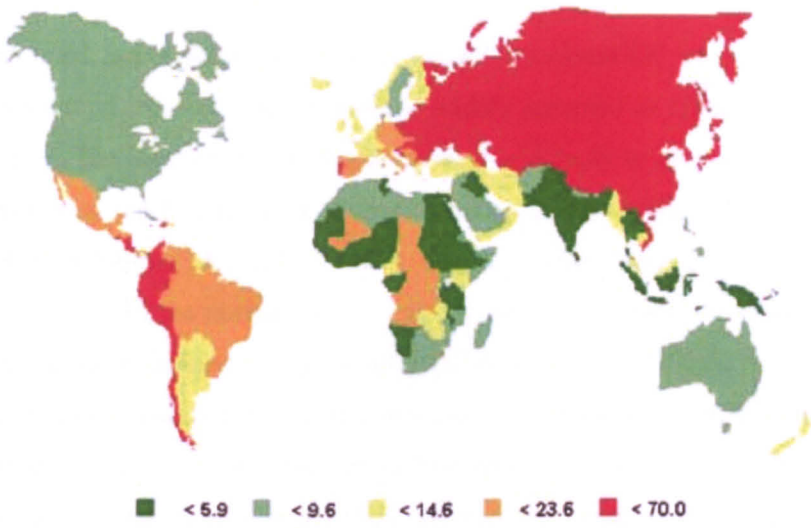


Figure 1.2: Geographical variations in GC death rate.
Age standardised world gastric cancer death rate is given per 100,000 (Parkin, 2004).

Despite a similar rate of infection among most Asian countries, the rate of GC is considerably lower in India and Thailand at 8-10 per 100,000 (Miwa *et al.*, 2002). Such a phenomenon is also observed in Africa, and has been described as the "African Enigma". It was hypothesised that the decreased rate of gastric cancer on this continent was due to common co-infections with helminths which drive a T helper 2 (Th2) immune response in contrast to the damaging T helper 1 (Th1) response that has been associated with the progression of *H. pylori* associated disease (Frenck & Clemens, 2003; Kuipers & Meijer, 2000) (The immune response to infection is described further in section 1.7). However, this was disputed in subsequent studies that demonstrated GC occurred at similar rates as seen in western countries, and it is believed that previous observations may have been a result of low life expectancy. Therefore death by other causes may occur before GC became apparent in the infected individual. Lack of medical facilities and insufficient assessment of disease may also be a contributing factor (Agha & Graham, 2005).

Gastric adenocarcinomas are defined as either well differentiated (intestinal-type) that are characterised by a corpus-predominant gastritis with atrophy and intestinal metaplasia, or undifferentiated (diffuse-type) which are characterised by pan-gastritis and no atrophy (Mbulaiteye *et al.*, 2009). Although the mechanisms

involved in the development of *H. pylori*-associated GC are still not completely understood, chronic inflammation is widely accepted as a major cancer-inducing factor. The mechanism of tumourigenesis is thought to involve DNA damage by cytokines and free radicals released during chronic inflammation (Hocker *et al.*, 1998; Scheiman & Cutler, 1999). Chronic inflammation can ultimately cause destruction of the normal gastric mucosa, which increases the risk of developing gastric adenocarcinoma by 90-fold (Kuipers *et al.*, 1995c). The histological changes that occur in the gastric mucosa during progression to intestinal-type GC are better characterised than for diffuse-type GC. Intestinal-type tumours evolve through stages of chronic gastritis, atrophy, intestinal metaplasia, dysplasia, and cancer, known as the Correa pathway (Figure 1.3) (Correa, 1995; Fox & Wang, 2001). Both atrophy and intestinal metaplasia (IM) are distinct steps in the pathway. Atrophic gastritis is defined as the loss of specialised glands (Genta, 1997b), whereas IM is defined as the replacement of gastric-type tissue with intestinal-type tissue, often diagnosed by the presence of tubular crypts lined with goblet cells (Correa *et al.*, 2010).

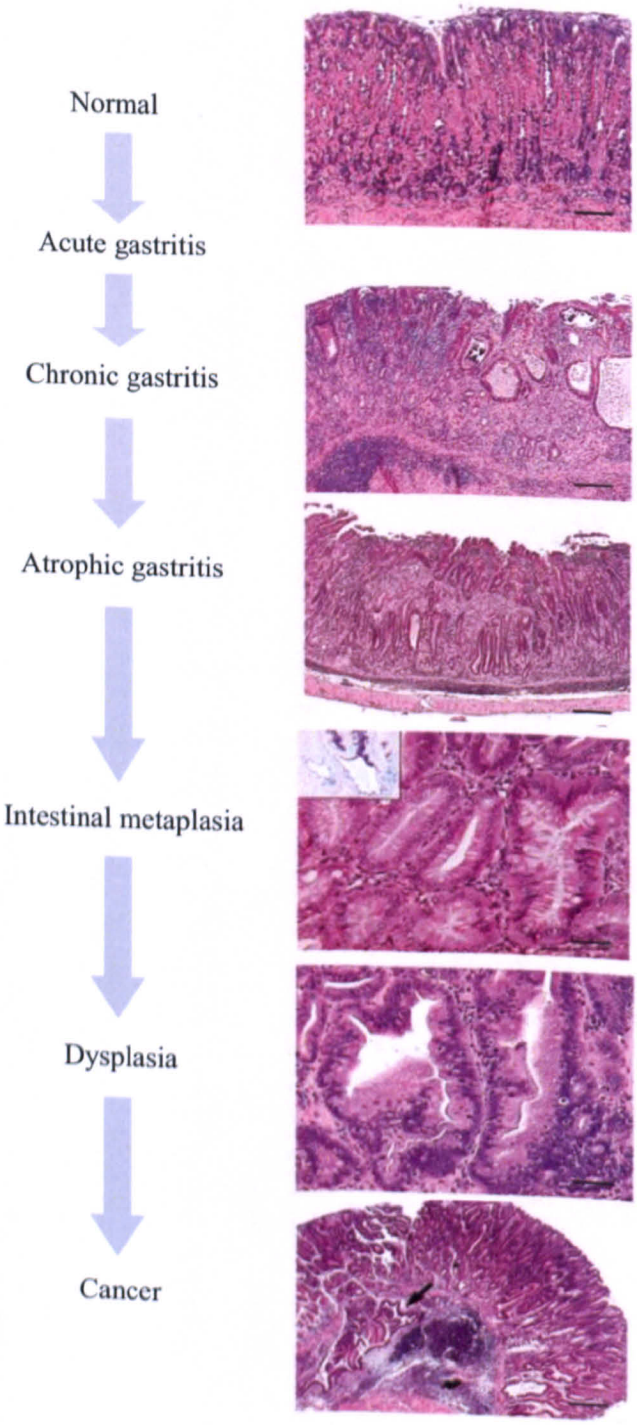

Normal
Acute gastritis
Chronic gastritis
Atrophic gastritis
Intestinal metaplasia
Dysplasia
Cancer

Figure 1.3: Proposed Correa pathway of pathological events in gastric adenocarcinoma, with histological presentation of stages involved.

Normal: Histology of the body of the stomach. Chronic gastritis: Moderate to severe inflammation. Atrophic gastritis: Chronic inflammation with focal fibrosis and complete loss of oxyntic parietal and chief cells. Intestinal metaplasia: Gastric epithelial metaplasia to an intestinal phenotype characterized by columnar elongation, goblet cells, and production of mixed acidic (blue, intestinal-type) and neutral (red, gastric-type) mucins as shown by pH 2.5 Alcian blue/PAS stain (inset). Dysplasia: High-grade glandular dysplasia characterised by irregular size and shape. Cancer: Gastric intraepithelial neoplasia, here with intramucosal invasion (arrow). Scale bars: 160 mm (first panel); 400 mm (second and third panels); 80 mm (fourth panel; inset, original magnification, ×400); 40 mm (fifth panel); 800 mm (sixth panel). (Fox & Wang, 2007)

Bacterial factors have been associated with *H. pylori*-related carcinogenesis. The most pathogenic strains of *H. pylori* are widely accepted as determinants in GC development (Prinz *et al.*, 2001). The role of these factors in disease progression is discussed later (section 1.6).

More recently, studies have proposed preferential damage to parietal cells by the bacterium, thereby altering the maturation of the stem cell (Correa & Houghton, 2007). Others have reported that *H. pylori*-induced inflammation leads to recruitment of peripheral or bone-marrow derived stem cells into the gastric mucosa, which then transform into malignant cells. Furthermore, bone-marrow derived stem cells were identified as the origin of malignancy in a mouse model (Houghton *et al.*, 2004), and *H. pylori* has also been identified inside gastric stem cells (Giannakis *et al.*, 2008). These studies have demonstrated an important factor in the development of gastric cancer and describe another possible mechanism by which *H. pylori* induces GC.

Genetic polymorphisms in genes coding for inflammatory cytokines also contribute to the progression of disease to GC. Interleukin-1β (IL-1β) is a crucial inflammatory mediator upregulated in the presence of *H. pylori* that is important in amplifying the inflammatory response to infection, and is a potent inhibitor of gastric acid secretion (El-Omar, 2001). IL-1β is key to the development of gastric cancer as it is capable of modulating the function of several gastric epithelial cell types, inducing the release of gastrin from G cells and inducing the release of histamine from ECL cells (Mahr *et al.*, 2000; Prinz *et al.*, 1997). The importance of IL-1β acid secretion was demonstrated by Takashima *et al*, who found IL-1β stimulation decreased acid output in a Mongolian gerbil model, and the addition of an IL-1R antagonist restored gastrin levels and acid output (Takashima *et al.*, 2001). This was also demonstrated in a transgenic mice model in which IL-1β overproduction was targeted to the stomach. These mice developed severe gastritis and adenocarcinoma in the absence of *H. pylori*, and infection with the bacterium accelerated the appearance of pathology, which was prevented by the inclusion of IL-1 receptor antagonist (Tu *et al.*, 2008).

Three polymorphisms in the *IL1β* gene, leading to increased IL-1β production, have been described at positions -511, -31 and +3954 (El-Omar *et al.*, 2000). All have been associated with an increased risk of developing GC in a number of

populations tested (El-Omar *et al.*, 2000; Figueiredo *et al.*, 2002; Machado *et al.*, 2001). In addition, a polymorphism in the IL-1 receptor, IL-1RN*2, has also been identified that results in prolonged responses to inflammatory stimuli and increases the risk of developing disease (Hsu *et al.*, 2004). Furthermore, the risk of developing disease is compounded with the presence of multiple polymorphisms (Figueiredo *et al.*, 2002; Machado *et al.*, 2001). However, numerous studies have failed to show a correlation between polymorphisms of the *IL1β* gene and GC. Such discrepancies have been attributed to genetic differences between populations, to genetic polymorphisms in the gene promoter and transcription factors (Chen *et al.*, 2006a; Lee *et al.*, 2007b), or diet (Shen *et al.*, 2007).

After the identification of polymorphic *IL1β*, other cytokine genes were also demonstrated to display polymorphisms in the gene. Tumour necrosis factor-α (TNFα) is also a known inhibitor of acid production, although much weaker than IL-1β. Several polymorphisms in the *TNF-α* gene have been associated with increased risk of developing *H. pylori*-associated gastroduodenal disease (El-Omar *et al.*, 2003). The most studied is the *TNFα* -308 polymorphism, which is associated with high TNFα transcription and found to correlate with increased risk of developing gastric cancer (Rad *et al.*, 2004). This association was also observed with the *TNFα* -238 polymorphism in children (Wilschanski *et al.*, 2007), and with the combination of the *TNFα* -857, -863 and -1031 polymorphisms, all of which confer high transcriptional promoter activity (Sugimoto *et al.*, 2007).

IL-10 is an anti-inflammatory cytokine involved in down-regulation of IL-1β and TNFα, as well as other inflammatory mediators. Three single nucleotide polymorphisms in the *IL10* gene have been identified at positions 1082 (G to A), 819 (C to T) and 592 (C to A). 8 possible haplotypes exist, of which the GCC haplotype is associated with a higher expression level of *IL10*, which favours an anti-inflammatory response. However, the ATA haplotype is associated with reduced *IL10* expression and consequently leads to a TH1-driven hyper-inflammatory response which causes excessive damage to the gastric mucosa (El-Omar *et al*, 2003). Importantly, some studies have failed to show an association

between *IL10* polymorphisms and increased risk of developing disease (Garcia-Gonzalez *et al.*, 2007; Hellmig *et al.*, 2005).

Many studies have shown that although polymorphisms in a single gene are associated with a two-three fold increased risk of developing gastric cancer, the presence of polymorphisms in multiple genes, which result in proinflammatory responses, substantially increases the risk by 27-fold (El-Omar *et al*, 2003).

1.2.3 Mucosa-associated lymphoid tissue (MALT) lymphomas

MALT lymphomas are also associated with *H. pylori* infection, occurring in less than 1% of infected individuals. MALT is not present in the uninfected stomach and appearance of this tissue type is dependent on the presence of *H. pylori*. The bacterium stimulates the infiltration of B cells in the mucosal stroma, which slowly proliferate. In some cases they develop into a low grade MALT lymphoma and may sometimes undergo transformation to large-cell B cell lymphoma (Zucca *et al.*, 1998). The risk factors associated with the development of MALT lymphoma are not well understood, but appear most commonly in the presence of pan-gastritis (Miehlke *et al.*, 2001). Almost all MALT lymphoma patients are infected with *H. pylori*, and significant studies have shown that eradication of the bacterium with antibiotic treatment can cause complete regression of the lymphoma in 70-90% of cases, although this is not the case for high-grade lymphomas (Morgner *et al.*, 2000).

1.2.4 Other *H. pylori* associated diseases

There is increasing evidence for potential beneficial roles of *H. pylori* infection in the human host, with the absence of infection being associated with an increased risk of developing various diseases. One of these is the inverse association between *H. pylori* infection and gastroesophageal reflux disease (GERD), Barrett's oesophagus, and oesophageal adenocarcinoma (Islami & Kamangar, 2008; Vaezi *et al.*, 2000; Vicari *et al.*, 1998). One hypothesis for this relationship is that infection with *H. pylori* leads to reduced gastric acidity, making refluxate less damaging to oesophageal epithelium than that of an uninfected individual. It is also possible that infection alters the expression of gastric hormones that may have effects on oesophageal tissue. However, the exact mechanisms involved in

this association are still poorly understood. It is thought that inflammation of the corpus induced by *H. pylori* colonisation suppresses acid production and hence protects against GERD development (Francois *et al.*, 2008; Roper *et al.*, 2008). This protective role of *H. pylori* was noted particularly with more virulent *cagA* positive strains (Fallone *et al.*, 2000; Lai *et al.*, 2005). However, many other studies were unable to report similar trends (Ferrandez *et al.*, 2006; Tsukada *et al.*, 2005).

Another intriguing finding is the rising incidence of asthma and related disorders as the rate of *H. pylori* infection has declined. Asthma is generally considered as a disorder arising from dysfunctional immune responses to common allergens. The absence of the bacterium has been associated with increased incidence of childhood onset allergies, but was not found to be related to later-onset of disease. This association was found to be strongest with *cagA* positive infections (Blaser *et al.*, 2008; Chen & Blaser, 2008). One hypothesis for this inverse association is that during *H. pylori* infection, populations of T-cells, including regulatory T-cells, are recruited that affect the activities of T cells present at other mucosal sites (Harris *et al.*, 2008; Robinson *et al.*, 2008). Also, regulatory T-cell responses, which are important for suppression of autoimmunity, allergy and inflammation (Thompson & Powrie, 2004), are notably induced during *H. pylori* infection, and may explain the protection against allergy observed with this infection (Robinson *et al.*, 2008). The relationship between *H. pylori* and the immune response is discussed further in Chapters 4, 5 and 6.

1.3 DIAGNOSIS OF *H. PYLORI* INFECTION

Many diagnostic tools exist for the detection of *H. pylori*, and are classified as either invasive if they require detection of the bacterium in gastric tissue obtained during endoscopy, or non-invasive if they are based on peripheral samples, such as blood, breath or stool samples (Table 1.1). The decision as to which technique to employ is based on a number of factors including patient convenience and safety, reliability, cost, and time taken to perform the test. The choice of diagnostic method may vary between clinics, but generally for hospital-based care, many patients undergo endoscopy which is then combined with either histology or a rapid urease test for the detection of *H. pylori*.

Diagnostic method	Sensitivity/ specificity	Remarks
Invasive tests :		
Histology	>95%	The "gold standard" for diagnosis of *H. pylori* infection. The Sydney scoring system is the most frequently used for quantification of bacterial load. Requires expert pathologist. Low bacterial numbers and atypical morphology of the bacterium may result in false negatives.
Culture	90-95%	An alternative to histology for diagnosis. Bacterial viability can be lost rapidly if optimal transport and culture conditions are not used. Selective supplements can be used to minimise culture of other organisms. Allows for testing of antimicrobial sensitivity.
Biopsy urease tests	>95%	Cost effective and rapid method. The commercial agar based CLO™ test is commonly used. Sensitivity is dependent on bacterial load, and reduced numbers may result in false negatives.
PCR	>95%	For diagnosis, this method may be more time-consuming and expensive. The presence of PCR inhibitors in gastric tissue may reduce sensitivity and contamination by exogenous *H. pylori* DNA may lead to false positive results. Allows for strain genotyping.
Non-invasive tests:		
Urea breath test (UBT)	>95%	This is a reliable test to evaluate the success of eradication treatment of *H. pylori*. The use of computer analysis eliminates errors in human judgement. There is little impact of transport conditions, making this test accurate and reproducible. However, the need for expensive equipment limits availability.
Serology	80-90%	Mainly used for epidemiological studies. Cross reactivity and immunological memory makes limits the reliability of the test, and therefore is not appropriate for routine screening of infection,
Stool antigen test	90%	This is a simple test, and samples are easily obtained. Detection of *H. pylori* from stool samples has been most successful using ELISA assays directed towards *H. pylori* antigens. However, this test may not be reliable for monitoring eradication therapy.

Table 1.1: Methods for the diagnosis of *H. pylori* infection.

Diagnosis of *H. pylori* infection is reviewed in detail by Megraud & Lehours, 2007

1.4 TREATMENT

Current treatment regimens specify that patients infected with *H. pylori* are treated with antibiotics and acid suppressants. Presently, 1st line therapy consists of two antibiotics, commonly clarithromycin and either metronidazole or amoxicillin, and a proton pump inhibitor (PPI) drug (Chey & Wong, 2007; Malfertheiner *et al.*, 2007). Eradication rates with 1st line therapy were reported to be greater than 90% in 2000 (Kearney & Brousal, 2000). However, recent studies have estimated these rates to have fallen as low as 70% in many areas, and even down to 60% in some regions (Kadayifci *et al.*, 2006). Reduced efficacy of 1st line therapy is likely due to increasing resistance to antibiotics. In the case of clarithromycin resistance, 1st line therapy is reported to be effective in as little as 18% of treated patients (Megraud, 2004). Increased use of clarithromcycin for the treatment of respiratory infections is the likely cause of *H. pylori* resistance to clarithromycin (Boyanova, 2009; Romano *et al.*, 2008). Metronidazole resistance is also common, and eradication rates have been reported to fall as low as 47% in areas with high levels of antibiotic resistance (Gerrits *et al.*, 2006).

Generally, 1st line therapy is reported to fail in approximately 20% of treated patients, highlighting the need for 2nd line therapy (Ford *et al.*, 2008). Many 2nd line therapies are in use, the most common involve treatment with bismuth-based quadruple therapy consisting of a PPI, bismuth, tetracycline and metronidazole, a combination reported to be effective in 70% of patients that failed to respond to 1st line therapy (Hojo *et al.*, 2001).

Treatment is very effective for the management of PUD, but the protection against gastric cancer probably depends on whether infection is treated early and the extent of mucosal damage at the time of treatment (Rauws & Tytgat, 1990).

1.5 VACCINES

H. pylori is successfully treated with current treatment regimens; however, antibiotic resistance is an increasing problem (Megraud, 2004). Therefore the development of a vaccine would be of great benefit, and numerous approaches have been investigated to develop effective vaccination against *H. pylori*.

Among the *H. pylori* antigens investigated, the Urease B subunit (UreB) has been extensively studied in a number of vector systems. Subcutaneous immunisation with UREB138, a region of the gene known to be important for urease activity, was found to reduce bacterial density in the gastric mucosa of mice (Morihara *et al.*, 2008). Vaccination with a recombinant fusion peptide of the urease B epitope and the cholera toxin B subunit encapsulated in liposome was found to protect mice from *H. pylori* infection both prophylactically and therapeutically. Furthermore, urease specific mucosal IgA was also detected in mice treated with the vaccine (Zhao *et al.*, 2007). Importantly, long-lasting immunity is also dependent on host factors. Prophylactic immunisation with recombinant *H. pylori* HpaA and cholera toxin was found to reduce bacterial density in BALB/c mice but not in C57BL6 mice, demonstrating the influence of host genetics in effective vaccination (Sutton *et al.*, 2007). However, vaccine models have yet to produce sterilising immunity.

1.6 *H. PYLORI* COLONISATION FACTORS AND VIRULENCE DETERMINANTS

Infection with *H. pylori* requires a complex interaction between both host and bacterial factors. Much diversity is observed for *H. pylori* leading to the existence of strains that vary in pathogenicity. The aggressiveness of the inflammatory response varies consequently, and a range of clinical manifestations can develop. This section reviews bacterial virulence factors that mediate colonisation and survival of the bacterium in the human host (Atherton, 2006; Blaser & Atherton, 2004).

1.6.1 Factors involved in colonisation

Successful chronic infection with *H. pylori* is dependent on colonisation and persistence of bacterial infection in a dynamic environment. Several bacterial factors have been implicated as essential for both initial and persistent colonisation of the gastric mucosa by the bacterium. Of interest are factors that are involved in initial colonisation of the acidic stomach by a bacterium that is not

considered acidophilic, and that expresses enzymes essential for bacterial survival that are susceptible to the acid environment (Sachs *et al.*, 2002).

H. pylori is known to colonise the protective mucous gel, which exhibits a pH gradient whereby increased pH is observed near the gastric epithelium, and a lower pH is seen towards the luminal side of the mucous gel. *H. pylori* tend to colonise close to the less acidic epithelium, and moves rapidly towards this layer by means of chemotactic motility using this pH gradient. Studies in Mongolian gerbils demonstrated that the bacterium was capable of orientating themselves within pH gradients, and in the absence of these gradients, the bacteria were found throughout the mucous layer (Schreiber *et al.*, 2004). These observations suggest *H. pylori* are capable of sensing and responding to changes in local acidity, and signal to the flagellar. Furthermore, the *H. pylori* genome encodes three membrane-spanning chemoreceptors (TlpA, TlpB and TlpC) that are thought to be essential for sensing changes in pH. Of these, TlpB was found to be essential for effective colonisation in a mouse model (Croxen *et al.*, 2006).

Essential to *H. pylori* acid tolerance is the urease enzyme. Urea, which is released as a result of the urea permease activity of the vacuolating cytotoxin A (discussed in section 1.6.4), is converted to ammonium and bicarbonate. This effectively neutralises cytoplasmic pH which buffers the periplasm and allows for survival in acidic conditions. Studies have demonstrated the importance of the enzyme in colonisation as knocking out the urease genes lead to failure of colonisation (Eaton & Krakowka, 1994).

1.6.2 *H. pylori* adhesins and outer membrane proteins

Once in the gastric mucous, *H. pylori* may adhere to the gastric epithelium, which is mediated by a number of bacterial factors.

1.6.2.1 BabA

Lewis b antigens (Le^b) expressed on gastric epithelial cells have long been known to play a key role in *H. pylori* pathogenesis (Lee *et al.*, 2006). Studies demonstrated specific binding of *H. pylori* to Le^b which was found to be a receptor for a *H. pylori*-specific adhesin (Boren *et al.*, 1993; Falk *et al.*, 1993) named blood group antigen-binding adhesin (BabA) (Ilver *et al.*, 1998). BabA may also mediate the adhesion of *H. pylori* to the gastric mucin MUC5AC (Van

de Bovenkamp *et al.*, 2003), a secretory mucin expressed in gastric surface and glandular mucous cells, which is an important constituent of the protective mucous gel (Ho *et al.*, 1995). Additional receptors may be involved in the adhesion of *H. pylori* as BabA-positive strains attach to the epithelium in the absence of Le^b expression (Walz *et al.*, 2005).

The adhesin is encoded by the *babA* gene, of which 2 alleles exist; *babA1* and *babA2*. The *babA1* allele lacks the ATG start codon due to a 10 base pair insertion in the signal sequence, and therefore does not encode a functional protein. A functional protein is only encoded by the *babA2* allele (Backstrom *et al.*, 2004). A number of mechanisms are predicted for the regulation of *babA*, the main mechanism involving chimera formation between *bab* genes in different loci present in the *H. pylori* genome; *babA*, *babB*, and *babC* (Backstrom et al., 2004; Pride & Blaser, 2002).

The prevalence of *babA2* positivity varies between 30 and 70% in western countries, although recent large scale studies demonstrated only 9.8% of western strains to be *babA2* negative (Fujimoto *et al.*, 2007). In contrast, studies in Taiwan and Japan found 100% prevalence of the gene in *H. pylori* isolates (Sheu & Wu, 2008; Yamaoka *et al.*, 2002b). *babA2* negative strains have been associated with mild gastritis and lower bacterial density compared to strains testing positive for the virulence factor (Fujimoto *et al.*, 2007). BabA has also been strongly associated with peptic ulcer disease and gastric cancer (Azevedo *et al.*, 2008; Gerhard *et al.*, 1999).

1.6.2.2 SabA

The adhesion of a *H. pylori* strain that had a *babA* deletion and was still able to colonise in the presence of Le^b antigen suggested that other adhesins were expressed on the surface of the bacterium. Sialyl-Lewis x and a (sLe^x and sLe^a) were later demonstrated to serve as receptors for *H. pylori* adherence through interactions with the sialic acid binding adhesin (SabA) (Mahdavi *et al.*, 2002). The expression of sLe^x and sLe^a is sparse on epithelial cells of normal, uninfected gastric tissue. However, a chronic inflammatory response to infection with *H. pylori* elicits sialylation of these Lewis antigens, increasing the level of sLe^x and sLe^a and hence adhesion through SabA interactions.

Expression of the *sabA* gene is regulated by a slipped strand mispairing mechanism and is thought to be mediated by the number of CT repeats in the 5' region of the gene, which create "on" and "off" patterns that confer functionality. However, later studies found that varying numbers of CT repeats did not correlate with SabA production (Lehours *et al.*, 2004; Sheu *et al.*, 2006). The ability of *H. pylori* to switch *sabA* status may implicate this gene in immune evasion during infection. Mahdevi *et al.* (2002) suggested a model for SabA binding, whereby *H. pylori* infection triggers sialylation of Le^x and Le^a allowing binding. At sites of intense inflammation, "on/off" switching results in loss of adherence, allowing the bacterium to escape the immune response and thus contributing to chronicity of infection (Mahdavi *et al.*, 2002). The prevalence of *sabA* varies geographically, with 80% of strains carrying the gene in Taiwanese populations (Sheu *et al.*, 2006), 86% in French populations (Lehours *et al.*, 2004), and 93% in Dutch populations (de Jonge *et al.*, 2004b).

Infection with *sabA*-positive strains has been associated with gastric cancer, intestinal metaplasia and atrophy, but was negatively associated with DUD and neutrophil infiltration. In a study examining patients from Columbia and the US, *sabA* was found to be the only predictor of gastric cancer in comparison of *babA* and *oipA* (an outer membrane protein [Section 1.6.2.3]) (Yamaoka *et al.*, 2006). However, *sabA* prevalence was not associated with *H. pylori* related disease in all populations tested, which may reflect global differences in the relationship between the virulence factor and disease status (Sheu *et al.*, 2006). *SabA* positive strains have been associated with increased bacterial density (Sheu *et al.*, 2003). Interestingly, this relationship was observed only in patients expressing sLe^x with little Le^b expression, but not in patients that expressed moderate levels of Le^b. This suggests that SabA has little impact on bacterial density when Le^b expression is strong allowing adhesion by BabA, but enhances colonisation in patients with weaker Le^b expression (Sheu *et al.*, 2003).

1.6.2.3 OipA

OipA is an additional outer membrane protein expressed by *H. pylori*. As with *sabA*, gene status is described as "on" or "off", and gene regulation is modulated by phase variation dependent on CT nucleotide repeats in the 5' region of the gene. *oipA* has been associated with increased expression of *IL8* both *in vivo* and

in vitro (Yamaoka *et al.*, 2000). The adhesin *oipA* has been associated with the vacuolating cytotoxin (VacA) and BabA, but most strongly with the cytotoxin associated gene A (CagA) which is a strong inducer of IL8. Therefore, the association observed between OipA and *IL8* expression may be a result of its association with CagA positivity. Importantly, mutagenesis of the gene reduced bacterial adherence but did not affect IL-8 secretion *in vitro* (Dossumbekova *et al.*, 2006). The prevalence of *oipA* differs between populations. 100% positivity was reported in Tunisian patients, 51% in Iran, and 33% in Turkish populations (Ben Mansour *et al.*, 2010; Dabiri *et al.*, 2009). *oipA*-positive status has been linked with duodenal ulceration and gastric cancer, but this may again be a result of the close association between *oipA* and other virulence determinants (Yamaoka *et al.*, 2000).

1.6.3 The Cytotoxin associated gene A (CagA) and Cag pathogenicity island (Cag PAI).

After the discovery of *H. pylori* as a causal agent in the development of ulcer disease (Marshall & Warren, 1984), numerous studies attempted to identify the bacterial agent involved in inducing disease in some infected individuals. Early studies described strains of the bacterium that were capable of inducing cellular rearrangements in gastric epithelial cells, which were also associated with more aggressive inflammatory responses in the infected host. The cytotoxin associated gene A (CagA) secreted by the bacterium was identified as a major bacterial agent responsible for these observations and was associated with increased risk of developing severe disease during chronic infection with *H. pylori* (Blaser *et al.*, 1995; Crabtree *et al.*, 1991b; Segal *et al.*, 1999).

CagA is a 120-140kDa highly immunogenic protein encoded by the *cagA* gene localised at one end of the *cag* Pathogenicity Island (*cag*PAI) and acts as a marker for the presence of the island (Tummuru *et al.*, 1993). The *cag*PAI is a 40kb DNA segment consisting of 27-31 genes, thought to have been acquired by *H. pylori* through horizontal transfer from an unknown origin (Akopyants *et al.*, 1998). Most genes in the island code for proteins with sequence similarity to components of type IV secretion systems, forming a syringe-like structure capable of penetrating gastric epithelial cells and allowing translocation of CagA into cells (Censini *et al.*, 1996). The entire island can be lost or restored through natural

transformation and is flanked by 39bp direct repeats. Strains of *H. pylori* that do not possess the *cag* PAI have a single copy of the flanking repeat in the glutamate racemase gene (Kersulyte *et al.*, 1999). More than 90% of East Asian *H. pylori* strains and roughly 60% of Western strains carry the *cag* PAI (Parsonnet *et al.*, 1997). Importantly, CagA varies in size and toxicity, and differences in the biological activities of bacterial strains isolated from patients in East Asia compared to Western isolates relates to the difference in gastric cancer incidence between these two geographical regions (Figure 1.2).

Translocation of CagA into the host cell is followed by tyrosine phosphorylation of the protein, which occurs at small repetitive motifs comprising a five amino acid sequence of Glu-Pro-Ile-Tyr-Ala (EPIYA). These motifs are present in multiple copies in the C-terminal variable region of the protein. Sequence analysis of the regions immediately flanking these motifs identified 4 distinct EPIYA segments in the CagA protein – EPIYA-A, -B, -C and –D. Western strains of *H. pylori* possess EPIYA-A, -B and –C segments, whereas East Asian strains consist of an EPIYA-D region in place of the C motif characteristic of Western strains (Higashi *et al.*, 2002b; Yamaoka *et al.*, 1998) (Figure 1.4). Most strains express CagA proteins with a conserved EPIYA segment core consisting of a single copy of each motif. However, many isolates express CagA proteins with multiple duplications of the EPIYA-C motif, and in some cases duplication of the EPIYA-A, -B or –D motifs have been reported (Higashi *et al.*, 2002b; Yamaoka *et al.*, 1998). Phosphorylation of these sites plays a critical role in the cellular responses that transpire after CagA translocation, including the induction of cellular morphological changes known as the "hummingbird" phenotype (Discussed further in section 1.6.3.1) (Argent *et al.*, 2004).

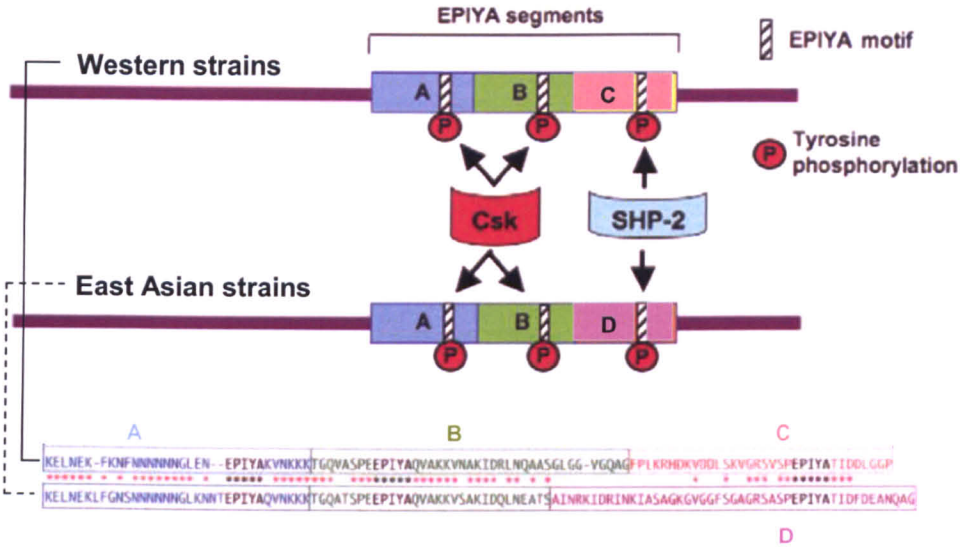


Figure 1.4: Structure of the EPIYA motifs of the *cagA* gene.

Repetitive Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs are found at the C-terminal region of the *cagA* gene. Sequence analysis of DNA flanking these motifs identified four distinct segments. EPIYA-A, -B, and –C motifs are found in Western strains of the bacterium. The presence of multiple EPIYA-C motifs is common and related increased risk of developing *H. pylori*-associated disease. East Asian strains of the bacterium carry the EPIYA-D motif in place of EPIYA-C, and are highly associated with the development of gastric adenocarcinoma. In some cases, duplication of the EPIYA-A and B motifs has also been reported. Once translocated into the host cell, host kinases phosphorylate the toxin at tyrosine residue in EPIYA motifs. Phosphorylated CagA is specifically bound by kinases Csk (at EPIYA-A and –B motifs) and SHP-2 (at EPIYA-C and –D motifs). (Adapted from Hatakeyama, 2008)

1.6.3.1 Cellular effects of CagA

Translocation of CagA into the host cell is followed by localisation of the protein to the inner surface of the plasma membrane. Mutagenesis of the EPIYA regions demonstrated an essential role of these motifs in localisation of CagA (Higashi *et al.*, 2005). Membrane-associated CagA is subsequently phophorylated at the tyrosine site in EPIYA-C motifs by the host Srk family protein tyrosine kinase, c-Src (Selbach *et al.*, 2002; Stein *et al.*, 2002). Activity of c-Src is regulated through

two tyrosine phosphorylation sites present in the c-Src protein; one conferring activity through auto-phosphorylation of the Y419 residue, and the other repressing activity after phosphorylation of residue Y530 by a C-terminal Srk kinase (Csk) (Selbach *et al.*, 2002; Stein *et al.*, 2002). Importantly, phosphorylated CagA (CagA-P) was found to interact at Src homology-2 (SH2) domains present in the Csk sequence. This interaction stimulates Csk activity and subsequently represses c-Src (Tsutsumi *et al.*, 2003). CagA phosphorylation, and hence activity, is therefore negatively regulated by the accumulation of CagA-P, which may represent a means of maintaining the bacterial infection in the host through modulating CagA production and consequently the inflammatory response that leads to cellular damage. A second host tyrosine kinase, c-Abl, was later discovered to also phosphorylate CagA, and is thought to maintain activation of CagA at late stages of infection (Tammer *et al.*, 2007).

The degree of CagA phosphorylation is dependent on the number of EPIYA-C motifs, with increased phosphorylation associated with multiple EPIYA-C motifs. The number of tyrosine phosphorylation sites also determines the biological activity of CagA, inducing more cytoskeletal rearrangement that leads to a severe "hummingbird" phenotype in more cells compared to strains expressing CagA proteins with fewer tyrosine phosphorylation sites. Extensive phosphorylation is also associated with an increased risk of developing GC (Argent *et al.*, 2004).

CagA-P interacts with proteins possessing Src homology-2 (SH2) domains. Noteably, CagA-P directly binds SH2 domain-containing protein tyrosine phosphatase (SHP-2), a bonafide oncogene, in gastric epithelial cells. This specific interaction activates SHP-2 phosphatase activity through a conformational change that relieves catalytic site inhibition (Higashi *et al.*, 2002a; Higashi *et al.*, 2002b). In most cases, SHP-2 is important in the regulation of signals involved in cell growth and cell motility (Neel *et al.*, 2003). SHP-2 interaction with CagA-P induces prolonged activation and dysregulation of the Erk/MAPK signal pathway, leading to induction of cyclin D1 and promotion of G1 to S phase progression. Dysregulation of such pathways represents a potential pathway involved in driving *H. pylori* induced gastric adenocarcinoma (Higashi *et al.*, 2002a; Higashi *et al.*, 2002b). Activation of this pathway is also mediated through binding of the adapter protein Growth factors receptor bound 2 (Grb2) to non-phosphorylated CagA (Mimuro *et al.*, 2002). The CagA-P-SHP-2 complex

also dephosphorylates Focal adhesion kinase (FAK) and inhibits the kinase activity of the enzyme, leading to disruption of focal adhesion points and increased cell motility, as well as cell elongation characteristic of the hummingbird phenotype (Tsutsumi *et al.*, 2006).

CagA-SHP-2 interactions are essential for induction of morphological changes in cells, giving cells the characteristic "hummingbird" phenotype. Importantly, the SH2 domains of SHP-2 bind with highest affinity to a consensus sequence that perfectly matches the sequence flanking the EPIYA-D motif in East Asian strains of *H. pylori*. However, a single amino acid mutation in the flanking sequence of EPIYA-C motifs reduces binding affinity. Consequently, EPIYA-D containing strains exhibit stronger binding and have a greater ability to induce hummingbird phenotypes (Higashi *et al.*, 2002a).

Hummingbird phenotypes are also induced through actin cytoskeletal rearrangements and polymerisation. These cellular changes were shown to be induced through dephosphorylation of actin binding proteins such as cortactin through CagA mediated inactivation of c-Src (Selbach *et al.*, 2003), or by activation of Rac1 and subsequent stimulation of WAVE protein and Arp2/3 complexes (Brandt *et al.*, 2007; Tammer *et al.*, 2007). However, the induction of the hummingbird phenotype by these interactions is controversial as a recent study demonstrated such cellular changes to be independent of Rac1 and Arp1/2 complexes (Bourzac *et al.*, 2007).

CagA also induces cellular responses independent of tyrosine phosphorylation, including the disruption of tight junctions and cell polarity in the epithelial layer (Amieva *et al.*, 2003). Tight junctions are essential for maintaining cell polarity by limiting the diffusion of integral membrane proteins between apical and basolateral membranes. CagA disruption of this activity involves direct interaction with partitioning-defective-1 (PAR1)/microtubule affinity regulating kinase (MARK) (Saadat *et al.*, 2007). PAR1 is thought to act as a master regulator of cell polarisation, and polarity is maintained by localisation of PAR1 to the basolateral membrane and localisation of the atypical protein kinase C (aPKC)/PAR3/PAR6 complex to the apical membrane, with tight junctions forming where the two proteins face each other. The kinase activity of PAR1 is essential for destabilising aPKC complexes that gather at the basolateral membrane and thus maintaining

tight junctions (Hurov *et al.*, 2004; Suzuki *et al.*, 2004a). Interaction with CagA inhibits this kinase activity causing junctional and polarity defects. Furthermore, *H. pylori* were found to preferentially adhere at junctions, and relocalisation of apical junction proteins zonula occludens-1 (ZO1) and transmembrane protein junctional adhesion molecule (JAM) to the site of bacterial adhesion was demonstrated (Amieva *et al.*, 2003). Cellular rearrangement of these proteins disrupted barrier function, and is thought to be related to the inhibition of PAR1 through CagA binding (Saadat *et al.*, 2007).

Unmodified CagA was also demonstrated to activate nuclear factor of activated T cells (NFAT) in gastric epithelial cells (Yokoyama *et al.*, 2005). Nuclear translocation of this transcription factor induces the transcription of genes involved in cellular differentiation. Notably, the induction of $p21^{Cip1}$ cyclin-dependent kinase inhibitor, which is involved in cell cycle inhibition, is thought to contribute to the induction of intestinal metaplasia (Crabtree & Olson, 2002). Direct interaction of CagA with c-Met effector phospolipase Cγ (PLCγ) either alone or as a complex with c-Met may activate the PLCγ-Ca^{2+}-calcineurin pathway, which leads to the activation of NFAT (Churin *et al.*, 2003).

Unphosphorylated CagA was also demonstrated to induce transcription of β-catenin-dependent genes, including cyclin D1. The transmembrane protein E-cadherin forms a complex with β-catenin, localising the protein to cell junctions where the complex plays a crucial role in maintaining cellular architecture, and also plays a role in signalling pathways involved in regulating cell growth and differentiation (Jamora & Fuchs, 2002; Takeichi, 1991). CagA was demonstrated to directly interact with E-cadherin, inhibiting E-cadherin/ β-catenin complexes from forming (Murata-Kamiya *et al.*, 2007). This results in cytoplasmic and nuclear accumulation of β-catenin, and subsequent activation of β-catenin-dependent genes and signalling, including the Wnt/β-catenin pathway which is involved in colorectal cancer (Segditsas & Tomlinson, 2006).

In addition, CagA has also been found to induce activation of the transcription factor NF-κB activation via the MAP kinase pathway. The activated transcription factor induces transcription of genes involved in proinflammatory immune responses, including *IL8* (Brandt *et al.*, 2005). However, studies reported the involvement of CagA in IL-8 induction to be controversial as *cagA* mutant *H. pylori* strains induced IL-8 secretion (Fischer *et al.*, 2001; Sharma *et al.*, 1998). It

was later shown that CagA was involved in late induction of IL-8 secretion, which may be dependent on polymorphisms in the CagA gene upstream of the EPIYA motifs (Brandt *et al.*, 2005). *In vitro* analyses demonstrated IL-8 secretion to be largely dependent on a functional *cag*PAI. Viala *et al.* (2004) reported the delivery of *H. pylori* peptidoglycan via a functional *cag*PAI, and subsequent recognition of the bacterial component by the intracellular receptor Nod1. This was followed by the activation of NF-κB and upregulated expression of *IL8*. Peptidoglycan was shown to induce IL-8 secretion during early infection, and the exact mechanisms involved in NF-κB activation are yet to be determined.

Overall, CagA activates numerous signalling pathways leading to cellular damage. However, regulation of CagA phosphorylation and other cellular proteins involved in CagA-induced cellular damage limits the potential damage to host cells and contribute to chronic infection with *H. pylori*. The cellular interactions of CagA are summarised in Figure 1.5.

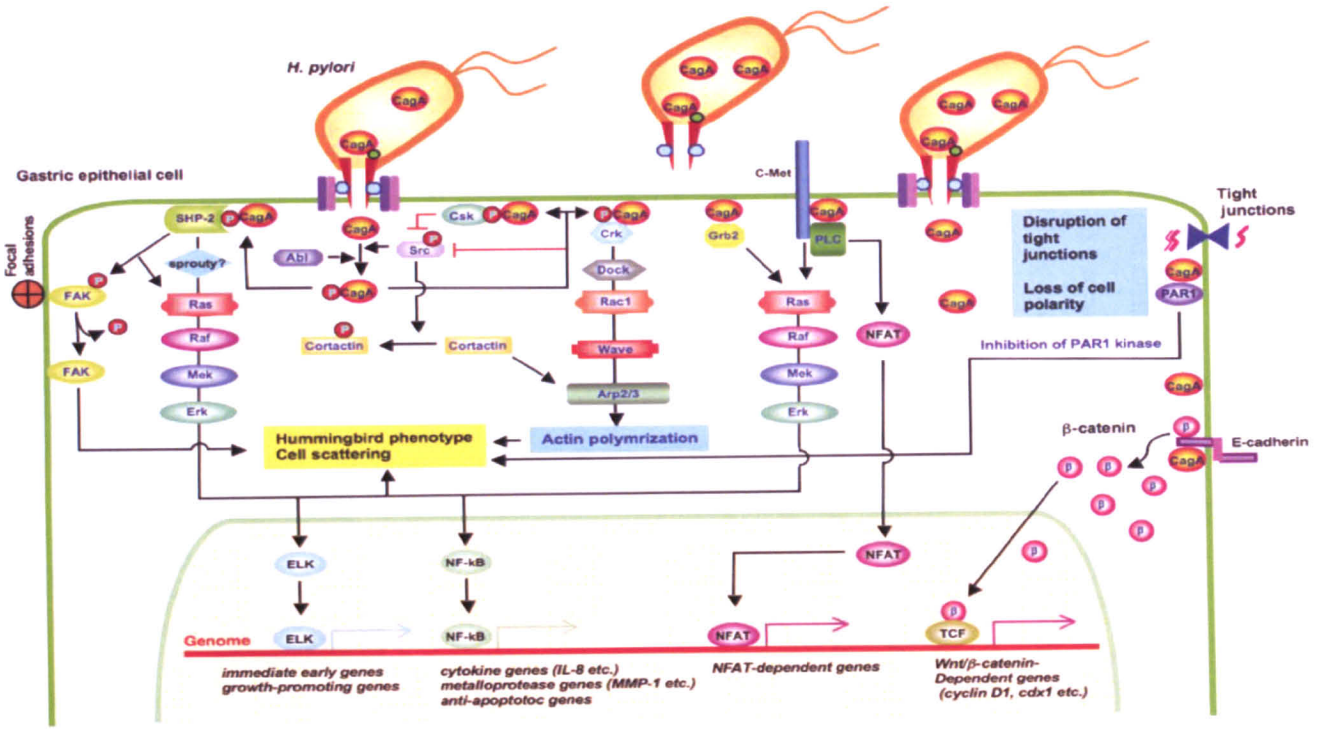


Figure 1.5: The cellular interactions of CagA.

Phosphorylation of CagA occurs through c-Src and Abl. CagA-P interacts with SHP-2, leading to induction of hummingbird phenotypes through activation of FAK and Erk/MAPK pathways. Cellular rearrangements are also induced through CagA interactions with Par-1, cortactin, and the Rac1/Wave pathway. CagA interactions with NFAT and E-cadherin also lead to the activation of genes involved in pro-inflammatory immune responses and regulation of cell-cycles (Hatakeyama, 2008).

1.6.3.2 CagA associated disease

There is a strong correlation between *cagA* positive strains of *H. pylori* and increased risk of developing peptic ulcer disease and gastric cancer (Blaser & Crabtree, 1996) (

Figure 1.6). Morphological transformation of cells and the loss of normal cellular architecture induced by chronic infection with *cagA* positive strains are likely to disrupt the gastric mucosa and induce inflammation. Furthermore, CagA induces cell proliferation and differentiation, which is likely involved in the development of gastric cancer. As mentioned, the risk of developing gastric cancer is notably higher in patients harbouring East-Asian strains due to the high affinity binding of EPIYA-D motifs to SHP-2. Interestingly, patients infected with East-Asian CagA-positive strains show varying degrees of gastric atrophy, suggesting other factors contribute to the development of disease (Azuma *et al.*, 2004). Increased numbers of EPIYA tyrosine phosphorylation sites are also associated with risk of developing gastric cancer (Argent *et al.*, 2004).

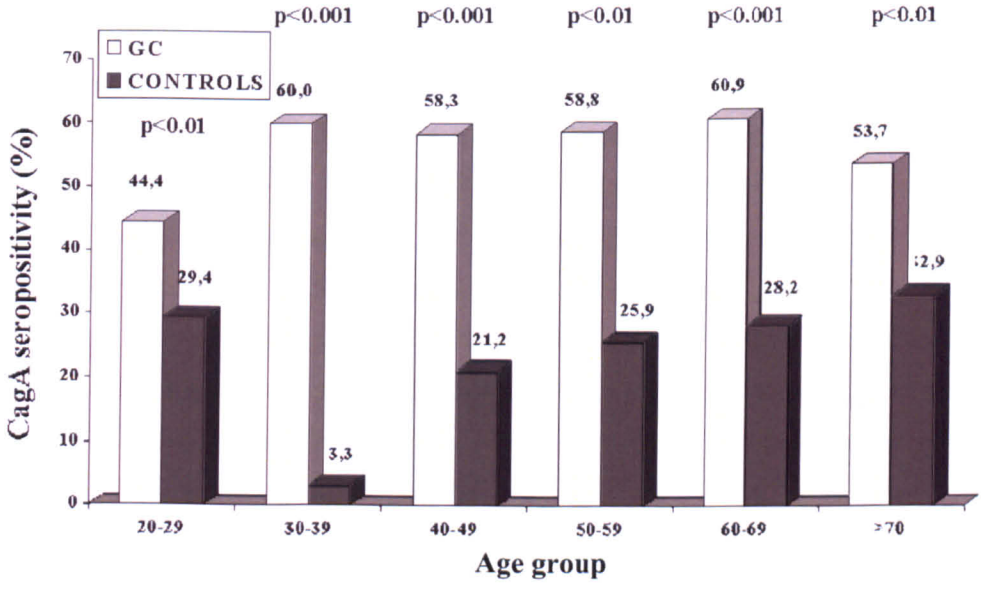


Figure 1.6: CagA association with the development of gastric cancer.

CagA seropositivity was significantly higher in patients with gastric cancer (GC) compared to controls, demonstrated for all age groups (Konturek *et al.*, 2009).

1.6.4 Vacuolating Cytotoxin A (VacA)

Soon after the discovery of *H. pylori*, broth culture filtrates of the bacterium were reported to induce vacuolation in the cell cytoplasm of cultured mammalian cells (Leunk *et al.*, 1988). The toxin responsible was later purified from culture supernatants and named the Vacuolating cytotoxin A (VacA) (Cover & Blaser, 1992). The *vacA* gene, which encodes the toxin, varies in size between strains, but is roughly 3.9kb and encodes a protoxin approximately 139kDa in size (Cover *et al.*, 1994). All strains of *H. pylori* have the *vacA* gene and most secrete VacA protein, however not all strains are able to induce cytoplasmic vacuolation of epithelial cells *in vitro* (Harris *et al.*, 1996). The reason for this is that *vacA* is naturally polymorphic, and the three main diverse regions are the signal (s) region, the intermediate (i) region, and the mid (m) region (Figure 1.7). All regions exist in two main allelic forms. The s-region was observed to play a role in vacuolating activity through altering channel formation in lipid bilayers *in vitro*, with s1-type strains having most activity whilst s2-type strains are non-vacuolating (McClain *et al.*, 2001). Analysis of the N-terminal region identified an additional hydrophilic 12 amino acid extension in s2-type toxins that was not observed in the hydrophobic N-terminus of s1-type VacA. Addition of this 12 amino acid extension to s1-type VacA blocked vacuolating activity, and removal of this region in s2-type VacA established vacuolating activity in the bacterial strain (Letley & Atherton, 2000; Letley *et al.*, 2003). Analysis of the s-region sequence determined further variations in s1-type strains, identifying three subtypes: s1a, s1b and s1c. No correlation with respect to geographic distribution of s1a and s1b subtypes was found; although the s1c subtypes were isolated from East Asian strains (van Doorn *et al.*, 1998). Variations in the mid-region were found to be important for cell specific targeting of vacuolation. In studies using various cell lines, m1-type *vacA* was found to bind and vacuolate RK13 and HeLa cells, whilst m2-type *vacA* was only capable of binding to and inducing vacuolation of RK13 cells. This difference in cell binding was attributed to differences in cell specific targets or receptor binding by m1 and m2-type VacA (Ji *et al.*, 2000). Receptor targets are discussed further below (Section 1.6.4.1). As with the s-region, sequence analysis of the m-region identified further polymorphic variation in m2-type VacA, giving rise to m2a and m2b subtypes of the toxin, the latter isolated in East Asian strains (van Doorn *et al.*, 1998).

A variation in a third region of the *vacA* gene located between the signal and mid region was recently observed and was termed the intermediate-(i-) region. The i-region type was also identified as a determinant of vacuolating activity, and was found to influence epithelial cell-type specificity similar to the m-region (Rhead *et al.*, 2007), although the mechanism for this effect is unknown.

Natural mosacism is observed for *vacA*, leading to the existence of all combinations of s-, m- and i-region type strains. This gives rise to strains with varying vaucolating capability (Atherton *et al.*, 1995; Letley *et al.*, 1999; Rhead *et al.*, 2007). Infections with strains of *H. pylori* which express active forms of the VacA cytotoxin have been highly associated with disease progression. In many populations, *vacA* s1/m1 type strains have been associated with peptic ulceration and gastric cancer (Atherton *et al.*, 1995; Basso *et al.*, 1998; Figueiredo *et al.*, 2001; Rudi *et al.*, 1998). This association was also observed for bacterial strains isolated from East Asian countries. However, *vacA* s1/m1 type strains are predominant in these regions, and some cancer patients were found to be infected with m2-type strains (Ito *et al.*, 1997; Shimoyama & Crabtree, 1998). Alternatively, of the 3 polymorphic regions of *vacA*, only the i-region was found to be a significant and independent marker of GC associated strains within an Iranian population studied (Rhead *et al.*, 2007). This association was also observed in a later study, which also reported a correlation between i1-type *vacA* and PUD (Basso *et al.*, 2008). In addition, i1-type strains were found to be associated with GU disease in an Iraqi population (Hussein *et al.*, 2008).

Overall, VacA induces cellular responses that lead to cell damage, and expression of the most virulent form of the toxin is associated with disease progression.

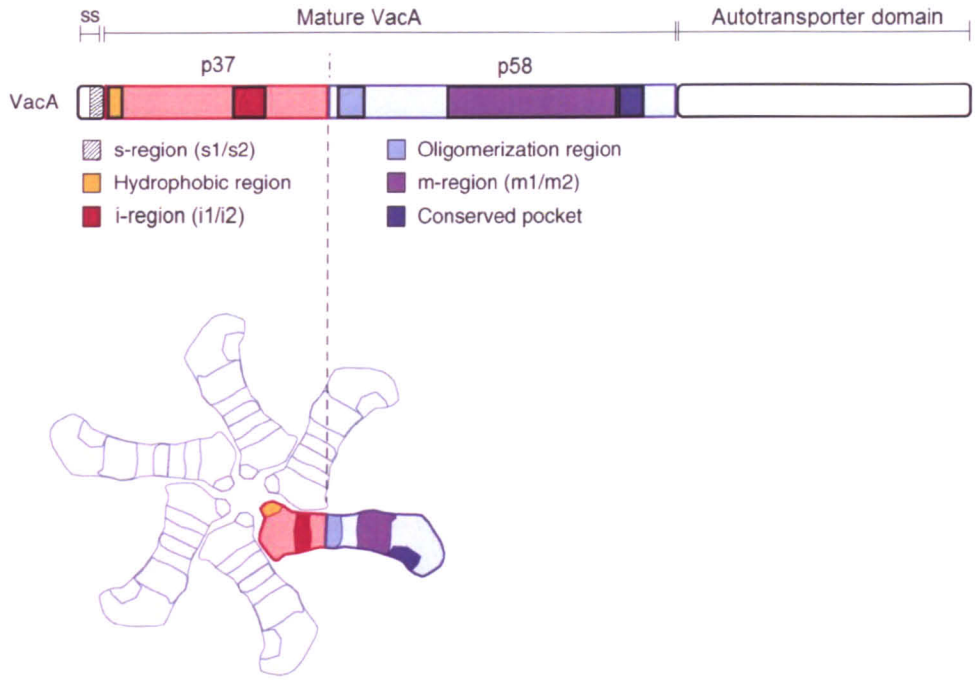


Figure 1.7: VacA protein processing

Schematic representation of the VacA cytotoxin. The *vacA* gene encodes a protoxin approximately 139kDa in size. An autotransporter domain in the C-terminus, and the N-terminal signal sequence are cleaved during secretion of the toxin, leaving the mature toxin ranging in size between 87-95kDa. Further processing can produce the p37 and p58 domains, which remain non-covalently associated. The mature toxin forms oligomeric structures, with hydrophobic regions and p37 domains forming the core that acts as a channel. The p58 domain is orientated towards the outside of the flower shaped structure (Adapted from Sewald *et al.*, 2008a).

1.6.4.1 Structure of VacA

As mentioned, the *vacA* gene encodes a protoxin of approximately 139kDa, which is secreted by the bacterium using an autotransporter (type V)-secretion system. Processing of the protein begins during secretion, during which a 33kDa autotransporter domain in the C-terminus, and 33 amino acid N-terminal signal region are cleaved. Further processing of the secreted VacA toxin results in cleavage of a 12kDa C-terminal fragment (Bumann *et al.*, 2002) that is thought to share similar properties as the α-protein of the autotransporter *Neisseria*

gonorrhoeae IgA1 Protease (Pohlner *et al.*, 1987). This protein is known to stimulate T-helper cell responses in peripheral blood monocytes (Jose *et al.*, 2000). However, the role of the cleaved VacA fragment of *H. pylori* is not known. The mature 87-95kDa toxin consists of an N-terminal domain (p37) where the signal and intermediate polymorphic regions are located, and a C-terminal domain (p58) consisting of the m-region (Figure 1.7). The two domains may be cleaved at an exposed protease-sensitive loop between them, but remain non-covalently associated. Studies suggest that the interaction between these two domains is important for VacA toxicity (Torres *et al.*, 2004).

The native VacA protein exists as a large oligomeric structure arranged as a flower-shaped complex, which is thought to consist of one or two rings, each comprising six to seven VacA molecules. Monomers in the complex are arranged to form a hydrophobic central anion selective channel, with p37 domains located near the central ring. The p58 domains are orientated towards the outside of the VacA oligomer (Figure 1.7). Purified oligomeric VacA has poor vacuolating activity *in vitro*, however under acidic conditions the structure dissembles and vacuolating activity of the toxin increases. Interestingly, at pH 2.0 VacA is highly resistant to proteolytic cleavage by the digestive enzyme pepsin (de Bernard *et al.*, 1995). Dissociated monomers can then form membrane-associated hexamers on the epithelial cell surface that act as anion-selective pores, causing egress of anions and urea from host cells (Cover *et al.*, 1997; Lupetti *et al.*, 1996) (Figure 1.8).

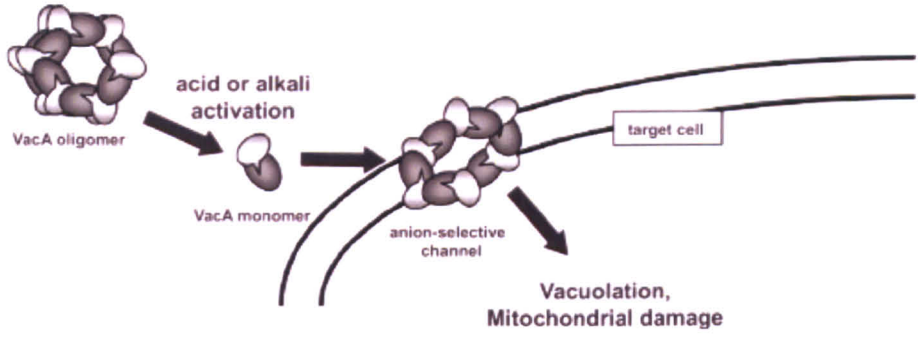


Figure 1.8: Oligomeric structure and membrane assembly of VacA.

A schematic view of the secreted oligomeric VacA toxin. Individual VacA monomers arrange to form a flower shaped complex consisting of 6-7 monomers, which has poor activity. Acid or alkali exposure results in the breakdown of the structure, releasing individual molecules that are biologically active. VacA active toxin monomers form a membrane-associated anion selective channel that allows for vacuolation (Wada *et al.*, 2004).

1.6.4.2 VacA binding to epithelial cells

Binding of VacA to specific cell targets is crucial for toxicity. A number of potential receptors have been suggested for binding of VacA. Numerous studies have identified transmembrane receptor-like protein tyrosine phosphatases α (RPTPα) and β (RPTPβ) as VacA-specific receptors on epithelial cell surfaces (Yahiro *et al.*, 1997; Yahiro *et al.*, 2003). Furthermore, glycosylation of a five amino-acid sequence of RPTPβ was found to be essential for VacA binding (Yahiro *et al.*, 2004). Recently, a T-cell receptor, the β2 integrin subunit of lymphocyte function-associated antigen-1 (LFA-1), CD18, was also identified as a VacA specific receptor (Sewald *et al.*, 2008b). The polymorphic m-region of VacA accounts for cell-type specific binding of the toxin. Whilst m1-type VacA was found to induce vacuolation in both rabbit kidney (RK13) and HeLa cell lines, vacuolation was not observed in HeLa cells with m2-type VacA (Ji *et al.*, 2000; Pagliaccia *et al.*, 1998). Analysis of the toxin sequence revealed amino acids 460-496 were involved in this cell-specific binding of m1-type VacA, and an insertion of 23 amino acids at position 475 was identified in the m2-type (Ji *et al.*, 2000).

1.6.4.3 VacA functions

Cellular vacuolation was one of the first characterised cytotoxic activities of VacA (Leunk *et al.*, 1988). VacA is internalised at lipid raft structures via a clathrin-independent pathway of endocytosis which requires cellular components such as the small GTPase Cdc42 (Ricci *et al.*, 2000). Once internalised, VacA monomers form an anion selective channel in endosomal membranes and are carried along the endocytic pathway to vacuolar-type ATPase (V-ATPase) positive late endosomes and lysosomes. Both late endosomal and lysosomal markers are found in vacuole membranes, suggesting that VacA may disrupt membrane trafficking at the level of late endosomes (Molinari *et al.*, 1997). The formation of vacuoles requires the full activity of V-ATPase, which acidifies the compartment and is potentiated by the presence of weak bases such as ammonium chloride (Cover *et al.*, 1993). VacA channels allow for an influx of chloride anions into the endosomal compartment, which results in a reduced membrane potential. This stimulates an increase in the proton concentration inside the vacuole through V-ATPase. An influx of weak bases through the vacuole membrane in response to the acidification of the compartment follows. These weak bases react with protons which prevents their escape. The resulting imbalance in osmotic pressure leads to the influx of water and swelling of the vacuole (Cover & Blaser, 1992). Rab7, a membrane traffic regulator, and Rac1, an actin-cytoskeleton associated protein, are small GTP-binding proteins that have also been recognised in playing a role in vacuole formation and are thought to be associated with VacA-induced vacuoles (Hotchin *et al.*, 2000; Papini *et al.*, 1997).

In addition to forming anion selective channels that induce vacuolation in endosomal compartments, VacA also increases epithelial permeability through formation of these channels at the apical surface of the gastric epithelium. This allows for ions and small neutral molecules to diffuse through epithelial cells towards the stomach lumen, particularly bicarbonate ions and pyruvate (Tombola *et al.*, 1999). Channel formation also permeabilises the membrane to urea, which is the essential substrate for the urease enzyme required for increasing local pH for protection of the bacterium (Tombola *et al.*, 2001). Weakening of tight junctions has also been suggested as a result of VacA toxicity, allowing the release of essential nutrients for bacterial growth (Papini *et al.*, 1998).

VacA has also been shown to localise to mitochondria resulting in disruptive effects on energy metabolism, including a decrease in mitochondrial membrane potential and reduced cellular ATP levels. An important response to VacA is the release of cytochrome c into the cytosol, an initiator of cellular apoptosis (Galmiche *et al.*, 2000), through the activation of Bax and Bak which are pivotal regulators of cytochrome c release (Yamasaki *et al.*, 2006).

Studies have implicated VacA as a key component in interfering with antigen processing and presentation. Two pathways exist for the generation of T-cell epitopes, either in early endosomes for loading on recycling MHC II molecules (the invariant chain [Ii]-independent pathway) or after antigen degradation in late endosomes for loading on newly synthesised MHC II molecules (Ii-dependent pathway). VacA was demonstrated to selectively inhibit the Ii-dependent pathway of antigen presentation, resulting in an impaired T cell response due to defective antigen production (Molinari *et al.*, 1998).

VacA also impairs T cell proliferation via a mechanism that leads to inhibition of *IL2* expression, a cytokine essential for T cell viability and proliferation. This mechanism involves blocking nuclear translocation of the transcription factor NFAT, which is essential for *IL2* expression (Boncristiano *et al.*, 2003). Activation of NFAT is induced through two independent pathways, one involving the influx of calcium and the other through the activation of stress related tyrosine kinases (Rao *et al.*, 1997). VacA was found to block NFAT activation by interfering with both pathways. The formation of VacA anion selective channels disrupts plasma membrane potentials, which likely drives decreased calcium ion influx. Alternatively, a channel-independent activation of the stress activated kinase p38 was found to inhibit NFAT activation, triggered by VacA association with a cell surface receptor (Boncristiano *et al.*, 2003).

It is clear that VacA is involved in numerous mechanisms to avoid and suppress an immune response. However studies have suggested a proinflammatory response that is elicited by this cytotoxin. Boncristiano *et al.* (2003) reported that VacA treatment of both macrophages and neutrophils showed substantial activation of p38 that consequently increased the expression of cyclooxygenase-2

(Cox-2), which contributes to inflammation by production of proinflammatory prostanoids. Increased COX-2 expression also induces gastrointestinal tumorigenesis through several mechanisms including the inhibition of apoptosis (Fujimura *et al.*, 2006). Hence, VacA may play a role in *H. pylori* induced gastric inflammation. The cellular effects of VacA are summarised in Figure 1.9.

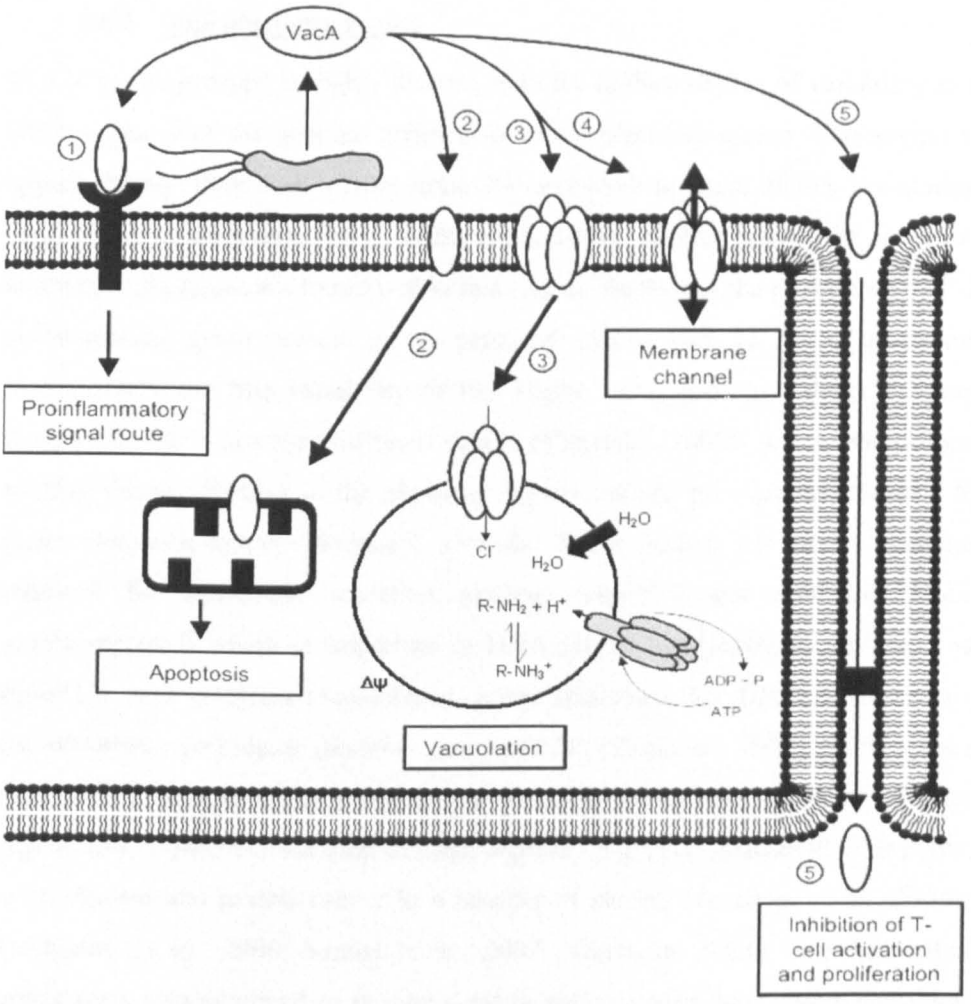


Figure 1.9: VacA intracellular interactions.

VacA interacts with cellular processing via a number of mechanisms that aid chronic colonisation of *H. pylori*. (1) VacA binds to cell membrane receptors and initiates a proinflammatory response; (2) the toxin is taken up by cells and trafficked to the mitochondria where it induces apoptosis; (3) Pore formation in cellular compartments reduces membrane potential and increased proton concentrations, leading to water influx and vacuolation; (4) membrane channel formation, resulting in leakage of nutrients to the extracellular space; and (5) VacA passes through tight junctions and inhibit T-cell activation and proliferation. (Adapted from Kusters *et al.*, 2006).

1.6.5 The plasticity region

The *H. pylori* genome is highly diverse, with the highest degree of variability in a DNA segment of the genome referred to as the plasticity region. This region is approximately 45kb in *H. pylori* strain J99 and 68kb in strain 26695, accounting for 3-4% of chromosomal DNA (Alm *et al.*, 1999). Almost half of the *H. pylori* strain-specific genes are found within this region. Analysing the prevalence of J99 strain-specific genes present in the plasticity region over 72 *H. pylori* strains demonstrated the true variability of this region, with gene prevalence ranging from 13 to 100% amongst different strains (Yamaoka, 2008). A number of open reading frames (ORFs) in the plasticity region encode proteins homologous to genes encoding known functional proteins. These include *vir* factors that are required for functional secretion systems (*jhp0917* and *jhp0918*), DNA topoisomerase I which is important in DNA replication (*jhp0919*, *jhp0920* and *jhp0931*), and integrase/recombinase genes important for DNA modification, recombination and repair (*jhp0941* and *jhp0951*) (Yamaoka, 2008). Studies have identified a relationship between the prevalence of certain genes in the plasticity region and *H. pylori*-associated disease. *Jhp0947* has been associated with peptic ulcer disease and gastric cancer in a number of studies (de Jonge *et al.*, 2004a; Occhialini *et al.*, 2000; Santos *et al.*, 2003; Yamaoka, 2008). Although other genes were also observed to be prevalent in patients with these disease profiles, such an association was not observed in different populations tested and little is known with respect to the plasticity region and clinical outcomes in East Asian strains (de Jonge *et al.*, 2004b; Rizwan *et al.*, 2008; Santos *et al.*, 2003).

1.6.6 Duodenal ulcer-promoting gene A (DupA)

In a recent study, the prevalence of genes *jhp0917* and *jhp0918* were found to be significantly higher in patients diagnosed with DUD. Furthermore, the prevalence of both genes was closely associated, with only 2% of tested strains being negative for just one gene. In this study, a 1 base pair insertion (C or T) at the 3' region of *jhp0917* was found which resulted in a frame shift leading to a continuous gene with *jhp0917* and *jhp0918*. This gene was named the duodenal ulcer promoting gene A (*dupA*) (Lu *et al.*, 2005). Bioinformatic analysis showed the gene encoded an intact *virB4* homologue, with the N-terminus (encoded by the

C-terminal region of *jhp0917*) showing homology to members of the FtsK/SpoIIIE family, a putative ATP-binding P-loop motif that has been implicated in intercellular chromosomal DNA transfer. The central domain of *dupA* (encoded by *jhp0917* and N-terminal region of *jhp0918*) shares homology with the TraG/TraD family, which are NTP hydrolases thought to be essential for DNA transfer in bacterial conjugation (Lu *et al.*, 2005).

The functions of DupA are not fully understood, but based on its homology to the ATPase VirB4, DupA is hypothesised to play a role in the correct assembly of another type IV secretion apparatus, or the delivery of effector molecules through such a system. Lu *et al.* (2005) also demonstrated increased IL-8 secretion with *dupA* positive strains *in vivo* and *in vitro*. Recently, *dupA* was found to be polymorphic with varying gene size in different *H. pylori* isolates. Analysis of the function of these isolates demonstrated the extended *dupA1* allelic form to induce IL-12 secretion in peripheral blood mononuclear cells (PBMCs) which was not observed with *dupA2* type strains as used by Lu *et al.* (2005). Furthermore, *IL8* expression was found to be increased for *dupA* positive strains in gastric tissue, but not for gastric epithelial cell lines, suggesting DupA may directly stimulate IL-8 secretion from other cell types, or may indirectly induce the expression of other cytokines known to stimulate epithelial cell IL-8 secretion (Hussein *et al.*, 2010). Importantly, it is not known which of the *dupA* alleles encode functional proteins.

The original study describing *dupA* demonstrated an association between the presence of this gene and duodenal ulceration. In addition, a possible protective role of *dupA* in the development of gastric cancer was suggested (Lu *et al.*, 2005). Evaluation of *dupA* and clinical outcome of infection has been studied further in a number of populations. An association between *dupA* positivity and DUD was confirmed in North India (Arachchi *et al.*, 2007), Iraq (Hussein *et al.*, 2008), and China (Zhang *et al.*, 2008). However, this was not the case in patients examined in Iran (Douraghi *et al.*, 2008; Hussein *et al.*, 2008), Belgium, South Africa, China and North America (Argent *et al.*, 2007). Interestingly, these studies did confirm the protective association between *dupA* and the development of gastric cancer (Argent *et al.*, 2007; Douraghi *et al.*, 2008). However, no association was found in other populations (Gomes *et al.*, 2008), demonstrating distinct geographical

variations in the prevalence of the *dupA* gene and its association with the clinical outcome of infection.

1.6.7 *H. pylori* neutrophil-activating protein (HP-NAP)

H. pylori neutrophil-activating protein (HP-NAP) is an oligomeric protein of 150kDa, with a central cavity for iron accumulation. It was initially identified as a promoter of endothelial adhesion of neutrophils, and also found to be a possible adhesin to mucin (Namavar *et al.*, 1998). The *napA* gene is found in all *H. pylori* strains, and differences in the level of gene expression may explain the differing ability of individual strains to induce inflammation. However, HP-NAP synthesis was found in 80% of *H. pylori* strains, regardless of whether patients presented with disease or not (Thoreson *et al.*, 2000). Overall, cellular effects of HP-NAP lead to the induction of T helper1 polarised immune responses that play a major role in maintaining chronic infection. This virulence determinant drives the inflammatory response through a number of mechanisms (illustrated in Figure 1.10). HP-NAP is capable of crossing the epithelial layer to the underlying tissue where it comes into contact with a number of leukocytes. An important feature of HP-NAP is the ability of the virulence factor to induce extravasation of rolling leukocytes across the endothelial layer through direct contact with cells. β2 integrin expression, essential for tight adhesion of leukocytes to endothelial cells, is also upregulated through contact with HP-NAP, leading to the recruitment of inflammatory cells towards the site of infection (Polenghi *et al.*, 2007; Satin *et al.*, 2000). HP-NAP was also found to stimulate mast cells and macrophages to secrete TNFα, which is important for increasing the adhesiveness of endothelial cells, representing an indirect mechanism by which HP-NAP induces the recruitment of leukocytes (Amedei *et al.*, 2006; Montemurro *et al.*, 2002).

In addition to these functions, HP-NAP was also found to stimulate monocytes and dendritic cells to promote an IL-12 and IL-23 rich milieu known to drive differentiation of antigen-stimulated T-cells towards a proinflammatory Th1 phenotype (Oppmann *et al.*, 2000; Trinchieri, 2003). Furthermore, HP-NAP was shown to drive naive T cells (Th0 cells) to express a Th1 polarised profile and also downregulate the Th2 antibody response (Amedei *et al.*, 2006; D'Elios *et al.*, 2009).

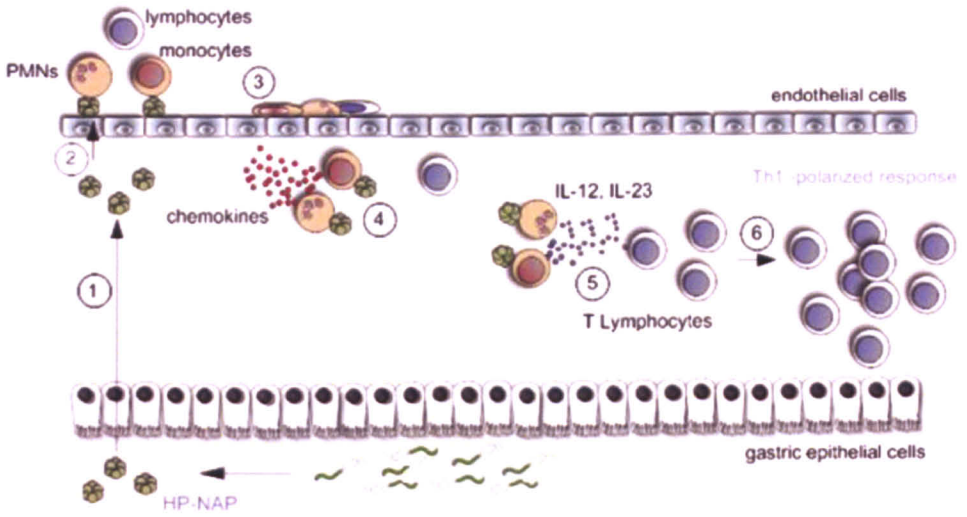


Figure 1.10: Cellular effects of *H. pylori* HP-NAP.

Schematic representation of HP-NAP functions in driving the inflammatory response to *H. pylori* infection. Once secreted by the bacterium, (1) HP-NAP crosses the gastric epithelial lining and (2) the endothelium, and (3) stimulates the adherence of leukocytes to endothelial cells, (4) followed by extravasation of cells. (5) HP-NAP also stimulates the secretion of cytokines IL-12 and IL-23 from activated neutrophils and monocytes, (6) thus creating an IL-12/IL-23-enriched milieu which is responsible for driving the differentiation of T helper cells towards the Th1 phenotype (Adapted from de Bernard & D'Elios, 2009).

1.7 THE IMMUNE RESPONSE TO *H. PYLORI*

The immune system uses a complex array of mechanisms to eliminate pathogenic microbes, whilst controlling inflammation to avoid responses that produce excessive damage to self-tissue. The immune response to infection can be categorised as innate immunity, which involves rapid responses by non-specific recognition of infecting particles and represent the initial response to infection, or adaptive immunity, which involves highly specific immune responses that come into play later over the course of an infection. Synergy between innate and adaptive immunity is essential for a fully effective immune response capable of eliminating microbial infections.

1.7.1 Innate immune responses to H. pylori

Innate immunity is central to the immune response to *H. pylori* infection, and acts as a front line defence against the bacterium. A number of components are involved and are discussed below.

1.7.1.1 Epithelial cells

The epithelial surface provides the first line of defence against infection. Tight junctions between these cells create an effective seal against the external environment. Mucosal epithelia are further protected by the expression of surface mucins, a collection of glycoproteins that create a thick gel covering the cell surface that is constantly shed and replaced. These cells also secrete antimicrobial peptides (Section 1.7.1.6). In this way, bacterial adhesion is greatly hindered. However, *H. pylori* have developed to bypass this protective barrier by means of flagella, along with the helical shape of the bacterium, to effectively burrow through the mucous gel to the epithelial cell surface, and also through down-regulation of certain antimicrobial peptides (Chapter 8, section 8.1). Evasion of this protective barrier is followed by adhesion to the cell surface through non-specific receptors that recognise common bacterial components.

Importantly, epithelial cells also secrete cytokines in response to infection, which are essential in the initiation of innate and ultimately adaptive immune responses. One of the key responses to *H. pylori* infection, and specifically to the presence of CagPAI is the induction of *IL8* expression (Brandt *et al.*, 2005), which is

important for the recruitment for the recruitment of neutrophils to the lamina propria.

1.7.1.2 Cell surface receptors

Attachment to gastric epithelial cells induces an inflammatory response via a number of mechanisms. Activation of such responses depends on recognition of small conserved molecular motifs on microbes known as pathogen-associated molecular patterns (PAMPs), which include LPS and peptidoglycan. These microbial components act as ligands for cell surface pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), activation of which is followed by induction of intracellular signalling pathways that lead to the activation of transcription factors such as NF-κB essential for inflammatory cytokine gene expression (Netea *et al.*, 2004).

Studies addressing the role of TLRs in mediating gastric inflammatory responses have been conflicting. Several lines of evidence have demonstrated the roll of TLR4, which recognises bacterial LPS, in *H. pylori* immune recognition. TLR4 RNA was detected in human gastric epithelial cell lines (Su *et al.*, 2003) and in human gastric pit cells (Ishihara *et al.*, 2004). However, other studies have demonstrated TLR4 not to be important in recognising *H. pylori* (Backhed et al., 2003; Mandell et al., 2004). In any case, *H. pylori* LPS is a poor activator of innate immune responses compared to other Gram-negative bacteria (Moran, 1999; Muotiala *et al.*, 1992). *H. pylori* recognition was found to be predominantly mediated by TLR2, which recognises peptidoglycan, rather that TLR4 (Mandell *et al.*, 2004). Furthermore, TLR2 was found to be important in *H. pylori* induced NFκB activation, demonstrating the importance of this receptor for immune recognition of the bacterium (Smith *et al.*, 2003). As with TLR4, evidence for the role of TLR2 in *H. pylori* recognition is controversial. TLR2 expression was not detected in human gastric tissue (Mandell *et al.*, 2004).

TLR5, important for the detection of flagellin, has also been implicated in *H. pylori* infection (Smith *et al.*, 2003). However, other studies have found little, if any, stimulation of this receptor. Such discrepancies in the reported involvement of TLR5 may relate to variable receptor expression patterns on different gastric cell lines (Gewirtz *et al.*, 2004; Lee *et al.*, 2003).

Nuclear-binding oligomerisation domain proteins 1 (NOD1) and 2 (NOD2) are intracellular PRRs which recognise cytosolic PAMPs and are expressed primarily in antigen presenting cells (APCs) and epithelial cells. These receptors are responsible for activating NF-κB pathways, which ultimately initiate transcription of inflammatory responses. NOD1 is a key component in activating inflammation during *H. pylori* infection in response to peptidoglycan injected by the CagPAI. In particular, NOD1 activation in epithelial cells is associated with up-regulation of *IL8* expression (discussed in section 1.6.3.1) (Viala *et al.*, 2004).

1.7.1.3 Neutrophils

Neutrophils are phagocytic, multi-lobed leukocytes normally found in the blood, which migrate into tissue to sites of infection in response to chemotactic factors. These cells function to internalise and kill microbes, releasing bactericidal peptides and reactive oxygen metabolites. In the gastric mucosa, epithelial cells are stimulated in a CagPAI-dependent manner to secrete IL-8, a chemokine important in recruitment of neutrophils to the site of infection (Suzuki *et al.*, 2004b). Stimulation of neutrophils by *H. pylori* results in further release of IL-8 from these cells which leads to recruitment of more neutrophils in the gastric mucosa (Shimoyama *et al.*, 2002). Numerous studies have shown *H. pylori* infection to induce an active inflammation, characterised by infiltrating neutrophils (example slides given in appendix 11.1) (Basso *et al.*, 2008; Demirturk *et al.*, 2001). Neutrophilic activity is dependent on the generation of reactive oxygen species (ROS) for effective killing of phagacytosed bacteria (Sawyer *et al.*, 1989). Enhanced ROS production in the gastric mucosa is highly associated with the development of *H. pylori*-associated disease (Suzuki *et al.*, 1996; Zhang *et al.*, 1996), and furthermore, correlates with CagA positive infections and increased neutrophil infiltration of the gastric mucosa (Danese *et al.*, 2001). Increased neutrophilic infiltration is also induced by *H. pylori* secreted HP-NAP (discussed in detail in section 1.6.7). Therefore, the phagocytic activity of neutrophils along with degranulation of stored proteins (e.g. metalloproteinases and serine proteases) that are capable of tissue damage (Rautelin *et al.*, 2009), contribute to the immune response against *H. pylori*.

1.7.1.4 Macrophages

Macrophages patrol the tissue for microbial infection. They are phagocytic cells and therefore, like neutrophils, are essential for initial responses to infection. These cells express a wide range of PRRs which are key for the recognition of microbes. Resistance to phagocytic killing by *H. pylori* has been observed in macrophages. *In vitro* studies demonstrated delayed phagocytosis in macrophages, and the accumulation of viable bacteria in large megasomal structures, protecting intracellular bacteria. The survival of *H. pylori* in megasomes was shown to be dependent on the *cag*PAI (Allen, 2001).

Macrophages' activity is also dependent on the release of nitric oxide (NO) through activation of inducible nitric oxide synthase (iNOS), which aids in killing intracellular bacteria. *H. pylori* infection is associated with increased activation of iNOS in macrophages (Fu *et al.*, 1999). This enzyme produces nitric oxide (NO) from the terminal nitrogen atom of L-arginine. However, *H. pylori* prevents NO synthesis by producing arginase, which converts L-arginine to urea and L-ornithine. In this way, *H. pylori* regulates NO production by competing for the substrate essential for its synthesis (Gobert *et al.*, 2001; von Bothmer *et al.*, 2002). Recently, activation of ERK signalling by *H. pylori* resulted in apoptosis of machrophages through the activation of c-Myc and ornithine decarboxylase (ODC) *in vitro* and in macrophages of the gastric mucosa, thus demonstrating a further mechanism by which *H. pylori* may interfere with host immune response (Asim *et al.*, 2010).

In addition, macrophages play an essential role in bridging the innate and adaptive immune response by presenting processed antigen on their cell surface, ready for recognition and activation of naive T-cells. Stimulation of macrophages by *H. pylori* induces the production of pro-inflammatory cytokines IL-6, IL-1β and TNFα (Crabtree *et al.*, 1991b; Gionchetti *et al.*, 1994). Furthermore, *in vitro* stimulation of macrophages resulted in the secretion of IL-12 (which stimulates T cells to secrete IFNγ) (Rad *et al.*, 2007), and IL-23 (which stimulates Th17 cell secretion of IL-17 (discussed in section 1.7.2.3)) (Amedei *et al.*, 2006), both of which are important to the immune response to *H. pylori* infection. The migration inhibitory factor (MIF), which is secreted by macrophages and other cell types, is associated with increased expression of chemokines, and therefore increases the

migration of macrophages to the site of infection. Recently, increased expression of MIF was observed in *H. pylori* infected mice, suggesting a role for this cytokine in the development of gastritis (Wong *et al.*, 2009). The importance of these cells in gastric inflammatory responses was also demonstrated after depletion of macrophages in mice reduced gastritis after *H. pylori* infection (Kaparakis *et al.*, 2008).

1.7.1.5 Dendritic cells

Like macrophages, dendritic cells (DCs) act as phagocytic cells, and are critical for the activation of adaptive immune responses though their ability to present antigen on the cell surface. They are found in tissue as immature cells, and mature on antigen stimulation of PRRs. Human dendritic cells were found to be activated by *H. pylori* in culture, and may produce a host of cytokines, including IL-6, IL-8, IL-10, IL-12, IL-1β and TNFα (Hafsi *et al.*, 2004; Kranzer *et al.*, 2004; Kranzer *et al.*, 2005). The cytokine profile produced by activated DCs determines the nature of the immune response. *H. pylori* infection has also been reported to induce IFNγ and IL-17 which drive a Th17, and hence, a pro-inflammatory response to infection (Khamri *et al.*, 2010). Recently, *H. pylori* were found to be ingested by dendritic cells (Rad *et al.*, 2007), and furthermore, were shown to reduce DC function in a CagA-dependent manner *in vitro*, with reduced ability to recognise LPS and reduced naive T cell differentiation to a Th1 phenotype (Tanaka *et al.*, 2010). Therefore, dendritic cells are essential for development of adaptive immune responses to *H. pylori*, and the bacterium has developed to inhibit such responses in dendritic cells.

1.7.1.6 Antimicrobial peptides

In order to fight potentially harmful microbes, multi-cellular organisms have developed specific defence mechanisms to evade such infections. Many species have evolved to produce and secrete small peptides known as antimicrobial peptides (AMPs) which are cationic peptides that exhibit a wide range of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, protozoa and viruses implicating them as direct effectors of the host immune response, acting as an early barrier in host defence. A number of different AMPs exist that are heterogeneous with respect to their primary and secondary structure,

tissue distribution, their effects on host cells, and antimicrobial potential. The expression of the mature peptide may be constitutive or inducible depending on species, tissue type and cellular lineages (Islam *et al.*, 2001; Selsted & Ouellette, 2005).

The most extensively studied families of AMPs are the defensins and cathelicidins, which are structurally and evolutionarily distinct, but similar in distribution and abundance (Chen *et al.*, 2006b).

1.7.1.6.1 Defensins

The defensin family of AMPs are small, arginine-rich peptides with molecular weight of 4-5kDa. They contain 6-8 conserved cysteine residues that form 3-4 disulfide bonds, and are categorised based on the position of the cysteine residues as well as gene sequence. The family is subdivided into the α-, β- and θ-defensins based on the distribution of cysteine residues (Ganz, 2003).

Of the 6 identified α-defensins, HNP 1-4 were isolated from neutrophil cells, and constitute between 5-7% of neutrophil protein content, acting intracellularly with phagolysozomes to kill engulfed bacteria (Ganz & Lehrer, 1994). The second major source of α-defensins are intestinal Paneth cells, which in humans, express HD5 and HD6 (Jones & Bevins, 1992; Jones & Bevins, 1993), although other intestinal cells have been reported to express HD5 (Cunliffe *et al.*, 2001).

The β-defensin subfamily consist of three β-strands flanked by an α-helical segment in the N-terminal fragment of the protein (Pazgier *et al.*, 2006). The first isolated human β-defensin was hβB1 (Bensch *et al.*, 1995), for which expression was subsequently detected in numerous epithelial cells including the gastrointestinal tract. As with hβB1, hβB2 and hβB3 expression is abundant in numerous epithelial cells (Table 1). Recently, hβD4 was identified and found to be expressed highly in testis and the gastric antrum. Subsequent studies observed expression of this defensin in lung, kidney and uterus, as well as neutrophils (Garcia *et al.*, 2001). The hβD5 and hβD6 peptides were isolated in 2002, and found to be expressed specifically in human epididymis (Yamaguchi *et al.*, 2002). Importantly, a number of other human β-defensin genes have been identified by

sequence analysis, although functional proteins are yet to be isolated (Schutte *et al.*, 2002).

The θ-defensins are structurally dissimilar to the α- and β-defensins as they are cyclised. They have been isolated in several species of Old world monkeys, but not in humans (Nguyen *et al.*, 2003). Although mRNA orthologs have been identified, mutations that introduce stop codons in the sequence abolish peptide production (Cole *et al.*, 2002).

1.7.1.6.2 Cathelicidins

Cathelicidins are a large family of AMPs commonly found in mammalian species. These AMPs are not characterised based on structure, but rather by the way in which they are produced and stored in cells. All members of the family are synthesised as a large precursor peptide with the antimicrobial activity ascribed to the C-terminal portion of the protein. Heterogeneity in the C-terminus yields peptides with varying secondary structure and hence bactericidal activity. These AMPs are categorised broadly into 3 groups, the most common being the α-helical amphiphatic structure, whist the other groups are flat β-sheet structures that are stabilised by disulphide bonding. The only homologue that has been described in humans is the α-helical LL-37 (Agerberth *et al.*, 1995). LL-37 is processed from the proprotein hCAP-18 encoded by the *camp* gene (Gudmundsson *et al.*, 1996). LL-37 was initially identified in granules of myeloid cells, but has since been detected in epithelial layers of the skin, respiratory tract and gastrointestinal tract (Frohm *et al.*, 1997; Hase *et al.*, 2003). LL-37 is actually stored as the proprotein in these cells until activated by serine protease cleavage, and after cleavage of hCAP-18, the N-terminal domain may also exhibit antimicrobial activity (Zaiou *et al.*, 2003). LL-37 has been found to be active against a broad range of microbes with a wide overlap in specificity (Durr *et al.*, 2006). Apart from the antimicrobial functions of LL-37, immunomodulatory functions have also been described (Durr *et al.*, 2006).

The role of defensins and cathelicidins in *H. pylori* infection is discussed further in chapter 8.

	Antimicrobial spectrum	Tissue/cell distribution	Ref
α-defensins			
HNP-1, -2, -3, -4	Gram + and – bacteria Fungi, Parasites, Yeast, viruses.	Leukocytes and intestinal epithelial cells	(Cunliffe *et al.*, 2002; Ganz & Lehrer, 1994; Selsted & Ouellette, 2005)
HD5 and HD6	Gram + and – bacteria Yeast	Intestinal epithelial cells	(Cunliffe *et al.*, 2001; Jones & Bevins, 1992; Jones & Bevins, 1993)
β-defensins			
HβD1	Gram- bacteria	Epithelial cells e.g. cells derived from the kidney, female reproductive tract, respiratory tract	(Chen *et al.*, 2006b)
HβD2	Gram + and – bacteria Yeast	Epithelial cells e.g. lungs, trachea, kidney and salivary glands	(Chen *et al.*, 2006b; Mathews *et al.*, 1999)
HβD3	Gram + and – bacteria Yeast	Epithelial cells e.g. skin, tonsils, respiratory tract and female reproductive tract	(Chen *et al.*, 2006b; Harder *et al.*, 2001)
HβD4	Gram + and – bacteria Yeast	Neutrophils. Epithelial cells e.g. testis, gastric antrum, uterus, lung and kidney.	(Chen *et al.*, 2006b; Garcia *et al.*, 2001)
Cathelicidins:			
LL37	Gram + and – bacteria. Yeast.	Neutrophils, monocytes, T- and B-cells, NK cells, epithelial cells e.g. liver, stomach, skin and intestine.	(Durr *et al.*, 2006)

Table 1.2: The expression and spectrum of activity of human antimicrobial peptides.

A number of antimicrobial peptides (AMPs) are secreted by epithelial cells. Most widely investigated are the α- and β-defensins, and cathelicidins. The tissue distribution and antimicrobial spectrum for common members of these AMP families are listed.

1.7.1.6.3 Mechanism of action of AMPs

Although known to have immunomodulatory properties (Yang *et al.*, 1999), the key function of AMPs is their antimicrobial activity. The cationic characteristics and amphipathic nature of AMPs allows them to target a fundamental difference between membranes of microbes and multicellular organisms, that is the electrostatic charge of the outer membrane. Electrostatic interactions act over a relatively long distance, therefore explaining their specificity for bacterial anionic phospholipids in the outer most lipid bilayer of bacterial cells, compared to the zwitterionic amphiphiles present in mammalian membranes (Zasloff, 2002). Analysis of the primary structure of hβD1-3 indicates a considerable variation in the net charge between defensins, with the more positively charged hβD3 having the greatest antimicrobial activity. The importance of the overall charge of these peptides was revealed in mutational studies which found no difference in antimicrobial activity when single amino acid substitutions did not alter the net charge or hydrophobicity of the peptide, although bacterial susceptibility and the rate of killing were altered (Hoover *et al.*, 2003; Kluver *et al.*, 2005). Increasing the net charge and hydrophobicity increases activity, whereas peptides with reduced charge and hydrophobicity were virtually inactive (Kluver *et al.*, 2005). The structure of AMPs is also considered important to function, given the conserved spacing between cysteine residues.

AMPs are thought to kill target microbes by disrupting their membrane integrity via a mechanism known as the "Shai-Matsuzaki-Huang model". This model involves association of the peptides with the outer membrane of the bacterium, followed by displacement of the lipid bilayer resulting in membrane disruption and pore formation, leading to cell leakage and death (Figure 1.11).

The importance of these AMPs in defence becomes apparent when regulation or function is disrupted, as describing in a growing list of diseases. This includes Crohn's disease, which is associated with a decreased expression of α-defensins (Wehkamp *et al.*, 2007), and psoriasis which is associated with increased expression of AMPs (Lande *et al.*, 2007; Yamasaki *et al.*, 2007).

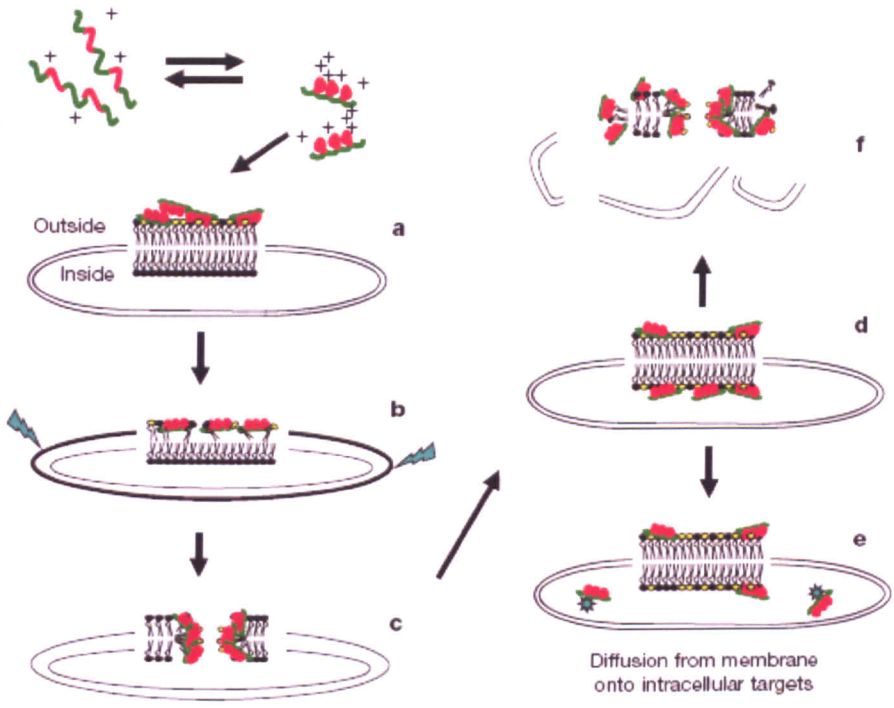


Figure 1.11: The Shai-Matsuzaki-Huang model for antimicrobial peptide mediated bacterial lysis.

The proposed mechanism for antimicrobial peptide action by the Shai-Matsuzaki-Huang model involves (**a**) the attachment of peptides to form a carpet on the outer membrane of the bacterium. (**b**) This is followed by the integration of the peptide into the membrane and thinning of the outer leaflet. The surface area of the outer leaflet expands, resulting in strain within the bilayer (jagged arrows). (**c**) This allows for pore formation, (**d**) and transport of lipids and peptides into the inner leaflet. (**e**) This leads to destruction of intracellular targets once peptides have diffused into the intracellular space, (**f**) or can result in the collapse of the membrane and cell destruction (Zasloff, 2002).

1.7.2 Adaptive Immune response

The adaptive immune response, in contrast to the non-specific innate immune response, is highly specific in targeting pathogenic microbes and clearing infections that have breached the innate response. Humoral and cell-mediated responses constitute the adaptive immune response, both of which are activated during *H. pylori* infection. However, despite this vigorous response, adaptive immunity is ineffective at clearing the infection. Instead, a severe chronic gastritis develops, which is associated with an increased risk of developing disease (Blaser & Atherton, 2004). *H. pylori* plays a central role in regulating these immune responses through virulence determinants expressed by the bacterium, which have been discussed previously.

1.7.2.1 Priming of the adaptive immune response

As mentioned, stimulation of epithelial PRRs ultimately results in the recruitment of leukocytes to the site of infection. In the case of *H. pylori* infection, these cells are recruited to the lamina propria (Crabtree, 1996; Mori *et al.*, 2001). Macrophages and DCs come into contact with *H. pylori* antigen, and are essential for activation of adaptive immunity. However, as *H. pylori* is generally considered a non-invasive pathogen present only in the mucous gel and attached to the epithelial cells, it is unclear how these APCs "sample" antigen. The loosening of tight junctions by *H. pylori* virulence determinants may disturb the integrity of the epithelial layer so that antigen can pass through to the underlying lamina propria and hence the patrolling APCs. However, non-virulent strains of *H. pylori* also induce gastritis on infection. Some studies have demonstrated an invasive capability of *H. pylori*, finding intracellular bacteria in patients with gastritis and *H. pylori*-associated disease (Genta *et al.*, 1996; Necchi *et al.*, 2007; Noach *et al.*, 1994b), and in naturally infected rhesus monkeys (Dubois *et al.*, 1994). Indeed, engulfed bacteria have been found within compartments of macrophages (Allen, 2001), and DCs were shown to bind and ingest *H. pylori* (Rad *et al.*, 2007).

Antigen sampling in the intestine is mediated through specialised aggregations of lymphoid tissue called Peyer's patches, which are not present in the stomach. The significance of these structures in the immune response towards *H. pylori* infection has been demonstrated in mice that lack Peyer's patches. These mice did not develop gastritis when infected with *H. pylori* (Nagai et al., 2007).

Furthermore, Peyer's patches were found to be important in the humoral response as well as cell-mediated responses (Kiriya *et al.*, 2007). It was also found that DCs activated by *H. pylori* in Peyer's patches instruct naive T-cells to differentiate into gut-homing T-cells, thus suggesting that the intestine is in fact a major source of antigen sampling and immune priming in *H. pylori* infection (Mora *et al.*, 2003).

There is now evidence to suggest that DCs are present at the gastric epithelial layer during *H. pylori* infection. Kao *et al.* (2010) found DCs near the surface of the epithelium in mice, and showed cytoplasmic projections of DCs extending between cells where they can sample luminal bacteria. In addition, DC-like cells have been identified in the *H. pylori* colonised human gastric epithelium (Necchi *et al.*, 2009), therefore providing evidence that priming of the adaptive immune response may occur in the stomach.

1.7.2.2 Humoral immune response

Humoral immunity is mediated by secreted antibodies produced by the cells of the B lymphocyte lineage (B cells). These cells also act as APCs, and activation of naïve B cells is mediated by the Th2 subset of T cells. Once activated, they differentiate into plasma cells capable of secreting large amounts of antibody.

The role of B cell responses to *H. pylori* infection is controversial. Although a strong IgG and IgA response has been demonstrated both systemically and in the gastric mucosa (Hayashi *et al.*, 1998; Mattsson *et al.*, 1998), the role of humoral immunity is marginal in protective immunity against the bacterium. In addition, it was shown that colonisation in B-cell deficient mice was normal during early infection, but bacteria were cleared in later weeks, demonstrating the importance of T-cell immunity for protective immunity. Furthermore, mice with normal B cell function were extensively colonised by *H. pylori* and developed mild gastritis during late infection, suggesting B-cell immunity was in fact detrimental to elimination of the bacteria and impaired the gastric inflammatory response (Akhiani *et al.*, 2004; Akhiani *et al.*, 2005).

1.7.2.3 Cell-mediated immune response

The T lymphocyte cell lineage (T-cells) is central to cell mediated immunity. T cells express the T cell receptor (TCR) and undergo clonal expansion upon

recognition of peptide fragments that are bound to major histocompatibility complex (MHC) class I or II molecules on antigen-presenting cells. T cells are classified according to the co-receptor expressed at the cell surface. Cells carrying the CD8 co-receptor ($CD8^+$ cells) bind to antigen-MHC class I complexes. As MHC class I binds antigen processed in the cytosol, $CD8^+$ cells are important in host defence against intracellular pathogens. These cells are capable of inducing apoptosis in host cells, mediated by releasing granzymes and perforin. $CD8^+$ cells are therefore referred to as cytotoxic T cells (Tc cells) (Castellino & Germain, 2006).

T cells expressing the CD4 co-receptor ($CD4^+$ cells) recognise antigen complexed with MHC class II molecules and, through cytokine secretion, promote and enhance the immune response of other cells. They are therefore referred to as T helper cells (Th cells) (Castellino & Germain, 2006). A number of Th subsets can develop from circulating naive $CD4^+$ cells and changes in the local microenvironment are important for determining which cell phenotype should arise. Proliferation of naive T cells through stimulation of TCR in an IL-12 rich environment gives rise to a Th1 phenotype (Figure 1.12). Th1 cells are characterised by the secretion of IFNγ, IL-2, and TNFα, and promote the functions of other immune cells including macrophages and neutrophils. Effector functions of Th1 cells are typically associated with the destruction of intracellular pathogens (Murphy & Reiner, 2002). Alternatively, TCR stimulation in an IL-4 rich environment is necessary for induction of Th2 cells (Figure 1.12). These cells typically secrete IL-4, IL-5, IL-9, IL-13, and IL-10, and promote humoral immunity inducing B cells to produce antibodies. Th2 cells are also associated with eosinophil activation and mast cell degranulation (Le Gros *et al.*, 1990; Murphy & Reiner, 2002; Swain *et al.*, 1990). These two subsets are commonly referred to as opposing arms of the immune response, and evidence suggest that chronic immune responses in humans are polarised to either Th1 or Th2 (Del Prete, 1998). Cytokines secreted by both subsets of cells have autocrine growth factor function on their secretive cells, and they simultaneously inhibit the expansion of the other subset, a function shown to be crucial to their regulation (Fernandez-Botran *et al.*, 1988; Gajewski & Fitch, 1988).

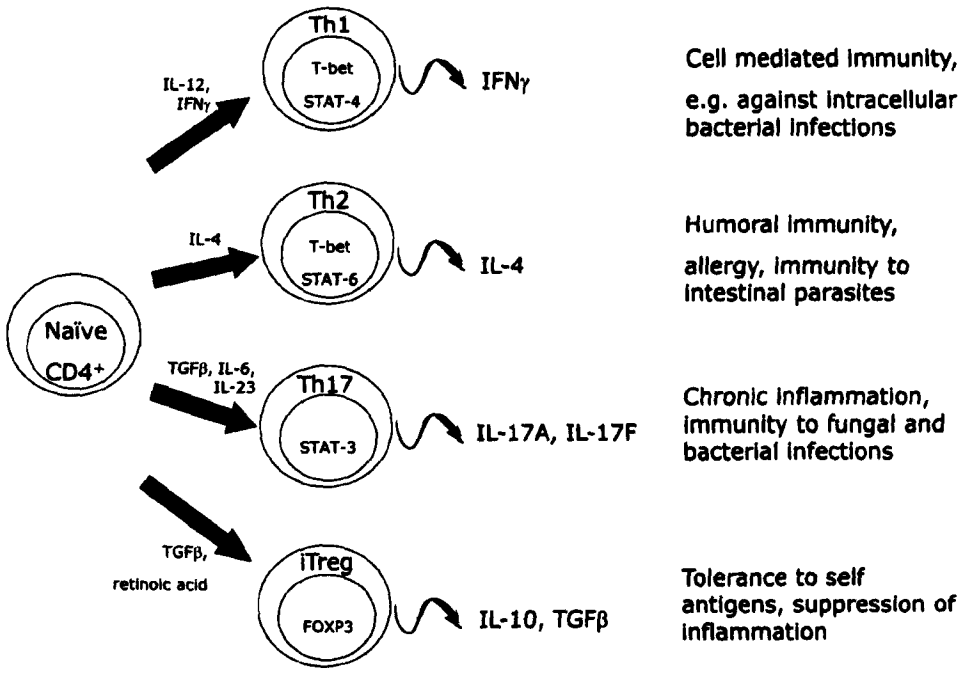


Figure 1.12: The differentiation of naive T cells.

Naive T cells can differentiate into a number of different effector T cells depending on the cytokine milieu during activation of the naive cell. In the presence of IL-4, they differentiate into Th2 effector cells important in humoral immunity, and secrete IL-4. They characteristically express the GATA-3 transcription factor and signal through STAT-6 dependent pathways. IL-12 is a potent inducer of the Th1 phenotype, which are characterised by the expression of the T-bet transcription factor, activation of STAT-4, and secretion of IFNγ. Th17 depend on TGFβ, IL-23 and IL-6, and secrete IL17. Tregs also require TGFβ for differentiation and secrete TGFβ and possibly other cytokines to mediate the function of this subset of $CD4^+$ cells (Adapted from Robinson & Atherton, 2010).

The mucosal inflammation induced by *H. pylori* is widely believed to be polarised towards a Th1-dominated phenotype (D'Elios *et al.*, 1997; Mohammadi *et al.*, 1996). Lymphocytes isolated from *H. pylori* infected mucosa were shown to have a Th1 cell phenotype, and gastric T cells produced little IL-4, demonstrating the dominance of Th1 cells (Bamford *et al.*, 1998). A number of factors contribute to Th1 immune dominance. The production of IL-8 is upregulated in epithelial cells during *H. pylori* infection, particularly in response to CagPAI (Viala *et al.*, 2004). IL-8 is essential in the initial response to *H. pylori* for the recruitment of IL-12 secreting neutrophils, which creates a cytokine milieu that induces Th1 differentiation, and strong production of IL-12 in *H. pylori* infected gastric biopsies has been found (Karttunen *et al.*, 1997). Furthermore, as discussed previously (section 1.6.7), HP-NAP is capable of inducing Th1 responses and down-regulating Th2 response (Amedei *et al.*, 2006). The Th1 response has been demonstrated to be specific for CagA, with the majority of gastric Th1 cells having specificity for this antigen, with prominent production of IFN-γ, but not IL-4 (D'Elios *et al.*, 1997). However, infections with non-virulent strains of the bacterium also induce an inflammatory response. Recently, *H. pylori* infection was found to activate dendritic cells and promote their maturation in a *cag* PAI- and VacA-independent manner (Kranzer *et al.*, 2005). Overall, cell-mediated immunity to *H. pylori* infection is believed to be polarised towards a Th1 phenotype, which contributes to the development of *H. pylori*-related disease profiles (D'Elios *et al.*, 2003).

More recently, a novel subset of T effector cells that differentiate from naive T cells was identified. These cells produce a range of cytokines including IL-6 and TNFα, but most abundantly secrete IL17 and were named Th17 cells (Aggarwal & Gurney, 2002). Th17 cells are a distinct population of T cells that are induced under the influence of IL-23, IL-6 and TGFβ (Figure 1.12) (Bettelli *et al.*, 2006). IL-17 has a number of biological roles, and is believed to stimulate a number of cells including fibroblasts, endothelial cells, epithelial cells, and macrophages to secrete chemokines for the recruitment of other cells (Aggarwal & Gurney, 2002; Ye *et al.*, 2001). Therefore, although Th17 cells are considered as cells of the adaptive immune response, cytokines secreted by these cells are important for the activation of innate immunity. IL-17 has been demonstrated to be upregulated

with *H. pylori* infection (Luzza *et al.*, 2000; Mizuno *et al.*, 2005). Importantly, IL-17 was found to be upregulated in the gastric mucosa and to stimulate the production of IL-8. Furthermore, up-regulation of IL-8 was observed in a gastric cell line, and neutrophil infiltration was observed in response to increased secretion of IL-8 (Luzza *et al.*, 2000). IL-17 may therefore be important in *H. pylori*-induced gastritis.

1.7.2.4 Regulatory Response

In recent years, there has been renewed interest into the regulation of immune responses, and it is now clear that a subset of T cells with regulatory functions is responsible for modulating these responses. These regulatory T cells (Tregs) are critical for the regulation of immune responses, maintenance of immunological self-tolerance and immune homeostasis, and the control of autoimmunity and cancer surveillance (Sakaguchi & Powrie, 2007). Tregs represent a minor subpopulation of $CD4^+$ T cells and are divided into two subpopulations which are defined as naturally occurring Tregs (nTregs) or inducible Tregs (iTregs).

Natural Tregs originate directly from the thymus and constitute 5-10% of the T cell population in the peripheral blood. They express high levels of CD25, the alpha-chain of the IL-2 receptor, and FOXP3 (Forkhead box P3), a transcriptional repression factor of the forkhead-winged helix family of transcription factors (Sakaguchi *et al.*, 2006), which, as discussed later, are markers that are characteristic of Tregs (Bluestone & Abbas, 2003). There is some evidence to suggest that nTregs are particularly important in maintaining tolerance to self-antigen (Hsieh *et al.*, 2006; Pacholczyk *et al.*, 2006).

Recently, it was found that the Treg cell response can also be shaped in the periphery, and naïve T cells differentiate into functional iTregs following stimulation in the presence of antigen and a specific cytokine milieu such as TGF-β, IL-10 and IL-4 (Figure 1.12) (Chatenoud & Bach, 2006). *In vitro* stimulation of naïve T cells in the presence of TGF-β was found to induce the development of $FOXP3^+$ cells with the suppressive characteristics of Tregs (Sakaguchi & Powrie, 2007; Zheng *et al.*, 2007). In addition, expansion of Treg cells was also demonstrated after TCR stimulation *in vivo* (Walker *et al.*, 2003; Yamazaki *et al.*, 2003).

Heterogeneity exists in the iTreg population, with numerous lineages that are characterised according to the cytokine expression profile of the cells. Tr1 cells have regulatory function and secrete high levels of IL-10 (O'Garra *et al.*, 2004; Thompson & Powrie, 2004). They can be generated from stimulation of naïve T cells that have been stimulated by antigen and APCs. This cell line differs from "conventional" Tregs as they are believed to be FOXP3 negative (Zhu & Paul, 2010). TGF-β producing Th3 cells are also iTregs (Weiner, 2001), and are believed to play a role in the induction of oral tolerance and prevent autoimmune disease after stimulation with self-antigen (Zhu & Paul, 2010). However, it is as yet unclear if these populations of cells are truly distinct. Many publications have described these populations but their classification overlaps.

As mentioned, Tregs are characterised by the expression of *FOXP3* (Sakaguchi *et al.*, 2006), which was shown to be important for both the development and function of Tregs (Chatila *et al.*, 2000; Khattri *et al.*, 2003; Ziegler, 2006). The function of FOXP3 involves its physical association with the Rel family transcription factors, NFATs and NF-κB, and in doing so, blocks their ability to induce expression of their target genes including key cytokine genes (Bettelli *et al.*, 2005). The importance of FOXP3 to Treg functionality becomes apparent with the autoimmune disease immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) in humans, which results from mutations in the *FOXP3* gene (Bennett *et al.*, 2001).

In addition to FOXP3, Tregs are also characterised by the high expression of CD25 (Sakaguchi *et al.*, 1995), and their survival and expansion is dependent on the presence of IL-2 (Setoguchi *et al.*, 2005). T cells expressing high levels of CD25 are considered regulatory (Baecher-Allan *et al.*, 2001). The $CD25^{hi}$ population of Tregs include both iTregs and nTregs, making it difficult to distinguish between the two subsets (Horwitz *et al.*, 2008b). Based on the expression of CD25 on the surface of Tregs, it is believed that a possible mechanism by which Tregs execute their suppressive capability is through the scavenging of IL-2, which is also essential for the differentiation of other T cells (Pandolfi *et al.*, 2000).

Cytotoxic T-lymphocyte antigen-4 (CTLA-4), is also highly expressed on the surface of Tregs, and is constitutively expressed on Tregs that express FoxP3. Furthermore, the relationship between TGFβ, CTLA-4 and FoxP3 was shown to be important in the generation of iTreg, but not nTreg (Horwitz *et al.*, 2008a). CTLA-4 is believed to block the differentiation of T cells through direct binding to the costimulatory molecules CD80 (B7-1) and CD86 (B7-2) expressed on APCs, which bind to CD28 on naive T cells for differentiation to effector T cells. CTLA-4 binds with higher affinity than CD28, therefore inhibiting the second signal, necessary for immune activation (Engelhardt *et al.*, 2006). Further evidence for the immune suppressive function of CTLA-4 was provided *in vivo* through blockade of CTLA-4, which resulted in spontaneous development of chronic organ-specific autoimmune diseases (Takahashi *et al.*, 2000).

Natural regulatory T-cells have been shown to act via the production of regulatory cytokines IL-10 and TGF-β (Annacker *et al.*, 2001; Asseman *et al.*, 1999; Hara *et al.*, 2001; Kingsley *et al.*, 2002; Mills & McGuirk, 2004; Suri-Payer & Cantor, 2001), and function through limiting effector T-cell function, and inhibiting recruitment of inflammatory cells such as neutrophils and monocytes (Rudensky & Campbell, 2006). The inhibitory roles of IL-10 and TGFβ are discussed further in Chapter 7 (section 7.1).

As *H. pylori* colonisation is persistent and provokes a chronic infection, the role of Tregs in suppressing the host immune response is of great interest. This concept is supported by the inability of *H. pylori* to maintain an infection in mice lacking Treg cells (Rad *et al.*, 2006; Raghavan *et al.*, 2003). Furthermore, elevated levels of both CD4+CD25^{hi} cells and *FOXP3* transcript can be found in the gastric mucosa of infected patients (Hida *et al.*, 1999; Lindholm *et al.*, 1998; Lundgren *et al.*, 2005a; Robinson *et al.*, 2008). Tregs were also found to home and accumulate in *H. pylori*-infected gastric tissue (Lundgren *et al.*, 2005b), demonstrating the importance of Tregs in the immune response mounted during infection with this bacterium. The regulatory response to *H. pylori* is discussed further in Chapter 7 (section 7.1).

1.8 AIMS

The chronicity of infection that develops in response to *H. pylori* infection is driven by a combination of host and bacterial factors. The interaction between these factors results in what may be considered as an equilibrium between the toxicity of the bacterium and the immune response that is mounted against the infection. In this balance, a shift towards an aggressive immune response may lead to reduced bacterial numbers, whereas a shift towards colonisation with less virulent strains of the bacterium may dampen the damaging inflammatory response. Due to the complexity of the host-*H. pylori* relationship, questions still remain about the driving forces that lead to lifelong infection. To address the question of factors that influence colonisation, the specific aims of this study were to:

- Design a quantitative assay for accurate assessment of *H. pylori* densities in the gastric mucosa.

- Determine the relationship between bacterial load and disease outcome in infected individuals.

- Determine the impact of bacterial virulence factors on bacterial load.

- Investigate proinflammatory host immune responses and their impact on *H. pylori* density.

- Investigate regulatory immune responses in relation to *H. pylori* density and disease profiles.

- Determine the influence of antimicrobial peptides on colonisation density.

Chapter 2

2. MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

Gastric biopsies were donated with permission by patients attending a routine upper gastrointestinal endoscopy at the Queens Medical Centre, Nottingham (mean age=53.7, age range=33-86 years, male:female ratio=0.76). Donors attending the clinic were referred based on a number of dyspeptic symptoms including acid reflux and chronic indigestion, but were otherwise healthy. Clinical endoscopic observations and disease severity were noted. Patients were discounted from the study if antibiotics, proton pump inhibitors (PPIs) or non-steroidal anti-inflammatory drugs (NSAIDs) were taken in the 2 weeks prior to endoscopic analysis. Tissue specimens from the antrum and corpus were taken with informed patient consent and Nottingham University Hospital Ethics Committee approval. Biopsies taken for this study are summarised in Table 2.1.

	BIOPSIES TAKEN	STORAGE CONDITIONS
For culture	1 x antrum 1x corpus	Isosensitest glycerol (15% v/v) medium, stored at -80°C
For CLO™ test and rapid urease test	1 x antrum	-
For histology	1 x antum 1 x corpus	Immediately fixed in formol-saline. Biopsies were paraffin wax embedded and 4μm sections were prepared
For qPCR and RT-PCR	1 x antrum 1x corpus	RNALater RNA stabilising reagent (Qiagen), stored at -80°C
For FACs analysis	4 x antrum	Sterile RPMI640 medium supplemented with 2% foetal bovine serum (FBS), 100U/ml penicillin G, 100μg/ml streptomycin sulphate and 250ng/ml amphotericin B.

Table 2.1: Biopsies donated by patients during routine upper GI endoscopy, and storage conditions.

2.2 BACTERIAL STRAINS AND CULTURE

2.2.1 *H. pylori* strains and culture

H. pylori clinical isolates were cultured from human gastric biopsies. Both clinical isolates and laboratory strains were cultured on Columbia blood agar plates containing 5% (v/v) horse blood (Oxoid) at 37°C in a 5% CO_2 humidified atmosphere. Isolated strains were stored at -80°C in isosensitest medium (Oxoid) containing 15% (v/v) glycerol, prepared according to manufacturer's recommendations of 23.4g media in 1L distilled water. The medium was sterilised by autoclaving at 121°C at 15psi for 15mins. Laboratory strains used are summarised in Table 2.2.

***H. pylori* strain**	**Relevant strain characteristics**	**Reference**
Tx30a	Wild-type (ATCC 51932) *vacA* s2/m2, *cag*PAI-	Leunk *et al.*, 1988
60190	Wild-type (ATCC 49503) *vacA* s1/m1, *cagA*PAI+	Leunk *et al.*, 1988
60190Δ*vacA*	As 60190 but disrupted by insertion of chloramphenicol/kanamycin resistance marker in the *vacA* gene	Argent *et al.*, 2008
60190Δ*cagA*	As 60190 but disrupted by insertion of chloramphenicol/kanamycin resistance marker in the *cagA* gene	Argent *et al.*, 2008
60190Δ*cagE*	As 60190 but disrupted by insertion of chloramphenicol/kanamycin resistance marker in the *cagE* gene	Argent *et al.*, 2008
11637	Wild-type (ATCC 43504) *vacA* s1/m1, *cagA*+	Constructed previously in our group
11637Δ*cagE*	As 60190 but disrupted by insertion of chloramphenicol/kanamycin resistance marker in the *cagE* gene	Constructed previously in our group
84183	Wild-type (ATCC 53726) *vacA* s1/m1, *cagA*+	Perez-Perez & Blaser, 1987
84183Δ*cagE*	As 60190 but disrupted by insertion of chloramphenicol/kanamycin resistance marker in the *cagE* gene	Argent *et al.*, 2008

Table 2.2: *H. pylori* laboratory strains used.

2.2.2 *Escherichia coli* culture

The Novablue *E. coli* strain (Novagen) was cultured on Luria-Bertani (LB) agar plates supplemented with carbenicillin (50μg/ml), Isopropyl β-D-1-thiogalactopyranoside (IPTG, 80μM), and X-gal (70μg/ml) as appropriate. LB broth was prepared with 10g tryptone (Oxoid), 5g yeast extract (Oxoid), and 10g sodium chloride (Sigma-Aldrich), made up to 1 litre with distilled water. The medium was sterilised by autoclaving at 121°C at 15psi pressure for 15 minutes. LB agar plates were made by the addition of Bactoagar (1.5% w/v [Oxoid]) to the broth.

2.3 GASTRIC CELL LINES

AGS cells, a human gastric epithelial cell line (ATCC CRL-1739^{TM}), were grown in F12 Ham's nutrient mixture medium (supplemented with L-glutamine, Invitrogen) with 10% FBS (Invitrogen), at 37°C in a 5% CO_2 humidified atmosphere. Once fully confluent, cells were washed twice with sterile phosphate buffered saline (PBS), and then treated with Trypsin-EDTA (Invitrogen) to release cells from the flask. F12 medium was added to the cells to wash away any remaining Trypsin-EDTA. Cells were collected by centrifugation (1500rpm, 3mins, at 21°C) and resuspended in F12 medium to appropriate dilutions, passed into new flasks with fresh medium, and incubated as before.

2.4 CELL QUANTIFICATION

2.4.1 *H. pylori* Cells

The concentration of *H. pylori* cells in a suspension was estimated spectrophotometrically using the Pharmacia Novaspec II, where an OD_{550} of 0.1 is equivalent to 1 x 10^8 colony forming units (CFUs)/ml (determined previously in our group).

2.4.2 Gastric cells

1 volume of gastric cells were stained with 3 volumes of Trypan blue solution (0.4 % w/v; Sigma) and quantified using a haemocytometer.

2.5 DNA PURIFICATION, MANIPULATION AND ANALYSIS

2.5.1 Purification of *H. pylori* genomic DNA

H. pylori cells cultured for 24 hours were harvested from blood agar plates and resuspended in 1ml sterile PBS. Cells were centrifuged (13000 rpm, 20°C, 5 minutes) and pellets were resuspended in 100µl of TE buffer (10mM Tris-Cl, 1mM EDTA, pH 8.0). 500µl GES reagent (5M Guanidine thiocyanate, 0.5M EDTA, 10% w/v Sarkosyl) was added and incubated for 5mins at room temperature before being transferred to ice. 250µl ice cold ammonium acetate (7.5M) was then added before further incubation on ice for 5mins. 650µl chloroform was added, mixed thoroughly and the layers separated by centrifugation (13000 rpm, 20°C, 5 minutes). The top aqueous layer was transferred to a clean 1.5ml microcentrifuge tube and the chloroform extraction repeated. Isopropanol was added to one volume of the aqueous layer, mixed thoroughly and incubated at room temperature for 5mins to precipitate DNA, which was then collected by centrifugation as before. DNA pellets were washed twice with 70% ethanol and allowed to air dry. Once dry, DNA was resuspended in 100µl nuclease free water (Invitrogen).

2.5.2 Purification of DNA & RNA from gastric biopsies

The All Prep DNA/RNA Mini Kit (Qiagen) was used, which allowed for the simultaneous purification of genomic DNA and total RNA from the same tissue sample. Thawed biopsies which had previously been treated with RNALater and stored at -80°C were transferred to RLT lysis buffer containing β-mecaptoethanol (350µl) and homogenised for 30 seconds using a T8 Ultra Turrax rotor-stator homogeniser (IKA, Werke & Co. Freiburg, Germany). Nucleic acids were then extracted according to the manufacturer's instructions. Briefly, DNA was first isolated by passing the tissue homogenate through an AllPrep DNA spin column, and then through an RNeasy spin column to selectively isolate RNA. DNA was eluted in TE buffer (100µl), and RNA in 30µl nuclease free water (Qiagen). Nucleic acid concentration was quantified using a NanoDrop ND-1000

spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The quality of extracted nucleic acids was assessed by the A_{260}/A_{280} ratio.

2.5.3 cDNA synthesis

cDNA synthesis of 100ng mRNA transcript was performed using 200U SuperScript II RNase H reverse transcriptase, 1x first strand buffer (250mM Tris-HCL, 375mM KCl, 15mM $MgCl_2$, pH 8.3 at room temperature), 10mM DTT, 1.25μM oligo(dT) primer (Invitrogen, Paisley, Scotland) and 200μM each deoxynucleotide triphospate (Roche) in a final volume of 20μl. Purified RNA extracted from gastric biopsies was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).
As a control for DNA contamination, first stage RT-PCR samples produced without reverse transcriptase were also synthesised.

2.5.4 Polymerase chain reaction (PCR) conditions and end point analysis

Reactions were prepared in a 50μl volume and contained 1xPCR Buffer (containing 1.5mM $MgCl_2$; Qiagen); 1.25U Hotstar Taq polymerase (Qiagen); 200μM each deoxynucleotide triphosphates (Roche); 0.1μM each of forward and reverse primer (provided by Sigma-Aldrich); up to 300ng of DNA template, and nuclease-free water to volume. Reactions were performed in a PCRexpress thermal cycler (Hybaid). Primers used in this study are listed in Table 2.3. PCR cycling conditions are shown in Table 2.4.
H. pylori strain genotyping was performed for *cagA*, *vacA* s-, m- and i-region, and *dupA* gene according to previous reports (referenced in Table 2.3).

PCR products were analysed on 2% (w/v) agarose gels, which were prepared with 1x Tris-acetate-EDTA buffer (TAE [40mM Tris-acetate, 2mM EDTA, pH 8.0]), and gels contained ethidium bromide (0.5μg/ml) for visualisation.
Samples were prepared with 6x loading buffer (0.25% bromophenol blue, 20% glycerol in TE, 0.1% SDS), and run at 70V (constant volume) and visualised under UV light.

Primer	Primer Sequence	Product Size (E)	Reference
GAPDH:			
hGAPDH1	GGTCCGAGTCACCGCCT	118bp (N/A)	In this study
hGAPDH2	TGGCGACGCAAAAGAAGATG		
16S rRNA:			
16S rRNAF	CGATGAAGCTTCTAGCTTGC	164bp (N/A)	Narayanan, 2005
16S rRNAr	ATAGGACATAGGCTGATCTC		
cagA:			
cag2	GGAACCCTAGTCGGTAAATG	450bp (N/A)	Rudi *et al.*, 1998
cag4	ATCTTTGAGCTTGTCTATCG		
vacA s region:		s1=259bp s2=286bp (N/A)	Atherton *et al.*, 1995
A3436	ATGGAAATACAACAAACACAC		
C1226	CTGCTTGAATGCGCCAAAC		
vacA m region:		m1=290bp m2=352bp (N/A)	Atherton *et al.*, 1995
VAGF	CAATCTGTCCAATCAAGCGAG		
VAGR	CTAGCGTCAAAATAATTCCAAGG		
vacA i region:		i1= 426bp i2= 432bp (N/A)	Rhead *et al.*, 2007
VACF1	GTTGGGATTGGGGGAATGCCG		
C1R	TTAATTTAACGCTGTTTGAAG		
C2R	GATCAACGCTCTGATTTGA		
dupA:			
DupAF113	GACGATTGAGCGATGGGAATAT	1018bp (N/A)	Argent *et al.*, 2007
DupAR1083	CTGAGAAGCCTTATTATCTTGTTGG		
FOXP3			
Forward	CAGCACATTCCCAGAGTTCCT	(2.07)	Robinson *et al.*, 2008
Reverse	GCGTGTGAACCAGTGGTAGAT		
TGFB1			
Forward	CAGAAATACAGCAACAATTCCTGG	(2.86)	Robinson *et al.*, 2008
Reverse	TTGCAGTGTGTTATCCCTGCTGTC		
IL10			
Forward	GCTGGAGGACTTTAAGGGTTACCT	(1.94)	Robinson *et al.*, 2008
Reverse	CTTGATGTCTGGGTCTTGGTTCT		

IL8			
Forward	CTCTTGGCAGCCTTCCTGA	(2.07)	Robinson *et al.*, 2008
Reverse	AGTTCTTTAGCACTCCTTGGCA		
GAPDH			
Forward	CCACATCGCTCAGACACCAT	(1.92)	Robinson *et al.*, 2008
Reverse	GGCAACAATATCCACTTTACCAGAGT		

Table 2.3: Primer pair sequences used for qPCR reactions and strain genotyping (E= reaction efficiency).

Step	Temperature (°C)	Length of time	Number of Cycles
Initialisation	95	15mins	1
Denaturation	95	30secs	35
Annealing	56	60secs	
Elongation	72	30secs	
Final Elongation	72	10mins	1

Table 2.4: PCR cycling conditions for amplification of *16S rRNA* and quantification of *H. pylori* density by RT-PCR.

2.5.5 Quantitative Real-Time PCR (qPCR)

2.5.5.1 Quantification of H. pylori *colonisation density*

Absolute quantification of *H. pylori* copy numbers was determined using standard curves (described in more detail in Chapter 3). Reaction mixtures contained 2.5µl of forward and reverse primers (0.1µM, provided by Sigma-Aldrich), 5µl of DNA template, and 12.5µl of 2xQuantiTect SYBR Green PCR Master Mix (included HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, dNTP mix, SYBR Green I dye, and ROX dye [Qiagen]). Reaction mixtures were made up to 25µl with nuclease-free water and were carried out using the Rotor-Gene 3000 system. Cycling conditions are listed in Table 2.4 with the addition of a final melt curve analysis between 72-95°C, increasing by 1°C for each step, to ensure primer specificity. No template controls (NTCs) were included in each run. Data was analysed using the Rotor-Gene 6 program, with melt curves showing single peaks for each gene amplified.

2.5.5.2 Reverse Transcriptase PCR (RT-PCR)

Quantification of mRNA transcripts was carried out using 4µl of cDNA template (prepared as previously described in section 2.5.3). Reactions were performed using a DyNAmo HS SYBR green qPCR kit (New England Biosciences, UK). Reaction mixtures contained 2.5µl of forward and reverse primers, and 10µl of DyNAmo HS SYBR green PCR Master Mix. Reaction mixtures were made up to 20µl with nuclease-free water. Amplification was carried out over 45 cycles of 15 seconds at 95°C, 30 seconds at 62°C, and 30 seconds at 72°C. No template controls were included in each run, and a melting curve analysis was performed as described above (section 2.5.5.1). First stage RT-PCR samples, produced in the absence of reverse transcriptase from each RNA sample were tested in parallel to ensure that products were not amplified from contaminating genomic DNA. Samples were run in duplicate and the results were analysed according to the method described by Pfaffl (Pfaffl, 2001). Relative gene expression levels were determined by normalising against *GAPDH* mRNA levels, and data was presented as a fold difference in comparison to a reference sample obtained from uninfected donors. For this uninfected comparator, cDNA was synthesised from purified

RNA pooled and extracted from biopsies donated by 10 *H. pylori* negative patients. A commercial human cDNA standard (BD Biosciences) was included as a positive control in all assays. Reaction efficiencies were measured with a standard curve developed using a 4-fold dilution series of a commercial human cDNA standard (BD Biosciences). Real-time PCR was performed on a Rotor-Gene 3000 (Corbett Research, Cambridge, UK).

Commercial primers were used for the quantification of human *hBD1* and *LL37* expression (Qiagen), and normalised with commercial *GAPDH* primers (Qiagen). Amplification was carried out over 40 cycles of 15s at 94°C, 30s at 55°C and 30s at 72°C. Reaction efficiencies were determined as previously mentioned (*hBD1*=1.97, *LL37*=1.83, *GAPDH*=1.93).

2.5.6 Preparation of human GAPDH plasmid construct pSAP1

An 118bp fragment of the human *GAPDH* gene was amplified by PCR using the hGAPDH1 and hGAPDH2 primer pair (Table 2.3) and the above protocol (Table 2.4). The PCR reaction was separated by agarose gel electrophoresis, and the corresponding band was excised from the agarose gel and weighed. The DNA fragment was then purified using the Wizard SV Gel and PCR Clean up System (Promega) according to manufacturer's instructions. DNA was eluted in 50µl of nuclease-free water.

The purified PCR fragment was then cloned into the pGEM-T Easy Vector System (Promega) according to the manufacturer's recommendations. Insert and vector were used in a 3:1 molar ratio. The amount of insert used was determined using the equation:

$$\left[\frac{\text{Vector concentration (ng) x Product size (kb)}}{\text{Size of vetor (kb)}}\right] \text{x insert:vector molar ratio}$$

10µl reaction mixtures contained 1x ligation buffer (Promega), pGEM-T Easy vector (50ng), and T4 DNA Ligase (3 Weiss units). Positive control reactions

containing control insert DNA (8ng; Promega), and vector only control reactions were also set up. Reaction mixtures were left to incubate at 4°C overnight.

1µl of the ligation reaction was transformed into *E. coli* Novablue competent cells (Novagen) according to manufacturer's recommendations. Cells were plated onto LB agar plates containing carbenicillin (50µg/ml), IPTG (80µM), and X-gal (70µg/ml), and incubated overnight at 37°C.

To test for the presence of the correct plasmid construct, white *E. coli* transformants were used to inoculate individual 5ml LB broth cultures supplemented with carbenicillin (50µg/ml) and incubated overnight at 37°C with shaking. Cells were harvested by centrifugation (4000 rpm, 5mins), and plasmid extraction was performed using the Qiaprep Spin Miniprep Kit (Qiagen) according to the manufacturer's recommendations. Plasmid DNA was eluted in 50µl of Buffer EB (10 mM Tris·Cl, pH 8.5).

Restriction digestion was carried out using the *NotI* endonuclease enzyme (New England Biolabs), which cuts either side of the insertion site, and excision sites were not present in the expected insert. Reaction mixtures were set up as follows: 5µl restriction buffer, 5µl BSA, 1µl restriction enzyme, 6µl DNA and 33µl nuclease free water (final volume of 50µl) Reactions were incubated at 37°C for 2 hours, and fragments were visualised by agarose gel electrophoresis (2% w/v) (section 2.5.4).

Positive clones were identified and verified by DNA sequencing at the Biopolymer Synthesis and Analysis Unit (Queen's Medical Centre, University of Nottingham). Sequence data was analysed using DNAstar software. A clone with the correct GAPDH insert was selected and named pSAP1

2.6 CO-CULTURE ASSAYS

All cells were cultured as described in section 2.3, trypsinised and seeded into flat-bottomed 24 well tissue culture plates at 5 x 10^4 cells/well, which were then incubated at 37°C, 5 % CO_2 for 24 hours. At 80% confluence, media was replaced with media containing 24 hour grown *H. pylori* cultures at an MOI 100. Cultures were incubated for a further 24 hours.

AGS cells were infected with *H. pylori* mutant and wildtype strains which were at the same passage number. After 24 hours of co-culture, cells were removed by scraping and treated with 1% Saponin (diluted in sterile PBS) to ensure a homogenous suspension of cells. A 10-fold dilution series was created in sterile PBS, and bacterial colony forming units (CFU) were determined by spotting 10µl volumes of the diluted suspensions onto Columbia horse blood agar plates, which were incubated as described above in section 2.2.1. Five 10µl spots were plated for each dilution, and CFUs were enumerated once colonies were visible, usually after 4 days incubation.

2.6.1 Cytokine assay

AGS cells were cultured and infected with *H. pylori* as described in section 2.6. For cytokine treatment, commercial recombinant protein was added at the time of infection at a concentration of 50ng/ml unless otherwise stated. Cytokines used included IL-1β, TNFα, TGFβ, IL-22 (all from Peprotech), IL-10 and IL-17 (AbSerotech). Untreated cultures were included as controls, as well as uninfected AGS cytokine stimulated controls. After 24 hours of culture, supernatants were harvested by pipetting and stored at -80°C for assessment of IL-8 secretion levels.

2.7 ELISA ASSAYS

IL-8 levels were assayed using a Biosource human IL-8 Cytoset™ enzyme linked immunosorbent assay (ELISA) kit (Invitrogen), according to the manufacturer's instructions. A standard curve was included on each plate and samples were measured in duplicate, diluted at half their original concentration. Serum gastrin-17, pepsinogen I and pepsinogen II levels were assessed using Biohit ELISA kits (Biohit). Optical densities were measured using a microplate reader (LabSystems

iEMS reader MF) at 450nm and 620nm and the concentration of cytokine in each sample was calculated by reference to the local standard curve.

2.8 HISTOLOGY

Gastric biopsy sections were prepared and stained by Dr Abed Zaitoun (Division of Histopathology, University Hospital, Nottingham) with either Haemotoxilin and eosin stain used to assess the degree of inflammation, activity, atrophy and intestinal metaplasia, or Toluidine blue stain which was used to assess the grade of *H. pylori* density. Scoring was carried out using the Sydney scoring system (Genta & Dixon, 1995; Genta, 1996; Price, 1991) and was done independently under the supervision of Dr Abed Zaitoun. Example slides are shown in appendix 11.1.

2.9 FLOW CYTOMETRY ANALYSIS

Four antral gastric biopsies were dissociated by rubbing through a sterile disposable 100 μm nylon cell strainer (BD Biosciences, Oxford, UK), washed and re-suspended in RPMI 1640 culture medium supplemented with 10% FCS, 100μg/ml penicillin and 100μg/ml streptomycin sulphate (all from Sigma) at 1 x 10^6 per ml. *H. pylori* whole-cell sonicated antigen from the SS1 strain (Lee *et al.*, 1997) was added to give a final concentration of 25μg/ml as described previously (Robinson *et al.*, 2005). As a positive control, PMA and ionomycin were added to give final concentrations of 20ng/ml and 1 μM respectively. Negative control tubes received no antigens or mitogens. The cultures were incubated for 1 hour at 37°C in 5% CO_2 before adding Brefeldin A (BFA) (Sigma) at 10 μg/ml and then replaced in the incubator for a further 14 hours. The positive control PMA/ionomycin tubes were incubated for only a further 5 hours after adding BFA.

Cells were pelleted at 250×*g* for 5 minutes at 4°C in preparation for staining with combinations of the following conjugated monoclonal antibodies for 30 minutes on ice: anti-human-CD4-phycoerythrin-Texas Red (ECD), anti-CD45-phycoerythin-cyanin 5.1 (PC5), and anti-human-CD25-PE (Miltenyi Biotech).

The cells were washed three times with 1ml of ice-cold PBA buffer (PBS containing 0.1% BSA and 0.05% sodium azide) supplemented with 2% FBS before fixing in 0.5ml 0.5% formaldehyde in PBS at 4°C overnight. The cells were then washed with 1ml PBA, and permeabilised by washing in 1ml PBA/0.1% saponin. Cells were washed with PBA/0.1% saponin/10% FCS before staining with anti-IL-10-FITC (R&D Systems) for 2 hours at 4°C. The cells were washed three times with PBS/0.1% saponin before fixing in 0.5% formaldehyde. The data on 100,000 events were acquired using a Coulter EPICS Altra flow cytometer, unstained cells and cells stained separately with each fluorochrome-labelled antibody were included to optimise compensation settings. Data analysis was performed using WinMDI version 2.8 (http://facs.scripps.edu/) or WEASEL v.2 (developed by Walter and Eliza Hall Institute of Medical Research). $CD45^+$ events were then analysed for CD4, CD25 and IL-10 staining with reference to appropriate isotype control antibody conjugates. Analysis of cell counts was assessed by a colleague, Darren P Letley.

2.10 PROGRAMS AND WEBSITES USED

All programs and websites used for data or sequence analysis are listed in Table 2.5.

Website or program used	Web address
Lasergene software by DNASTAR Inc. Was used for sequence data analysis	
Ensembl website used for *GAPDH* gene analysis	www.ensembl.org
Netprimer website for primer design	www.premierbiosoft.com/netprimer
BLAST search	www.ncbi.nim.nih.gov/BLAST

Table 2.5: Programs and websites used.

2.11 STATISTICAL ANALYSIS

Statistical analyses and box-and-whisker plots were prepared using MINITAB Release 14 software. Boxes represent the first to third quartiles, the median values are shown as horizontal lines within the bars, and the whiskers depict the lowest and highest observations within 1.5 times the first to third interquartile range. Statistical tests of unpaired data were carried out using the Mann-Whitney U-test, unless otherwise stated. Correlations were examined using the Spearman's rank correlation test. Analysis of *in vitro* data was tested using the Student T-test. A significant difference was taken at $p \leq 0.05$

Chapter 3

3. DEVELOPMENT OF A QUANTITATIVE ASSAY FOR *H. PYLORI* FROM HUMAN GASTRIC TISSUE

3.1 INTRODUCTION

The quantification of bacterial load has many applications, including monitoring the effectiveness of treatment regimes and vaccines. A number of techniques are available for measuring bacterial burden in gastric tissue with varying degrees of accuracy, making some techniques semi-quantitative at best.

3.1.1 Quantitative culture

The use of quantitative culture has been utilised in a number of studies with varying degrees of success. Previous comparative studies have investigated the accuracy of quantification of *H. pylori* density by culture in comparison to other quantitative tools such as histology and qPCR. However, the degree of correlation between different assays varies between studies, with some reports showing a strong correlation (Atherton *et al.*, 1996; Janzon *et al.*, 2009), whilst others have not (Auroux *et al.*, 1998; Tummala *et al.*, 2007). Furthermore, studies that have shown a good correlation between bacterial loads found reduced bacterial numbers with culture in comparison to qPCR (Janzon *et al.*, 2009; Nayak & Rose, 2007). The accuracy of quantitative culture is of course dependent on the viability of the bacteria in the sample, illustrating the importance of an optimal transport system and rapid processing of tissue after collection. Culture conditions may also impact the accuracy of quantification. A homogenous biopsy suspension is necessary as a cluster of organisms will grow as a single colony rather than several, leading to an underestimation of bacterial density, and an overgrowth of other organisms may be a problem if selective media are not used (Goodwin *et al.*, 1985). This may explain the differences in the efficiency of quantitative culture observed between comparative studies.

3.1.2 Quantification by UBT

The urease breath test (UBT) has also been used for the quantification of *H. pylori*. Previous studies have noted a positive correlation between the change in $^{13}CO_2$ and *H. pylori* colonisation density measured by qPCR (Perri *et al.*, 1998; Zagari *et al.*, 2005). Although the difference in $^{13}CO_2$ may give an indication of bacterial load, UBT does not provide absolute quantification. The UBT is non-invasive and measures density in the entire stomach, which may prove useful as a quick and easy method for monitoring treatment of the infection. However, UBT will not be appropriate for determining bacterial load in different anatomical sites of the stomach. This may be due to uneven intragastric distribution of the urea solution, which may result if the protocol for UBT is not strictly followed, including the positioning of the patient (Atherton, 1997). Also, urease activity of individual *H. pylori* strains may differ by several-fold, and therefore, urease activity can not accurately reflect bacterial density. Not surprising, studies have failed to show a correlation between bacterial load and UBT (Graham & Klein, 2000; Logan *et al.*, 1991; Tummala *et al.*, 2007).

3.1.3 Quantification by histology

Overall, histological estimation of *H. pylori* density is most commonly used. *H. pylori* can be visualised microscopically with several special stains including Toluidine blue and silver stains, which allow for better contrast between bacteria and the mucous in comparison to the standard hematoxylin-eosin (H&E) stain. Measure of bacterial load is usually based on the semi-quantitative Sydney system of classification (Dixon *et al.*, 1996). Some previous comparative studies have demonstrated reasonable correlation in quantification between histology and culture (Atherton *et al.*, 1996), as well as qPCR (Kobayashi *et al.*, 2002). However, quantification is dependent on the expertise of the examining pathologist and so estimations of bacterial load by histology can be subjective.

3.1.4 Quantification of nucleic acids

Bacterial density can also be quantified using molecular methods. Probe hybridisation methods, with the use of target-specific non-radioactive labelled probes such as digoxigenin (DIG), can be used. However, the sensitivity of

detection may be poor as large amounts of DNA are required (Tsai *et al.*, 2003). The use of PCR increases the sensitivity of detection as theoretically, a single copy of the target gene can be detected. Bacterial density can be estimated from band density of PCR products on acrylamide gel, and accuracy can be increased by normalising band density of the target gene against the density of a reference gene such as Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (discussed further in section 3.1.5.2). However, PCR has inherent limitations (von Wintzingerode *et al.*, 1997). A major drawback in analysing PCR amplicons after completion of the final PCR cycle (termed "end point" analysis) is that the number of amplicons detected may not reflect the actual proportion of the target gene. This results from biases introduced into the reaction that are associated with the amplification of a target from a mixed DNA template (Suzuki & Giovannoni, 1996). Moreover, it may be difficult to distinguish small differences between samples based on band intensities, making quantification by such methods limited.

To overcome the limitations of quantification observed with standard PCR, competitive PCR was developed, which has also been utilised for the quantification of nucleic acids (Becker-Andre & Hahlbrock, 1989). This technique uses an internal standard, which is co-amplified using the same primer set as the target template. The standard harbours a mutation that will result in a PCR product of different size to the template, making the two amplicons easily distinguishable. As the amount of competitive template added to the reaction is known, it is easy to calculate the amount of target product in the reaction.

Competitive PCR was previously shown to be a reliable method for the quantification of *H. pylori* (Furuta *et al.*, 1996; Monteiro *et al.*, 1997). The use of an internal standard for quantification reduces well-well variations in PCR amplification. In addition, although quantification involved assessing band intensity of PCR products on acrylamide gel, the inclusion of internal standards provided a means for absolute quantification (Furuta *et al.*, 1996). However, the DNA standard may also alter the final result through competing for reagents and primers during the PCR.

As mentioned, estimating bacterial load by end point analysis may not be sufficient for accurate quantification as the kinetics, and hence the efficiency of the reaction drops over the course of amplification, eventually leading to a plateau in the amount of PCR product produced. Ideally, the efficiency of a reaction should be 100%, which can be described as the synthesis of two complete molecules from every one template, and the number of detected amplicons is directly proportional to the initial number of target sequences. This occurs in the exponential phase of the reaction (Heid *et al.*, 1996). The sensitivity of PCR is due to the exponential amplification nature of the procedure (Equation 1).

Equation 1:

$$N_n = N_0 \times (1 + E)^n$$

Where N_n is the number of DNA molecules after n cycles of PCR, N_0 represents the initial number of template molecules, and E is the efficiency of amplification. The efficiency of a reaction is dependent on a number of factors including the length and the sequence of the DNA to be amplified, primer design, concentration of reaction buffer, and temperature, and even a minor change in E can result in dramatically different amplification kinetics. The efficiency begins to approach zero as PCR reaches the plateau phase. For this reason, it is difficult to directly quantify N_0 by measuring N_n at the end of the reaction (Heid *et al.*, 1996). Real-time PCR overcomes this problem by determining N_0 during the exponential phase of the reaction.

3.1.5 Real-time PCR

Real-time PCR is a kinetics-based quantitative technique which exploits fluorescence technology for the detection of newly synthesised PCR products. Fluorescence emission is detected at each PCR cycle throughout the reaction. During the initial few cycles of the reaction, where PCR products are too few to quantify accurately, very little change in the fluorescence signal is detected. This represents the baseline of the reaction. As the PCR progresses, the build up of newly synthesised amplified product results in increasing amounts of fluorescence emission (Heid *et al.*, 1996). The difference between the signal at each time point

and the signal of the baseline is calculated, and is described as ΔRn (Gibson *et al.*, 1996). Real-time PCR plots display this change in fluorescence emission against the cycle number (Figure 3.1).

For nucleic acids quantification, the fluorescence data are converted into numerical values. The fluorescence level at a defined threshold, typically 10 times the standard deviation of the average signal of the baseline fluorescence signal, is used (Ct). The Ct value is inversely proportional to the logarithm of the initial template concentration and is always in the exponential phase (Gibson *et al.*, 1996).

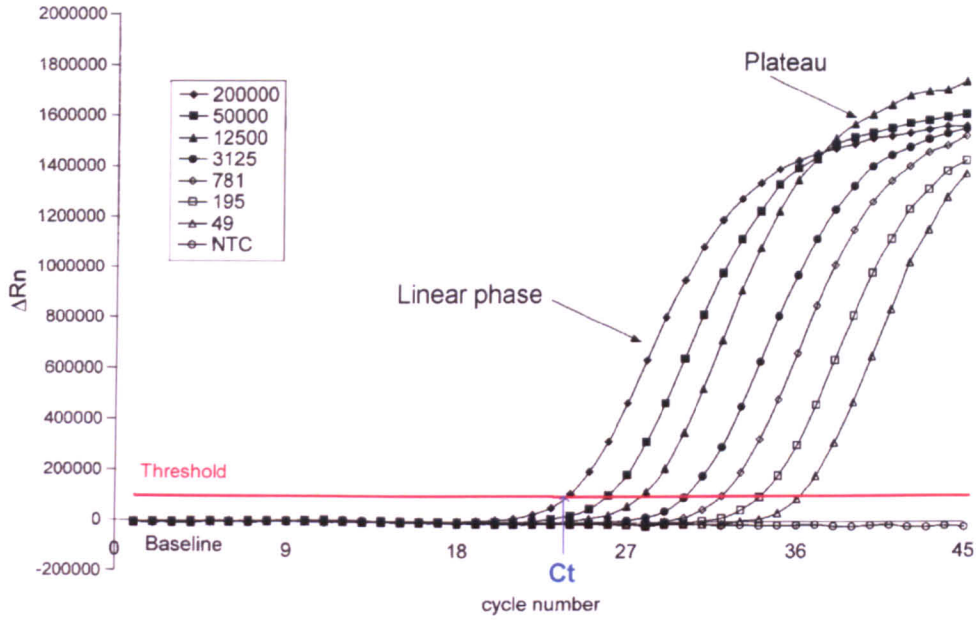


Figure 3.1: An example PCR plot illustrating the stages of the amplification reaction.

Amplification plots for a five-fold serial dilution of template cDNA, amplified by real-time PCR. The change in fluorescence (ΔRn) between the baseline signal and the signal detected at each cycle is plotted. The initial cycles of the reaction show little changes in fluorescence, and represent the baseline of the reaction. ΔRn increases as the reaction progresses, and crosses the threshold in the exponential phase of the reaction. The point at which ΔRn crosses the threshold is referred to as the Ct value, and is used to calculate the initial concentration of the template. The reaction then proceeds through a log phase, before increases in ΔRn slow down and eventually reach the plateau phase. Serial dilutions demonstrate the impact of the initial template concentration on Ct values, with higher concentrations of template having lower Ct values (Taken and adapted from Giulietti *et al.*, 2001).

3.1.5.1 Detection chemistries

The use of fluorescence-based detection increases the sensitivity of quantification, and enables the detection of gene number at a much wider range than end point analysis. Detection using fluorescence chemistry is largely based on fluorescence resonance energy transfer (FRET), which involves light emission from an excited reporter dye, which is absorbed and activates an acceptor dye that may emit light as a result. A number of different fluorescent technologies exist for real-time PCR. These are categorised into:

a) Primer-dye based signalling systems:

   This technology involves a fluorescent reporter that is attached to the primer. After binding to the target gene, amplification leads to the release of the fluorescent signal. With the LUX system, for example, the reporter is attached at the 3' end of the oligonucleotide. A 4-6 base pair extension at the 5' of the oligonucleotide binds to an internal sequence near the 3' end, effectively creating a stem loop structure that quenches fluorescence of the reporter dye. Following the melting and annealing of the primer to the template, the primer is made linear and incorporated into the newly synthesised DNA strand, which frees the reporter and results in an increase in fluorescence of up to 10-fold over the background (Nazarenko *et al.*, 2002). This technology allows for high specificity; however, the quality of the assay is dependent on the specificity of the primers for the target gene.

b) Probe based signalling:

   These assays involve a third oligonucleotide that also binds to the target sequence. The probe is bound by both the reporter dye and also the acceptor dye that quenches the fluorescence of the reporter. Annealing of the probe must occur before the binding of the primers. *Taq* polymerase binds immediately after and begins extension of the target gene. The 5' nuclease activity of the polymerase cleaves the probe that is bound and releases the reporter from the quencher, allowing emission of light which is detected (Holland *et al.*, 1991). A number of different probe-based technologies exist, including the Taqman system and molecular beacons. Multiple reporter dyes can be used with a variety of quenchers. These systems benefit

from the added specificity of using a third primer, making them useful for detection of target genes with low copy number. In addition, there is no detection of primer dimers. However, the addition of an extra primer increases the cost of the assay and complicates assay design (Giulietti *et al.*, 2001).

c) Free dye systems:

These systems incorporate a free dye into a newly synthesised double-stranded DNA product. This was initially demonstrated with ethidium bromide (Higuchi *et al.*, 1993). The most commonly used dye for real-time PCR is now SYBR green I (Schneeberger *et al.*, 1995). This dye is ideal as it has low background fluorescence, and when bound to the minor groove of double-stranded DNA, a dramatic increase in fluorescence output of almost 2000 times the initial unbound signal is seen. This system is beneficial as it is relatively cheap and the assays are easy to develop. However, this system is limited as all double-stranded DNA products will be susceptible to detection by SYBR green. This includes primer-dimers and any non-specific amplicons generated during the course of the reaction. Therefore, excellent primer design is necessary to minimise non-specific amplification. The use of heat dissociation analysis is also beneficial for determination of non-specific fluorescence detection. This involves incremental heating of the PCR product at the end of the reaction (Ririe *et al.*, 1997). The dissociation of double-stranded products as a result releases the dye and causes a decrease in fluorescence intensity. A negative first derivation curve of fluorescence intensity shows the melting temperature of the products in the reaction. Ideally a single peak should be present representing the specific PCR product that is amplified (see Figure 3.3C). Melting temperatures measured at the point of dissociation should be similar to the predicted melting temperature of the PCR amplicon, therefore making primer-dimers easy to identify, as their short oligonucleotide sequence will have a low melting temperature. The use of HotStart DNA polymerase may also help to eliminate extra-assay signals. The use of SYBR green at high concentrations has been shown to inhibit PCR, illustrating the need to optimise assays (Giglio *et al.*, 2003).

The choice of which technology to use is dependent on many factors. The need for high specificity would call for the use of probe based systems such as Taqman. However, these technologies can be expensive and low cost favours the use of SYBR green.

3.1.5.2 Normalisation of PCR assays and reference genes

Although quantification by real-time PCR is highly sensitive and specific, a number of errors can be introduced at various stages of the protocol. These include variations in the amount of DNA in the reaction, which may differ as a result of varying sizes of tissue samples, variability in the extraction procedure, or simple pipetting errors. For the quantification of RNA, additional error may be introduced during cDNA synthesis (Bustin & Nolan, 2004). Consequently, an appropriate method of normalisation is required to minimise these experimental errors.

The most basic and simple method for normalisation is correcting for sample size, for example by using tissue weight. However, correcting for sample size may not ensure that the samples contain the same cellular content. For example, adhesive cells used for *in vitro* culture assays can often clump together, making it difficult to obtain accurate cell counts. Therefore, this method of normalisation is not sufficient on its own. Correcting for the total amount of DNA extracted from samples may also be efficient. However, this is dependent on the accuracy of measuring DNA concentration. For the quantification of RNA, it is essential to use consistent concentrations of RNA for cDNA synthesis. However, this does not account for errors introduced during the synthesis of cDNA (Bustin & Nolan, 2004).

Normalisation to a reference gene is currently the most acceptable method to correct for minor errors. In theory, normalisation by amplification of a reference gene is the most accurate method as the reference gene will be subject to the same variation that affects the gene of interest. Ideally, reference genes are expressed constitutively in all cells and at different times of development. These genes are often termed housekeeping genes, and the most commonly used include β-actin,

GAPDH, and hypoxanthine-guanine phosphoribosyl transferase (HPRT) (Giulietti *et al.*, 2001). Importantly, the expression of the housekeeping gene must not be affected by the experimental procedure. There is evidence to suggest that both β-actin (Foss *et al.*, 1998) and GAPDH (Bustin, 2000; Freeman *et al.*, 1999) expression is influenced by the experimental treatment or conditions. It is now thought that use of multiple reference genes in an assay may reduce the error that may occur as a result of using a single reference gene (Vandesompele *et al.*, 2002). However, this may increase the cost of the experiment, and may not be suitable if a limited amount of sample is available for amplification.

3.1.5.3 Methods of quantification

Quantification of the results obtained by real-time PCR may be absolute or relative. Absolute quantification involves the use of standard curves. Standards can be based on a plasmid construct containing a portion of the gene of interest, synthetic oligonucleotides, or genomic DNA. Concentrations of the standard are measured by spectrophotometry, and copy numbers can be calculated using the molecular weight of the DNA or RNA. The design and development of a standard curve is essential for accurate quantification, as small variations between reactions in the initial phase are amplified greatly during PCR (Heid *et al.*, 1996). The quality of the standard curve can be estimated from parameters determined by the software used for real-time PCR. The efficiency of the reaction (E) provides an indication of how well the PCR reaction has proceeded. The integrity of the data fit to the standard curve (r^2) is a measure of the accuracy of the dilutions and the precision of pipetting. Therefore, these parameters can be used to estimate the accuracy of the standard curve and hence, the accuracy of quantification (Souaze *et al.*, 1996).

Relative or comparative methods are often used for measuring small physiological changes in gene expression levels, and measure the relative change in mRNA levels. They can be used to measure difference in gene expression compared to a control group, for example, an untreated or uninfected control. This method does not require standard curves with known concentrations of transcript, but does require reaction efficiencies of both reference and target gene to be similar, which is often determined using a standard curve (Bustin, 2002). Quantification involves

the comparison of Ct values and the units used to express relative quantities are irrelevant (Livak & Schmittgen, 2001).

3.1.5.4 Applications of Real-time PCR

An accurate tool for of the quantification of *H. pylori* is beneficial in a clinical setting for monitoring the effectiveness of treatment regimes and vaccines. Real-time PCR was previously used to demonstrate the effectiveness of a urease based vaccination against *H. pylori* in mice (Mikula *et al.*, 2003). Real-time PCR has also been used to determine the abundance and distribution of specific bacterial strains (Becker *et al.*, 2000; Suzuki *et al.*, 2000). Quantitative PCR is also important for assessing functional genes and their abundance within the environment (Denman *et al.*, 2007; Henry *et al.*, 2006; Okano *et al.*, 2004).

An accurate quantitative assay will be important for investigating the impact of *H. pylori* bacterial density on host responses and may also be useful in epidemiological studies on the acquisition and spread of *H. pylori*. Previous studies of bacterial colonisation density have reported an association with inflammation of the gastric mucosa (Blanchard *et al.*, 2003; Chen *et al.*, 2001), bacterial virulence factors and the presence of duodenal ulceration (Atherton *et al.*, 1996). These data demonstrate that quantification of bacterial density in the gastric mucosa may be useful in determining the severity of infection, and its influence on histological changes and clinical outcomes, and the use of real-time PCR based assays should provide a highly accurate means for determining bacterial load. The association of colonisation density with both host and bacterial factors will be discussed in the following chapters.

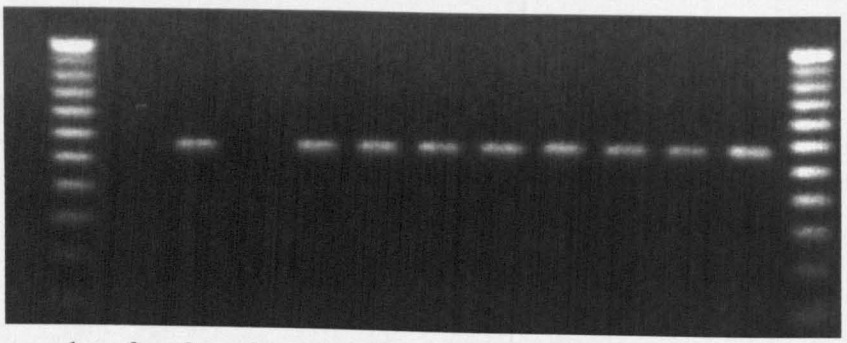
3.1.6 Experimental aims

- To develop a real-time PCR based quantitative assay for the accurate quantification of *H. pylori* from gastric tissue samples.
- To validate the assay developed, determine the accuracy of quantification, and the reproducibility of the assay.
- To compare the accuracy of the assay to other quantitative tools in order to determine the efficacy of the assay developed.

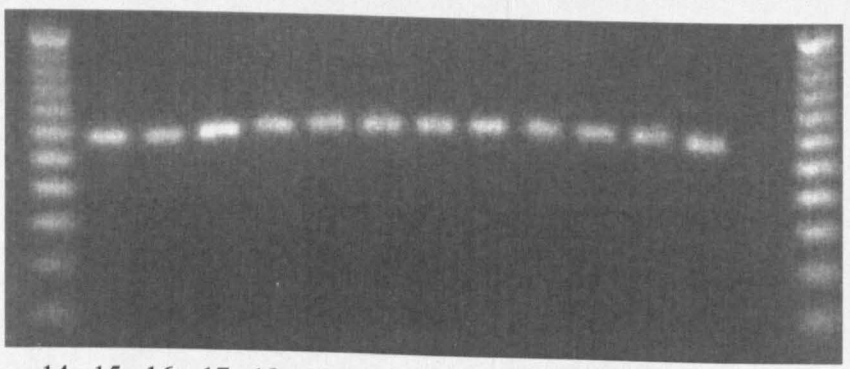
3.2 RESULTS

3.2.1 Development of a quantitative assay for detection of *H. pylori*

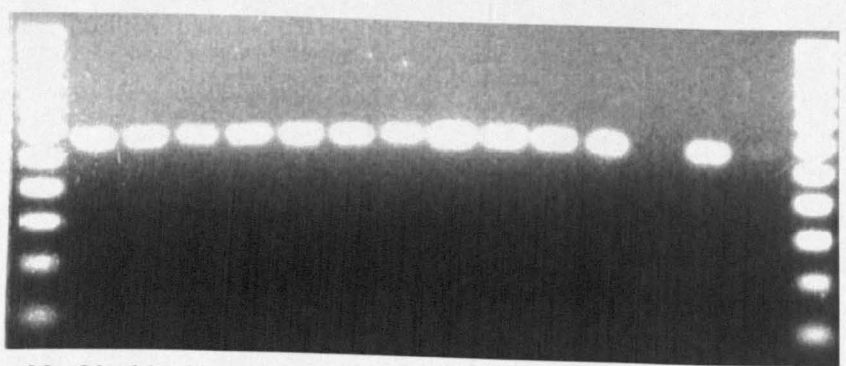
Absolute quantification of bacterial density was a requirement for this assay to provide the most accurate measure from human gastric tissue samples. A real-time PCR using SYBR green I fluorescence technology was employed. The *16S rRNA* gene was chosen for quantification of *H. pylori* genome copy numbers in DNA extracted from gastric tissue biopsies, as it is well conserved amongst all strains. Gene specific primers were designed, and BLAST analysis of the primer sequences confirmed specificity to *H. pylori* only. Primer detection specificity was tested on 30 clinical isolates. All strains tested were PCR positive for the gene, and only a single PCR product was observed (Figure 3.2).



1 2 3 4 5 6 7 8 9 10 11 12 13



14 15 16 17 18 19 20 21 22 23 24 25 26 27 28



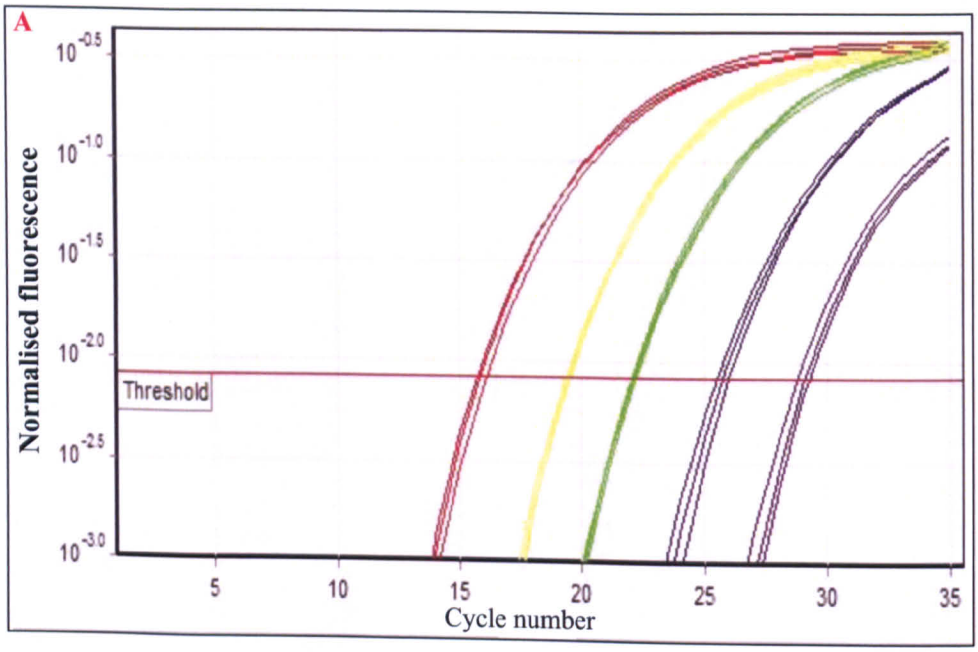
29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44

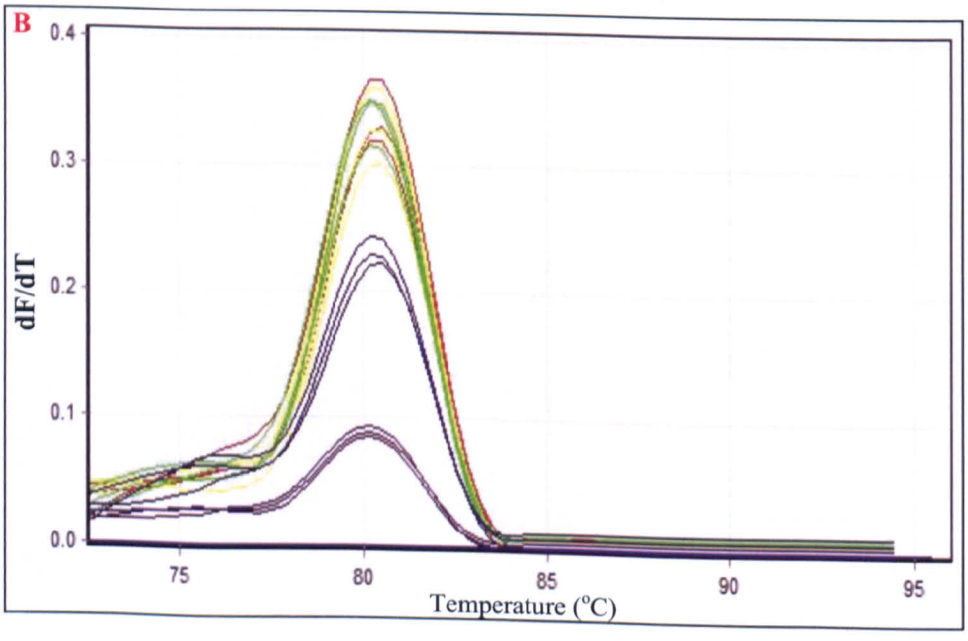
Figure 3.2: Gel images showing positive PCR amplification of *16S rRNA* from multiple *H. pylori* strains.

Clinical isolates of *H. pylori* were cultured from numerous gastric tissue biopsies donated by infected patients. The *16S rRNA* gene fragment was amplified from each strain to validate primer specificity in multiple strains of *H. pylori.* All PCR reactions were amplified alongside a no template control (NTC). *H. pylori* strain 60190 was used as a positive control, and a 1Kb ladder was included alongside. A product size of 164bp was expected.

Lanes: 1. Ladder, 2. Empty, 3. Positive control, 4. NTC, 5. Q16a, 6. Q36a, 7. Q55a, 8. Q64a, 9. Q102, 10. Q173a, 11. Q201a, 12. Q249b, 13. Ladder, 14. Ladder, 15. Q255a, 16. Q265a, 17. Q274a, 18. Q241a, 19. Q256b, 20. Q223b, 21. Q187a, 22. Q182a, 23. Q215b, 24. Q173a, 25. Q247b, 26. Positive control, 27. NTC, 28. Ladder, 29. Ladder, 30. Q136a. 31. Q173b, 32. Q117a, 33. Q14a, 34. Q7b, 35. Q242b, 36. Q106a, 37. Q102b, 38. Q240b, 39. Q245b, 40. Q77b, 41. Empty, 42. Positive control, 43. NTC, 44. Ladder.

To allow for absolute quantification, standard curves were designed for inclusion in each PCR to reduce run-to-run errors. Genomic DNA from the *J99 H. pylori* strain was purified and used as a standard. A 5-fold dilution series was prepared ranging from 20ng/µl to 10.24fg/µl, and all samples were tested in triplicate for every real-time PCR performed. From this, the sensitivity of the assay was determined, and a standard curve within a smaller range of 4ng/ul-6.4pg/µl was designed which encompassed the range of detection of *H. pylori* from tissue samples (Figure 3.3A). Analysis of the melt dissociation curve showed a single peak at 80.3°C, again demonstrating primer specificity (Figure 3.3B). Analysis of the resulting standard curve demonstrated excellent linearity of data (r^2=0.997), with a gradient of -4.676. However the efficiency of the reaction was reduced (E=0.64) (Figure 3.3C).


A
Normalised fluorescence
$10^{-0.5}$
$10^{-1.0}$
$10^{-1.5}$
$10^{-2.0}$
$10^{-2.5}$
$10^{-3.0}$
Threshold
5
10
15
20
25
30
35
Cycle number


B
dF/dT
0.4
0.3
0.2
0.1
0.0
75
80
85
90
95
Temperature (°C)

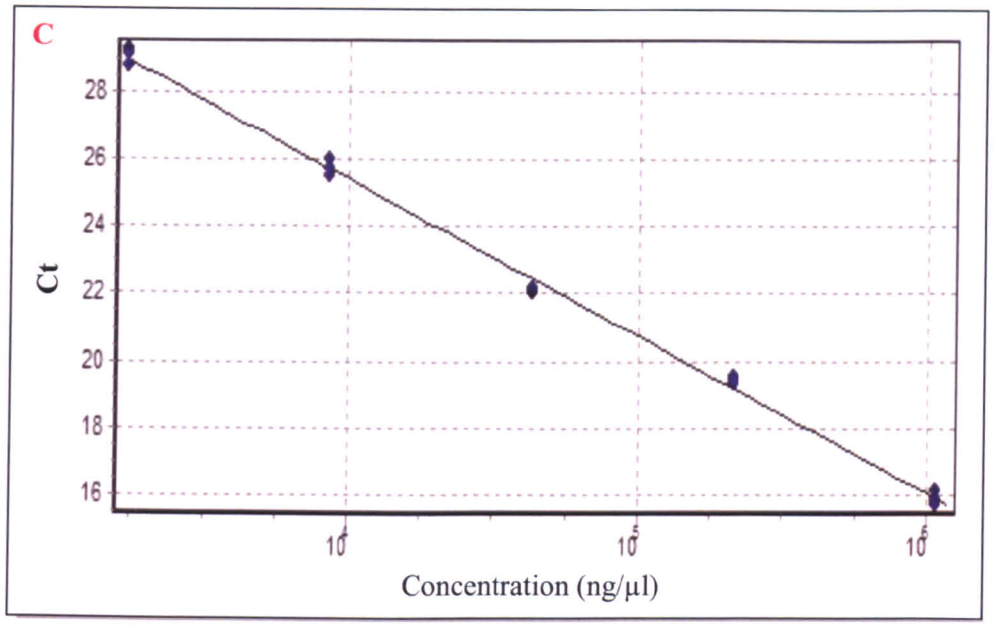


Figure 3.3: Real-time PCR plots and standard curve for *16S rRNA* using J99 genomic DNA

A standard curve was developed using a 5-fold serial dilution of *J99* genomic DNA ranging from 4ng/µl-6.4pg/µl. A- PCR amplification plots display the level of fluorescence detected in each PCR cycle. The threshold was determined by the Rotorgene 6 software. B- the dissociation curve shows the negative first derivation of fluorescence intensity versus temperature (dF/dT). Peaks represent the temperature at which PCR products dissociate. A single peak at 80.3°C was seen when using *16S rRNA* specific primers. C- a standard curve is plotted giving the Ct values obtained for each concentration of genome copy numbers, with r^2=0.997, E=0.64, and a gradient of -4.676.

4ng/µl, 800pg/µl, 160pg/µl, 32pg/µl, 6.4pg/µl

For absolute quantification of bacterial numbers, genome copy numbers in each dilution point of the standard curve were calculated using equation 2. A breakdown of this equation is provided in appendix 11.2 (taken from Roussel *et al.*, 2007). DNA concentrations were determined spectrophotometrically using the NanoDrop. Genome copy numbers calculated using this equation were defined as "estimated copy numbers".

Equation 2:

$$\text{Genome copies} = \left[\frac{\text{DNA concentration (pg/µl)}}{\text{Template size x gene copy number}} \right] \times 8.85 \times 10^{8}$$

After amplification, the Rotorgene 6 software was used to determine the number of target genes amplified, termed "actual copy number", for each dilution point in the standard curve. To assess the accuracy of detection, the percentage variation between estimated and actual values was calculated by the software using equation 3.

Equation 3:

$$\% \text{ Variation} = \text{Absorbance} \times \left[\frac{\text{Measured Concentration}}{\text{Estimated concentration} - 1} \right]$$

The degree of accuracy varied, ranging from 1.8 - 23.4%. However, a plot of estimated and actual copy numbers showed an excellent linearity (r^2=0.998) (Figure 3.4). This demonstrated excellent accuracy of the dilutions prepared for the standard curve, and that little error was introduced during pipetting and preparation of reactions. However, this does not show the accuracy of measuring bacterial load in infected samples using real-time PCR.

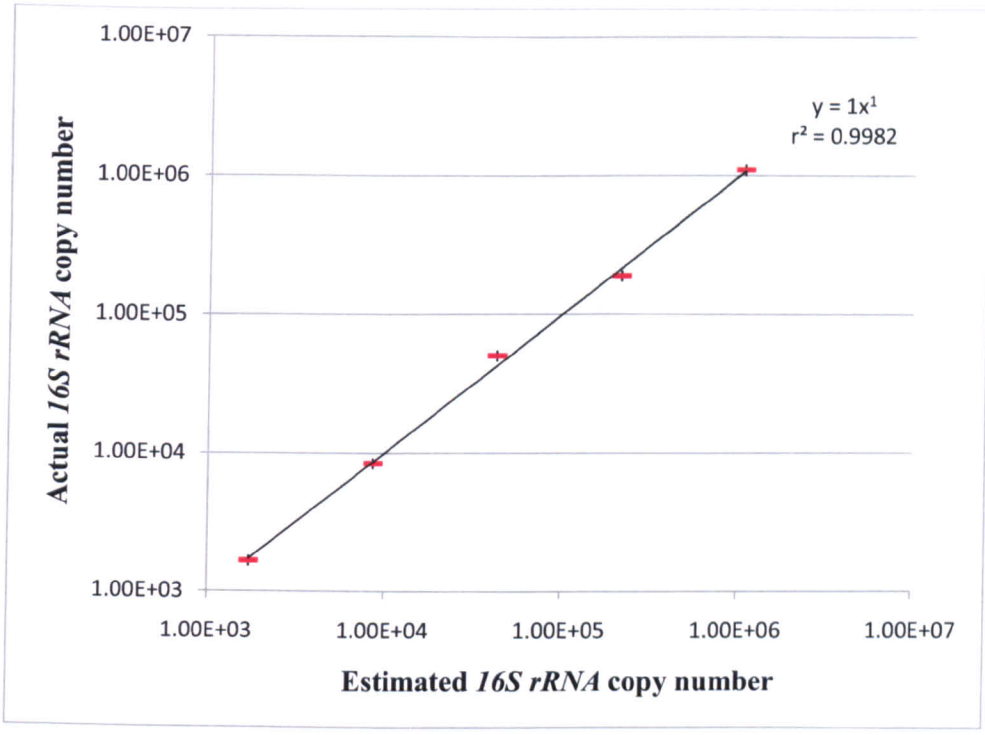


Figure 3.4: Graphical representation of the accuracy of the *16S rRNA* gene quantitative real-time PCR assay.

A standard curve was developed using real-time PCR for DNA concentrations ranging between 4ng/µl-6.4pg/µl for *16S rRNA* detection. The Rotorgene software was used to calculate gene copy numbers for each reaction (actual copy number), and compared with the estimated values calculated from the concentration of DNA added to each standard (estimated copy number). For each dilution point, the gene copy number calculated by the Rotorgene 6 software after amplification was plotted (defined as actual *16S rRNA* copy number). A trend line was added to demonstrate the accuracy of detection. A gradient of 1 and r^2 value of 0.998 are given.

3.2.2 Testing the accuracy of *H. pylori* real-time PCR

To test the accuracy of the real-time PCR assay, a culture of the *J99* strain was diluted to give bacterial concentrations ranging between $1x10^4$ to $1x10^8$ colony forming units (CFU), estimated by optical density. Genomic DNA was extracted from the diluted bacterial suspensions, and bacterial load was determined using the real-time PCR assay. The detection limit of the assay was $1x10^5$ bacteria. Detected copy numbers from the PCR were roughly 50 times lower than the amount of bacteria initially estimated. However, the degree of error in detection was consistent over all samples tested, and 10-fold difference were observed with good linearity between samples (r^2=0.999) (Figure 3.5). This suggests that the difference in bacterial load detected by PCR compared to the number of bacteria initially added to each bacterial suspension may be due to loss of bacterial DNA during the extraction process, demonstrating the impact of sample processing on the accuracy of quantification. However, it also suggests that the loss of bacterial DNA occurs in a highly linear and predictable manner.

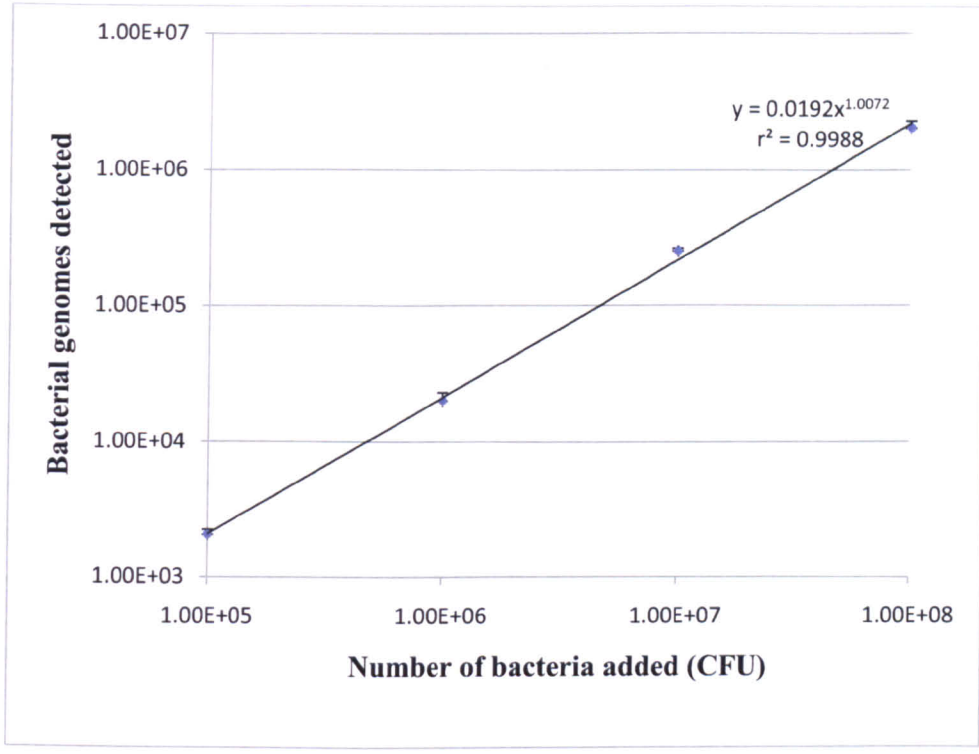


Figure 3.5: Accuracy of bacterial genome copy detection using real-time PCR.

A bacterial suspension of the *J99 H. pylori* strain was diluted to concentrations of $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, and $1x10^8$ colony-forming units (CFU), estimated by spectrophotometry. Bacterial genome copy number was assayed in triplicate using the *16S rRNA* real time PCR system developed. The equation of the trendline is given with r^2=0.9988.

As mentioned earlier (section 3.1.5.2), more accurate quantification benefits from normalising data for another measurable factor. For validation of the quantitative PCR, colonisation densities were therefore measured relative to the total amount of DNA in samples. An *in vitro* culture assay was developed, for which $1x10^6$ gastric epithelial cells (AGS) were infected with varying numbers of bacterial cells ranging from $1x10^5$ to $1x10^8$ CFUs. Bacterial genome copy numbers were measured in the resulting DNA extracted from AGS-bacterial cell mixtures. Normalised for total DNA, the PCR data showed approximately a 5 to 10-fold reduction in the bacterial genome copy number (gradient of 0.009) compared to the bacterial CFUs initially added (gradient of 1). Measurements between replicates appeared to be fairly reproducible; however, there was increased variability between replicates at the lower limit of sensitivity (Figure 3.6).

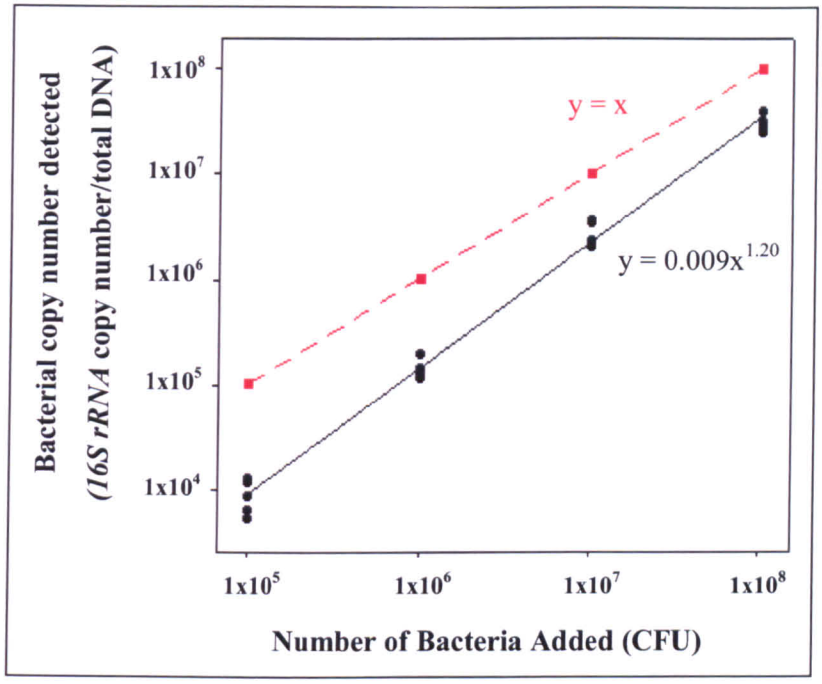


Figure 3.6: Accuracy of the quantitative *H. pylori* PCR in epithelial cell-bacterial cell co-cultures when data were normalised for the total amount of DNA present.

$1x10^6$ gastric epithelial cells (AGS) were infected with a *J99 H. pylori* bacterial suspension at concentrations of $1x10^5$, $1x10^6$, $1x10^7$, or $1x10^8$ CFU. Total DNA concentrations extracted from cell suspensions were determined by NanoDrop spectrophotometry. Bacterial genome copy numbers were measured for 6 replicates by real time PCR and data normalised for the total amount of DNA present. The red broken line illustrates the bacterial copy number/total DNA from spectrophotometric estimation of CFUs added, and the black, solid line shows the actual density measured by real-time PCR.

To test the accuracy of quantification from gastric tissue samples, uninfected biopsies were spiked with a bacterial suspension at concentrations ranging from $1x10^5$ to $1x10^8$ CFUs. As before, *H. pylori* genome copy numbers were calculated relative to the total DNA concentration. Again, lower densities were observed by PCR quantification (gradient of 19.5) in contrast to the initial number of bacteria added (gradient of 1). As bacterial load increased, the degree of error also increased, with more than a 10-fold difference at $1x10^8$ CFU. In this case, the sensitivity of the assay was also reduced with a detection limit of $1x10^6$ (Figure 3.7).

These two experiments showed that normalising for total DNA in samples introduced considerable error. Alternative strategies for normalising were necessary as gastric biopsy specimens vary in size. This must be taken into account.

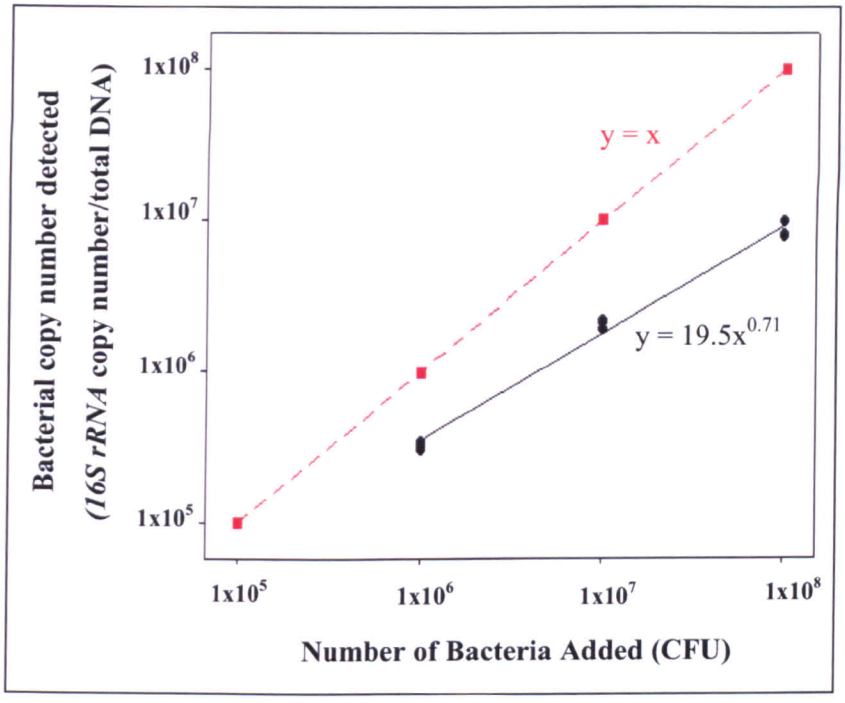
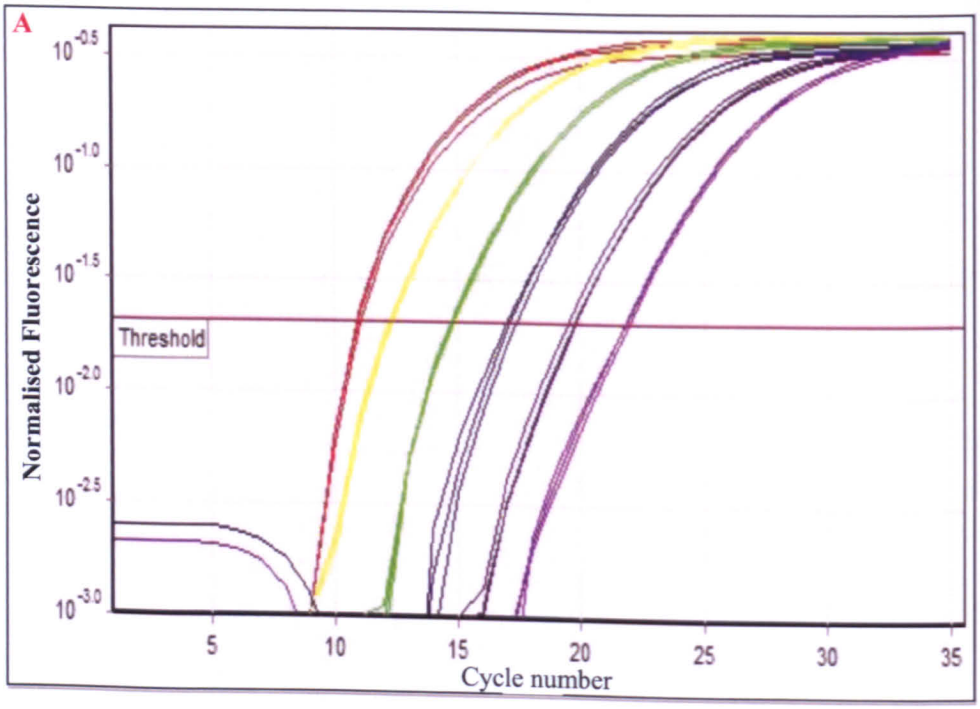


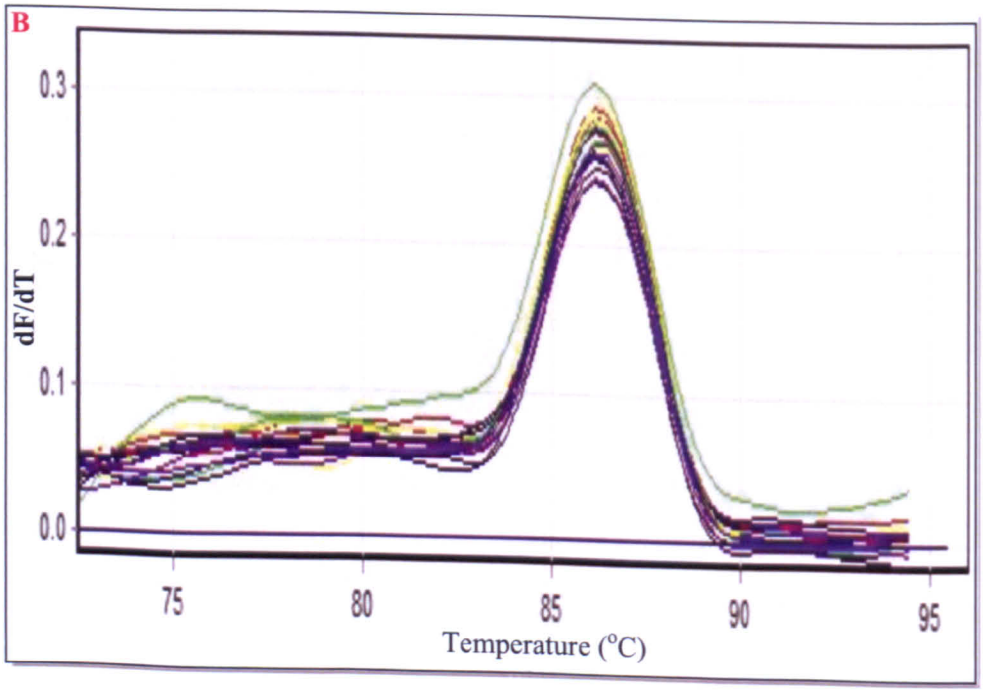
Figure 3.7: Detection of *H. pylori* in gastric biopsies in gastric tissue to determine the accuracy of quantification by real-time PCR.

Biopsies taken from uninfected patients were infected with 0, 10^5, 10^6, 10^7, or 10^8 colony-forming units of *H. pylori J99* bacterial cells. Biopsies were homogenised, DNA was extracted, and the bacterial copy number was measured in triplicate by PCR. Bacterial copy numbers detected in total DNA were plotted against the number of bacteria added (black solid line). A line showing the expected result is also shown (red broken line).

3.2.3 Normalising for human cell numbers in biopsies

As bacterial density measured relative to total DNA was inaccurate, an alternative strategy was required. Bacterial load would ideally be normalised against some measure of the number of human cells present in the tissue. For this, the human *GAPDH* gene was quantitatively co-amplified, thus giving a measure of bacterial load in terms of bacterial genome copy number per human genome copy number. Primers were designed with specificity confirmed by BLAST analysis of the gene sequence. Standard curves were developed using the pSAP1 plasmid construct containing the *GAPDH* amplicon as a standard. Serial dilutions were prepared within a range of 32pg/µl-10.24fg/µl (Figure 3.8A). A dissociation plot gave a single peak at 86.3°C illustrating the specificity of primers (Figure 3.8B). Standard curves demonstrated excellent linearity (r^2=0.996), with a gradient of -3.237, and the reaction proceeded with an excellent reaction efficiency (E=0.96) (Figure 3.8C).


A
Normalised Fluorescence
$10^{-0.5}$
$10^{-1.0}$
$10^{-1.5}$
$10^{-2.0}$
$10^{-2.5}$
$10^{-3.0}$
Threshold
5
10
15
20
25
30
35
Cycle number


B
dF/dT
0.3
0.2
0.1
0.0
75
80
85
90
95
Temperature (°C)

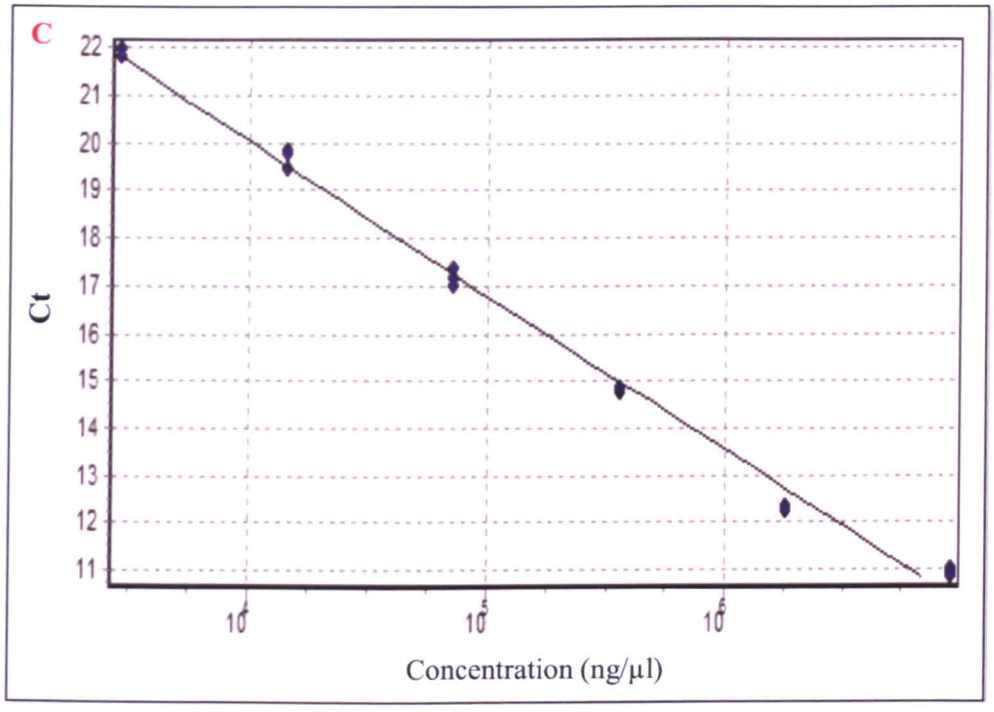


Figure 3.8: Real-time PCR plots and a standard curve for the *GAPDH* plasmid construct.

The pSAP1 plasmid construct was used for developing a 5-fold serial dilution ranging from 32pg/µl-10.24fg/µl. A- PCR amplification plots represent the degree of fluorescence detected at each PCR cycle for three replicates. B- the dissociation curve shows the negative first derivation of fluorescence intensity against temperature (dF/dT). A single peak at 86.3°C represents the melting temperature of the PCR product. C- a standard curve was plotted giving the Ct values for a given concentration of genome copy numbers, with an efficiency of 0.96, r^2 value of 0.996, and a gradient of -3.237.

32pg/µl, 6.4pg/µl, 1.28pg/µl, 256fg/µl, 51.2fg/µl, 0.2fg/µl

The percentage variance between the estimated concentrations of DNA measured using NanoDrop spectrophotometry, and the actual DNA concentration determined by amplification of DNA standards was calculated using the Rotorgene 6 software. The variance ranged from 1.5-36.7% (Figure 3.9). The gradient was 1 and the r^2 value 0.995, demonstrating a close fit between the data sets.

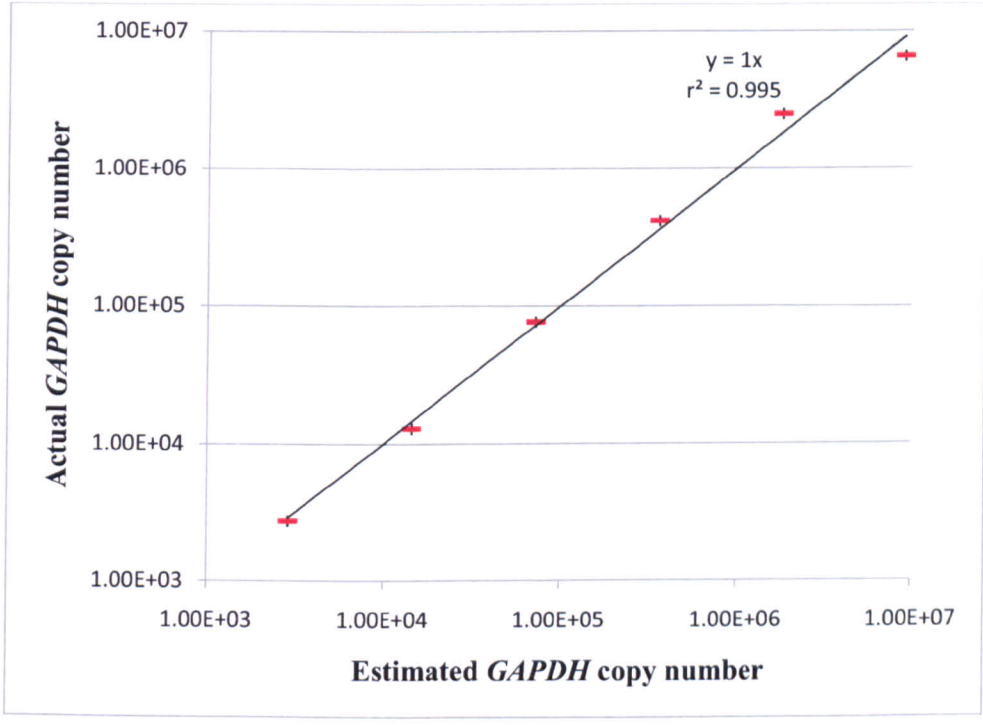


Figure 3.9: Graphical representation of the accuracy of *GAPDH* detection by the PCR assay.

A standard curve was developed using pSAP1 with DNA concentrations ranging between 32pg/µl-10.24fg/µl. The estimated gene copy number (calculated using equation 1) for each dilution point and the gene copy number calculated by the Rotorgene 6 software after amplification are plotted. Each dilution was tested in triplicate. A trend line is given with a gradient of 1 and r^2 value of 0.995.

To test the accuracy of detection when measuring bacterial load relative to human *GAPDH*, known numbers of *H. pylori J99* cells were added to AGS cells as described previously (Figure 3.6). The ratio of bacterial cells:human cells were calculated using the qPCR assay, this time normalising for *GAPDH*. This gave a much more accurate quantification, with bacterial loads measured by PCR (gradient of 0.84) agreeing closely with estimated densities initially added to the culture (gradient of 1) (Figure 3.10). Overall, quantification by real-time PCR was more accurate when data was corrected for the number of human cells rather than normalising against the total amount of DNA present.

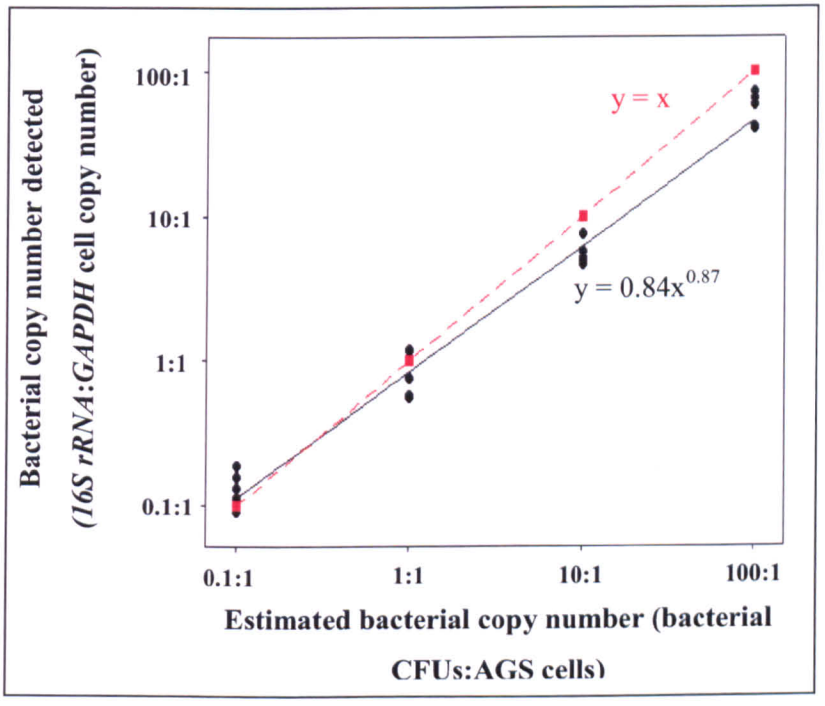
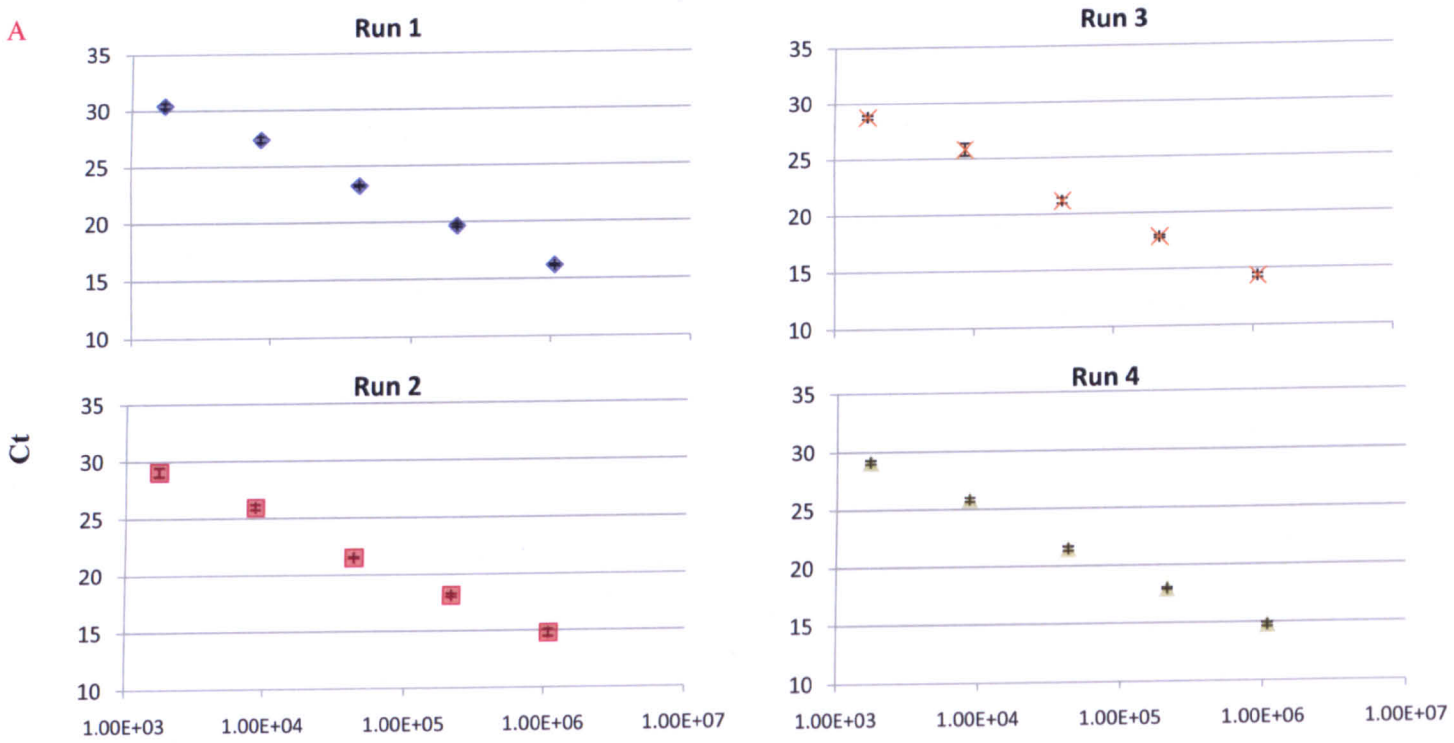


Figure 3.10: Accuracy of bacterial load measured in samples of AGS cells mixed with known numbers of *H. pylori J99*, normalising for *GAPDH*.
$1x10^6$ gastric epithelial cells (AGS) were infected with either $1x10^5$, $1x10^6$, $1x10^7$, or $1x10^8$ bacterial CFUs. Bacterial genome copy numbers were measured by real time PCR and normalised against human *GAPDH* cell copy number for 6 replicates. The red broken line shows the estimated bacterial:human cell ratio initially added, and the black, solid line represents the ratio of bacterial:human cells calculated after PCR amplification.

3.2.4 Testing the reproducibility of the real-time PCR assay

It is possible that the results of the assay may be affected by slight differences in run conditions. This was addressed by including standard curves in each reaction carried out. To assess whether reproducibility between reactions was sufficient, standard curves developed for quantifying both bacterial and human genome copy numbers were tested in quadruplicate and compared. Successive reactions of DNA standards for both genes showed excellent reproducibility (Figure 3.11). Intra-run reproducibility was assessed using the Rotorgene 6 software which calculated the percentage variance between replicates within a run. Variability ranged between 3.7-12.9% for *16S rRNA* standard curves, and 3.9-14.9% for *GAPDH.*

A


Run 1
Run 2
Run 3
Run 4
Ct
35
30
25
20
15
10
1.00E+03
1.00E+04
1.00E+05
1.00E+06
1.00E+07
16S rRNA copy number

B

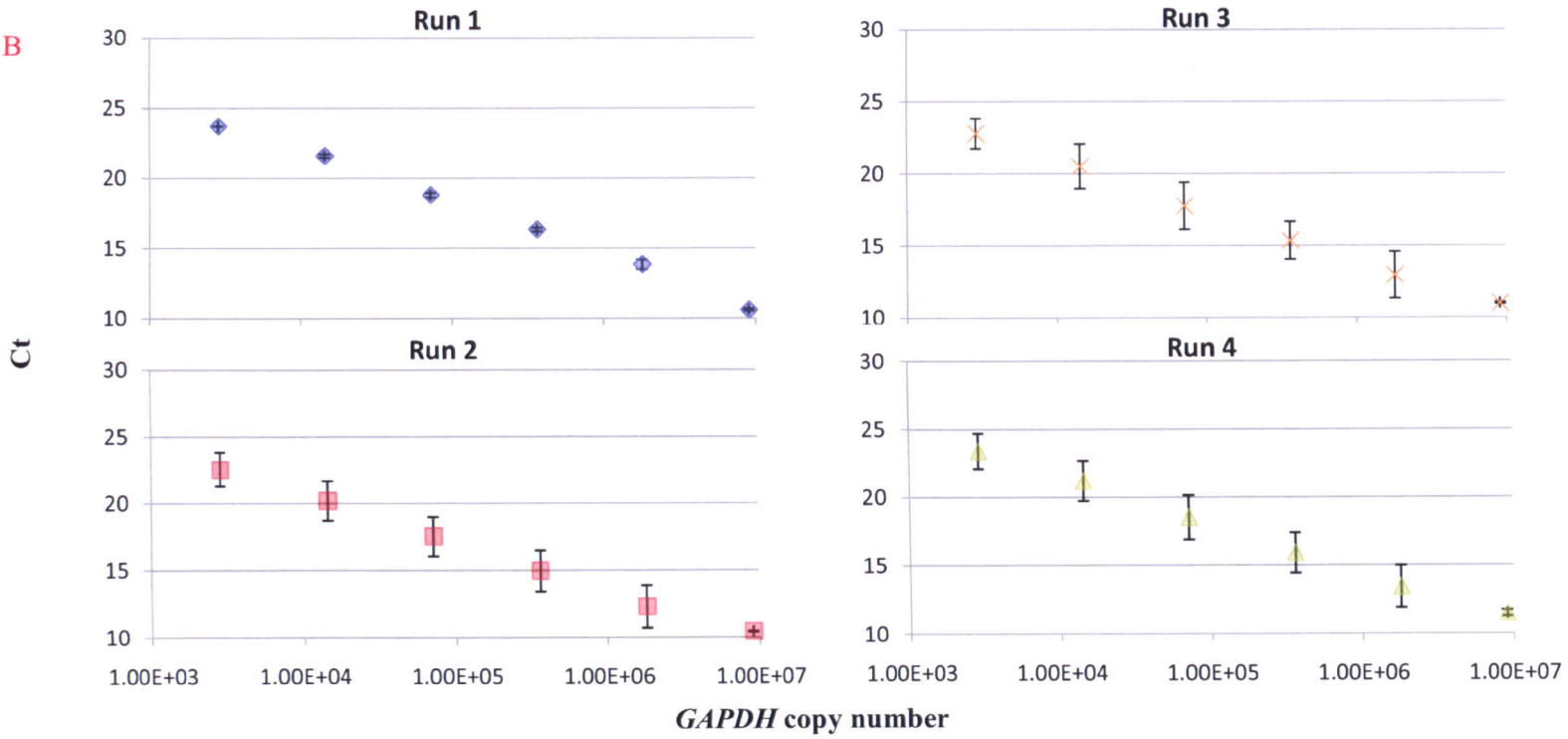

Run 1
Run 2
Run 3
Run 4
Ct
30
25
20
15
10
1.00E+03
1.00E+04
1.00E+05
1.00E+06
1.00E+07
GAPDH copy number

Figure 3.11: Analysis of standard curve reproducibility between successive PCRs.

PCRs for standard curves based on A- *H. pylori* strain *J99* chromosomal DNA for measuring *16S rRNA* gene copy number, and B- the pSAP1 plasmid construct for measuring human *GAPDH* gene copy number were repeated in 4 successive reactions. Each data point represents the average Ct values of 3 replicate reactions, with bars representing standard deviation.

Although excellent inter-run reproducibility was found for standard curves, the percentage variability was measured using Ct values. The Ct scale is exponential in nature, and small differences in Ct values may translate to large variations in actual bacterial numbers. Therefore, it would be important to measure the percentage variance between detected copy numbers in biopsy samples. Colonisation density was measured in 10 *H. pylori* infected tissue samples, taken from either the antrum or corpus region of the stomach. For all samples, both *GAPDH* and *16S rRNA* gene amplification was conducted in triplicate. Bacterial load was measured for the same sample in 2 individual reactions. The calculated bacterial load was found to be highly comparable between reactions, demonstrating good inter-run reproducibility (Figure 3.12).

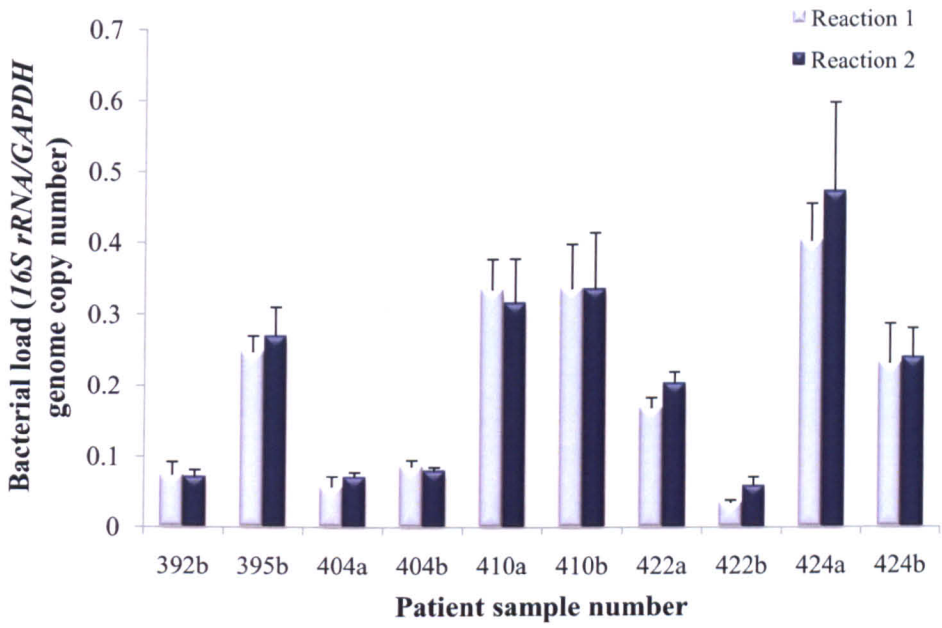


Figure 3.12: The reproducibility of bacterial load measured in *H. pylori* infected tissue between PCRs.

Colonisation density was measured in tissue samples taken from the antrum (denoted as "a") and corpus (denoted as "b") region of the stomach. Triplicate reactions for each sample were included in each PCR, and an average load was determined for each sample. PCRs were repeated using DNA samples that were left on ice between reactions. The average bacterial load for reaction one (light blue solid bars) and reaction 2 (dark blue solid bars) are given. Error bars depict the standard deviation amongst 3 replicates.

Of note was the observed effect of sample freeze-thawing on the quantification of bacterial load. Considerable variability was observed between reactions when samples were used after a single freeze-thaw cycle. (Figure 3.13). This illustrated the importance of using a consistent protocol for assessing colonisation density by PCR.

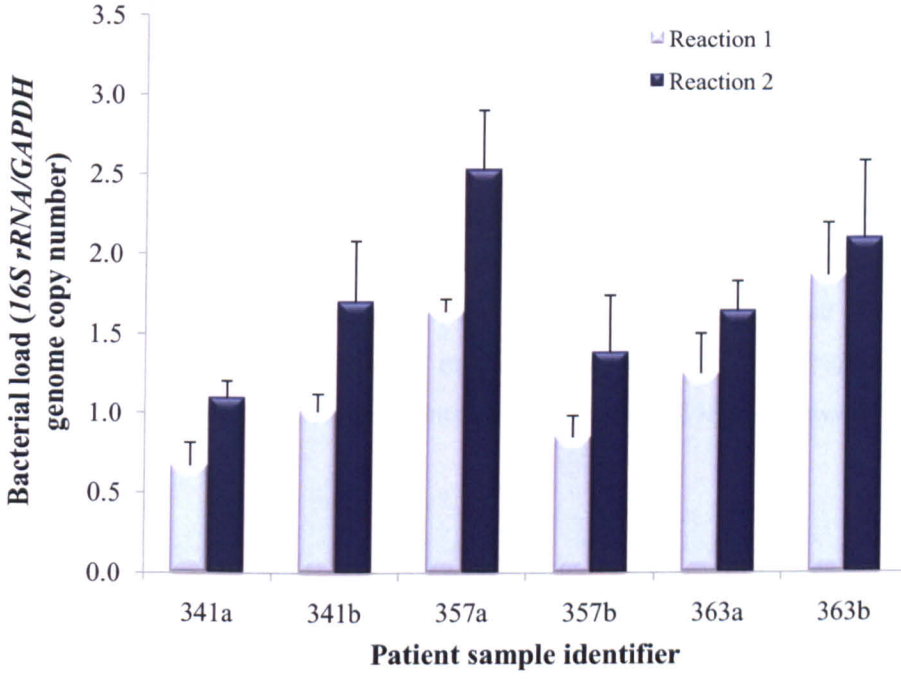


Figure 3.13: The effects of freeze-thaw on reproducibility of measured bacterial density.

Bacterial load was measured in tissue samples taken from the antrum (denoted as "a") and corpus (denoted as "b") region of the stomach. Triplicate reactions for each sample were included in each PCR, and an average load was determined for each sample. PCR reactions were repeated after a freeze-thaw cycle of sample DNA. The average bacterial load for reaction one (light blue solid bars) and reaction 2 (dark blue solid bars) are given. Error bars depict the standard deviation amongst 3 replicates.

3.2.5 Comparison of quantitative PCR with semi-quantitative histology

In the current study, it has been shown that qPCR is highly accurate and reproducible in determining bacterial load. As histology is commonly used for quantification of *H. pylori*, it was of interest to investigate whether this semi-quantitative tool is comparable to qPCR for the determination of bacterial load. Bacterial load was measured in gastric biopsies using the quantitative PCR assay developed here. Bacterial loads were stratified according to the grade of density measured by histology, with grades determined as mild (grade 1), moderate (grade 2), and severe (grade 3).

A weak positive association was found in the antrum between mild and severe colonisation (for score 1: median=0.3, n=9, IQR=0.03-0.60; for score 3: median=0.4; p=0.042, IQR=0.25-0.83). A trend towards a positive association was also observed in the corpus, although this was not statistically significant (Figure 3.14). Thus, quantification by the two methods agreed weakly, particularly in the antrum. Given the reproducibility of qPCR, this implies that histology is not a good method for quantifying bacterial load in the stomach.

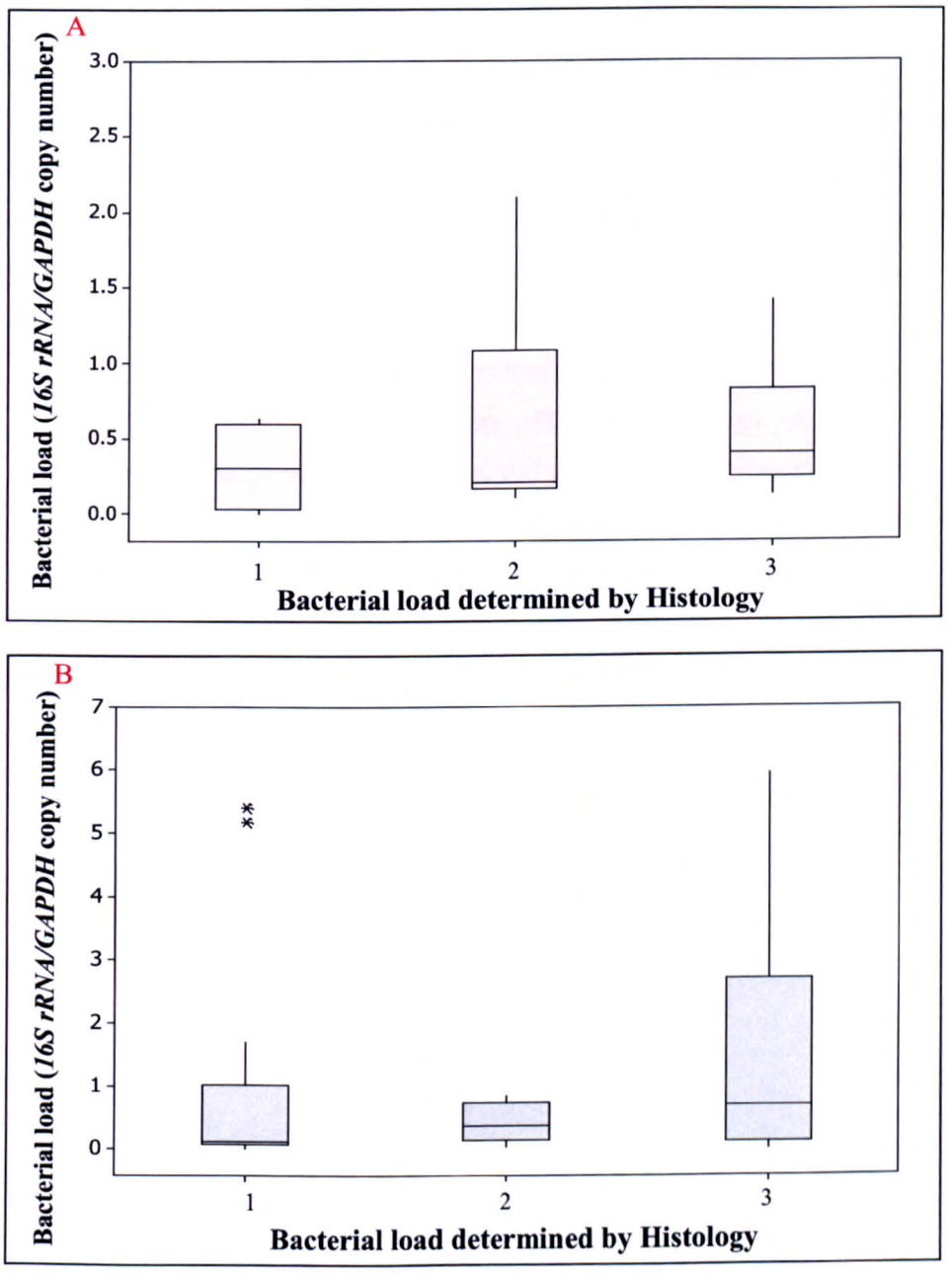


Figure 3.14: Comparison of bacterial load determined by histology and qPCR.

Bacterial load was quantified by qPCR in 34 antral and 38 corpus biopsies. Bacterial density measured by PCR was stratified according to the histological grade of *H. pylori* density for comparison in A- the antrum and B- the corpus.

Finally, histology and qPCR were compared as ways of measuring the pattern of bacterial colonisation in the stomach, as determined by calculating the antral:corpus ratio of colonisation. For histology, an antral predominant colonisation was described as a higher bacterial load in the antrum compared to the corpus, giving an antral:corpus ratio of greater than 1, and a corpus predominant colonisation was described as having an antral:corpus colonisation density lower than 1. A pan-colonisation was described as having equal bacterial loads in both the antrum and corpus, therefore giving a ratio of bacterial density of 1. Predicted patterns of colonisation by qPCR and histology were compared. Agreement was good, particularly for antral predominant colonisation where 12 out of 15 samples gave the same result with both methods. However, there was less agreement for corpus predominant colonisation (3 out of 4 agreed) (Figure 3.15). Thus, histology performs reasonably well for assessing whether colonisation is antral-predominant, corpus-predominant or pan-colonisation.

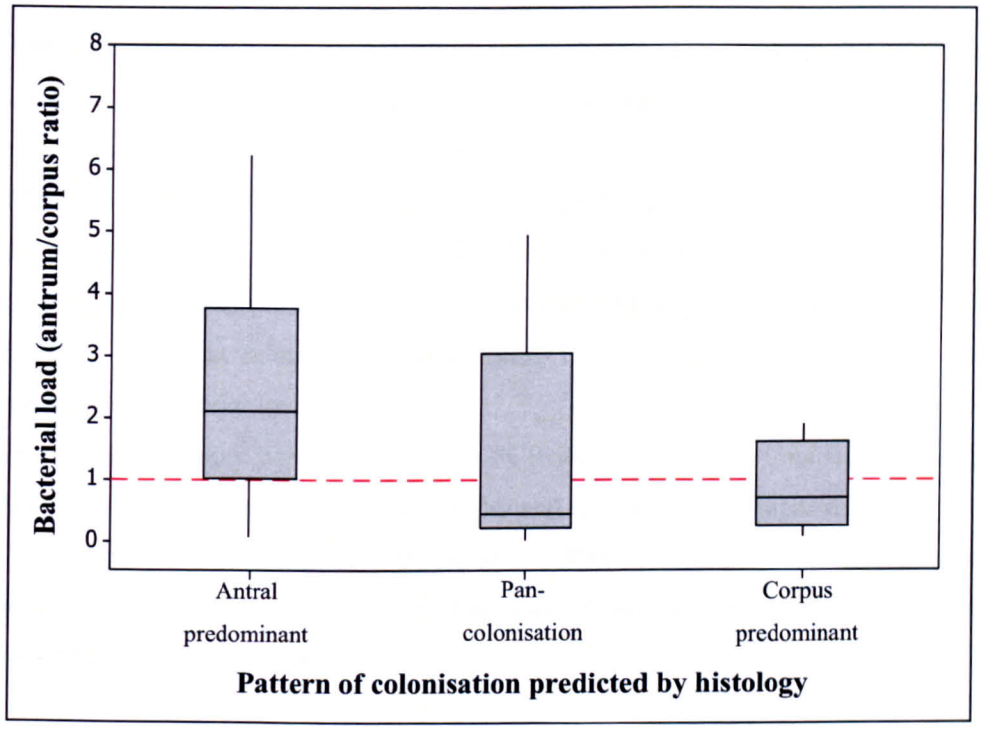


Figure 3.15: Comparison of the pattern of colonisation determined by PCR and histology.

The pattern of colonisation was determined using histology and qPCR by calculating the antral/corpus ratio of bacterial load. An antral predominant colonisation was described as a ratio greater than 1, pan colonisation was described as having a ratio of 1, and corpus predominant having a ratio of less than 1. The ratio of density measured by qPCR for each histological grade of *H. pylori* density was plotted to show the agreement between methods for predicting patterns of colonisation. A reference line at 1 on the y-axis is shown to highlight equal density in the antrum and corpus.

3.3 DISCUSSION

The relationship between *H. pylori* and the host is complex, with each having an impact on the other in many respects. It has been suggested that bacterial density may affect host responses (Blanchard *et al.*, 2003; Chen *et al.*, 2001), and be related to particular bacterial virulence factor profiles, as well as correlating with disease progression (Atherton *et al.*, 1996). Further investigation into the importance of colonisation density is necessary in understanding the extent of the bacterial and host relationship, highlighting the value of an accurate tool for quantifying bacterial load.

A quantitative assay based on real-time PCR was chosen. Previous studies using this tool have reported excellent sensitivity and specificity for diagnosis (He *et al.*, 2002; Kobayashi *et al.*, 2002; Mikula *et al.*, 2003). However other quantitative methods are still extensively used. The aim of this chapter was to assess the quantitative aspect of PCR.

3.3.1 Development of a quantitative real-time PCR

Successful amplification of all *H. pylori* strains requires amplification of a conserved gene that is specific to the bacterium. The *16S rRNA* gene was chosen for amplification as it has been previously used successfully and is a highly conserved gene.

Initial BLAST search analysis of primer sequences confirmed specificity to *H. pylori* strains only, as did PCR amplification of the gene from 30 clinical isolates. Primer specificity was confirmed by dissociation melt curves, which showed amplification of a single product, illustrating *16S rRNA* to be an ideal candidate for specific detection of *H. pylori*. Homology of the *16S rRNA* gene has however come under question as cross-reactivity with human tissue samples has been reported (Chong *et al.*, 1996). A stretch of 546bp region was identified with *H. pylori* specificity, which was recommended for specific primer design (Liu *et al.*, 2008). Primers used in this study were designed in a region of the gene that had 100% homology between *H. pylori* strains *J99*, *26695*, and *43504*. The use of *16S*

rRNA as a target gene for quantification has been successful in other studies, showing high sensitivity, as low as 10^1 copies, although reproducibility between replicates was reduced at the lower limit of sensitivity. Compared with other diagnostic tests, 100% specificity and sensitivity has been reported (Kobayashi *et al.*, 2002; Roussel *et al.*, 2007).

Other target genes have been used for quantification of *H. pylori* with real-time PCR, for example genes coding for urease subunits and *23S rRNA*. Specificity has previously been an issue with amplification in *16S rRNA* based assays, as false positive detection has occurred, although sensitivity has been reported as good (He *et al.*, 2002; Lascols *et al.*, 2003; McDaniels *et al.*, 2005; Mikula *et al.*, 2003).

Measuring bacterial load most commonly utilises absolute quantification with the use of standard curves, which was applied in this study. Standard curves can be based on a number of different templates including genomic DNA or plasmid vectors containing a fragment of the target gene. Importantly, the standard used must have similar reaction efficiencies to the sample DNA tested for accurate quantification. Most often, discrepancies in efficiency result from PCR inhibitors present in sample DNA, particularly when using tissue. However, this effect was found to be reduced when samples are diluted (Roussel *et al.*, 2007). For this study, standard curves based on amplification of the *16S rRNA* gene were designed using genomic DNA and gave excellent linearity between replicates (r^2=0.997), although the efficiency of reaction was reduced (E=1.64). The range of detection reached a lower limit of 10^3 copies per reaction, which gave excellent sensitivity of detection. However, two samples tested in this data set were false negative results, which highlight the limit of sensitivity on occasion. Diluting samples may help to determine whether these false negatives were a result of PCR inhibitors. Although linearity was excellent, it is not a measure of accuracy in detection. Intra-run reproducibility was excellent, and comparison of the calculated copy number for standards against estimated standard values was excellent, demonstrating that low efficiency of the reaction did not greatly affect quantification of bacterial genome copy numbers. However, spiking of uninfected tissue with bacteria showed further reduction in sensitivity, raising the question of

PCR inhibitors in gastric tissue that may affect amplification efficiencies and hence quantification.

Although accurate detection was observed within standard curves, bacterial load is always reported relative to another measureable factor. For example, this may be in terms of per gram of tissue, per total DNA, or per host genome copy number. Importantly, the accuracy in measuring this relative marker is essential for accurate final quantification. Measuring colonisation density relative to tissue weight is the least accurate as variability introduced during tissue processing is not accounted for. Therefore, normalising data against a measure of DNA is far more reliable (Roussel *et al.*, 2007). Normalising bacterial load against the amount of total DNA proved to be inaccurate in the current study, reaching almost a 10-fold difference at the lowest limit of sensitivity. For this reason, bacterial loads were measured relative to the amount of host genomic DNA.

For the amplification of host genomic DNA used for normalisation of bacterial copy numbers, the *GAPDH* housekeeping gene was chosen. Standard curves were designed using the pSAP1 plasmid construct consisting of an amplified portion of the human *GAPDH* gene. Reaction efficiencies may vary depending on the standard used. Amplifications using plasmid vectors are known to proceed with higher efficiency, in comparison to genomic DNA. As absolute quantification required knowing the whole genome size, a plasmid construct was used for definite template size. Standard curves showed excellent efficiency (E=1.04) and linearity (r^2=0.993), and the measure of gene copy numbers for each standard point correlated well with estimated values. However, to determine the extent of the difference in reaction efficiencies as a result of using a plasmid construct, a standard curve based on human genomic DNA should have been tested for comparison; although the use of genomic standards has previously been shown to be comparable with plasmid standards (Roussel *et al.*, 2007).

Overall, reliable standards are essential for accurate quantification. The accuracy of DNA standards can be affected by a number of factors including the process of

purification and the presence of PCR inhibitors that may be co-purified with DNA. It has been suggested that providing standards are prepared in the same manner as test samples, PCRs will proceed with the same efficiency, making for a more accurate quantification (Dumonceaux *et al.*, 2006).

A clear complication in quantification of *H. pylori* relative to host genomic DNA is that colonisation is restricted to the mucosal surface. Host genomic DNA accounts for the number of cells throughout the tissue sample and final quantification may therefore be affected by the variable depth of tissue biopsies. Ideally, the best method for quantification would be to measure bacterial load relative to an epithelial specific marker. However this would require quantification of messenger RNA, and measure of a constitutively expressed host gene, which is yet to be characterised. This reflects back to quantification of bacteria per epithelial cell number by histopathology.

H. pylori infection is known to upregulate inflammatory responses, increasing the number of PCR targets, which may also impact final quantification. Hence, RNA quantification may be a more accurate measure of host cells. However, a reference gene for which expression is unchanged during inflammation is essential. It is also recommended that more than one reference gene is used for each assay. Other genes that have previously been used include *β-actin* and *SART 1*, for which expression was found to be constitutive (Camilleri *et al.*, 2007).

3.3.2 Reproducibility of the quantitative assay

Reproducible quantification is essential to determine efficacy of the assay. Good intra- and inter-run reproducibility was observed for standard curves in this study. However, reproducibility between test samples must also be measured. Here, samples were tested twice in subsequent PCR reactions, and excellent accuracy was observed in bacterial load measured in each reaction, as was observed with standard curves. Reactions that were repeated whilst DNA samples were left on ice between reactions showed extremely agreeable results. However, after just a single freeze-thaw cycle, density of infection was much more variable between

successive reactions. Hence, samples were treated in a consistent manner for measurement of bacterial loads.

3.3.3 Methods used for the quantification of *H. pylori*

Histological analysis of *H. pylori* infection is widely used, although the use of the commonly applied Sydney system is semi-quantitative. Comparison of bacterial load measured by histology and qPCR did show a significant trend in antral samples between mild and severe colonisation. Although not statistically significant, the same trend was observed in the corpus. The lack of statistical significance may be due to small sample numbers. Previous comparative studies between the two quantitative methods showed a similar result. Kobayashi *et al.* also reported a significant correlation between mild and severe colonisation density in antral and corpus samples for a larger data set (Kobayashi *et al.*, 2002; Lascols *et al.*, 2003). The qPCR assay developed in the current study showed excellent reproducibility, implying that histology is a poor method for quantification, perhaps because only a small part or the mucosal surface is examined. However, reproducibility experiments for histology were not performed, which limits our ability to fully interpret the comparison.

Several studies have used histology to determine the patterns of colonisation density (Jonkers *et al.*, 1998; Louw *et al.*, 1993), described as antral-predominant, corpus-predominant and pan-colonisation. The accuracy of histology in comparison to qPCR in this regard was also investigated. A better correlation between histology and PCR was observed. This was most apparent when looking at antral predominant colonisation for which 80% concordance was observed. This illustrates that previous studies using histology should not be discounted.

Some studies have employed the use of 4 point grading systems in an attempt to improve quantification (Twisk *et al.*, 2001). Actual bacterial counts within a small field of the tissue cross section, normalised by the number of gastric epithelial cells, may prove more accurate. However, this may be time consuming as a number of different fields would have to be analysed for an accurate analysis. Accurate examination of slides is widely dependent on the expertise of the pathologist, and may be subjective. Studies have shown inter-observer variability

when evaluating *H. pylori* gastritis (Aydin *et al.*, 2003; El-Zimaity *et al.*, 1996). In the present study, all slides were examined by an individual, qualified pathologist to minimise variability in grading. Immunohistochemistry with a specific *H. pylori* antibody may also be used, and has been shown both to improve variability in results between pathologists in comparison to routine histology, and also proved to have higher sensitivity and specificity for detection (Camorlinga-Ponce *et al.*, 2003; Jonkers *et al.*, 1997; Orhan *et al.*, 2008).

Previously, bacterial load has been widely measured using culture (Chen *et al.*, 2001; Rad *et al.*, 2006), but was not considered in this study as in our laboratory, we frequently failed to culture *H. pylori* when other methods have shown it to be positive. Also, previous comparative studies have shown quantitative culture to be less sensitive in comparison to PCR, with a log difference in detected bacterial loads (Lascols *et al.*, 2003; Roussel *et al.*, 2007). One major limitation associated with quantitative culture is the reliance on viable bacteria. However, this does highlight a drawback to qPCR, in that this method does not account for dead bacteria in the gastric tissue on collection. Use of ethidium bromide monoazide (AEM) has been suggested for detection of viable cells alone by PCR, as AEM only penetrates dead cells, and covalently binds to DNA preventing amplification (Nogva *et al.*, 2003).

It is important to note that treatment with proton pump inhibitors (PPI) and antibiotics may affect bacterial load, and may result in false negative results with culture, histology, urea based tests and PCR. PPIs have a weak anti-bacterial effect (Figura *et al.*, 1997), and their action interferes with the pH of the stomach thus changing the local environment. In turn, the distribution of *H. pylori* in the gastric mucosa may be changed, in particular leading to disappearance of the bacterium in the antrum (Megraud *et al.*, 1991). This effect depends on the dose and length of treatment. Changes to the bacterial niche may also trigger a change from a typical morphology to a coccoidal form of *H. pylori*, which may also impact diagnostic testing, and would hence affect quantification. It has also been reported that PPIs may have antiurease properties that may interfere with urease

based testing (Tsuchiya *et al.*, 1995; Yakoob *et al.*, 2005), although this has not been shown in all studies conducted (Tang *et al.*, 2009).

Overall, an ideal quantitative system allows for accurate absolute quantification with excellent specificity, sensitivity and reproducibility. However, no individual system has been reported to fit such criteria. The system developed in the current study was highly specific for *H. pylori* detection. Excellent reproducibility was observed, both between replicates within a run, and between subsequent PCR reactions. Furthermore, the quantitative assay developed performed better in comparison to semi-quantitative histology.

As mentioned, quantitative tools are essential in a number of applications. In particular, qPCR can be used for determining factors that may affect colonisation in the gastric mucosa, including bacterial virulence factors and the host response to infection. This is the focus of the following chapters.

Chapter 4

4. THE RELATIONSHIP BETWEEN *H. PYLORI* COLONISATION DENSITY AND DISEASE.

4.1 INTRODUCTION

4.1.1 *H. pylori* colonisation density in the gastric mucosa

H. pylori-associated disease occurs in only a small proportion of the infected population and a number of factors are known to drive the progression of disease (Kusters *et al.*, 2006).

The development of specific disease profiles has been attributed to the patterns of gastritis and acid secretion in the stomach, with DUD associated with antral-predominant gastritis and hyperchlorhydria, whilst inflammation of the whole stomach and low acidity drives development of GUD (Figure 4.1).

H. pylori can induce changes to both acidity and inflammation by means of virulence factor expression, for instance the acid neutralising urease enzyme, and CagA which induces a proinflammatory host response. Gastric acid secretion appears to play a key role in colonisation by the bacterium. Logan *et al.* demonstrated that acid suppression resulted in a shift of *H. pylori* colonisation that was accompanied by increased bacterial load in the corpus and reduced acid secretion (Logan *et al.*, 1995). With this in mind, it is possible that the development of a particular ulcer disease may relate to localisation of the bacterium in the gastric mucosa. Previous studies have demonstrated such a relationship to exist, reporting an antral predominant pattern of colonisation with DUD, and corpus predominant colonisation with GUD, a result that was also observed in children (Jonkers *et al.*, 1998; Louw *et al.*, 1993). However, these reports differ with respect to the degree of colonisation measured in the antrum and the corpus for both DUD and GUD. Louw *et al.* (1993) reported antral colonisation to be more common in patients with DUD compared to GUD, with

no difference in corpus colonisation for either disease, suggesting that antral colonisation may act as a determinant of disease progression. However, they also showed that higher grades of colonisation were observed in the corpus of patients with GUD compared to those with DUD. Conversely, Jonkers *et al.* (1998) demonstrated increased bacterial load in the corpus for GUD compared to DUD. A later study by Tham *et al.* (2001) demonstrated increased bacterial colonisation in patients with DUD in comparison to those with GUD and gastritis in the antrum and corpus.

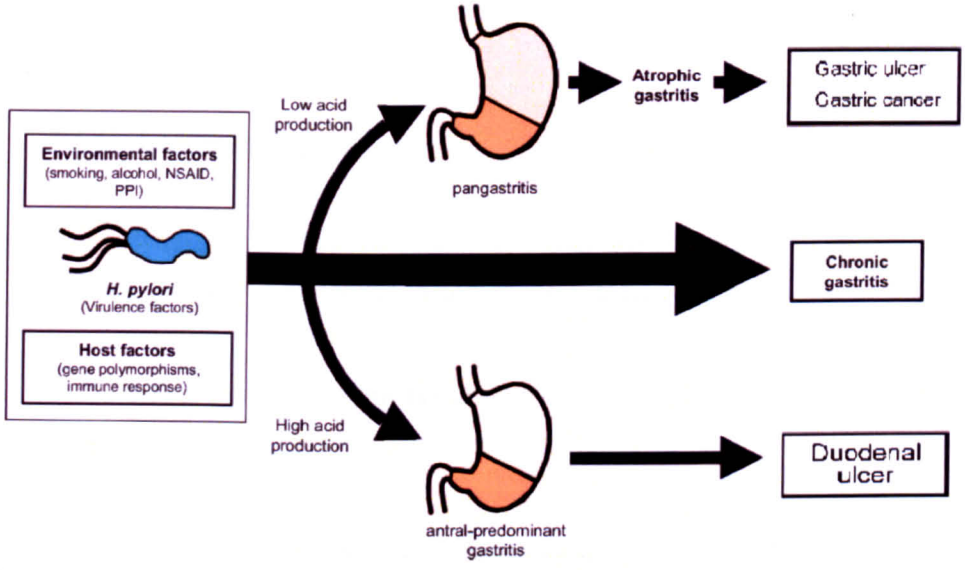


Figure 4.1: Factors that that drive disease outcome in *H. pylori* infection.
A combination of environmental, bacterial and host factors contribute to the pattern of gastritis that develops in response to *H. pylori* infection. An antral-predominant gastritis has been associated with the development of peptic ulcer disease, whereas a pan-gastritis is known to increase the risk of developing gastric cancer (Adapted from Kusters *et al.*, 2006).

H. pylori infection is an important risk factor for the development of gastric adenocarcinoma. The progression of *H. pylori* associated malignant disease follows changes in the gastric mucosa that involve stages of gastritis, atrophy and

intestinal metaplasia of the gastric mucosa which may develop into gastric adenocarcinoma (Correa, 1995; Fox & Wang, 2001). The appearance of atrophy and IM are associated with a change in the local environment that disfavours bacterial growth and colonisation is often not detectable in patients that have developed gastric cancer (Peek & Blaser, 2002). The degree of gastric atrophy and the presence of intestinal metaplasia (IM) have indeed been found to negatively correlate with bacterial density (Kekki *et al.*, 1991; Kuipers *et al.*, 1995c; Valle *et al.*, 1996). Presence of this pathology was also found to relate to peptic ulcer disease, with reduced occurrences of both atrophy and IM in the antrum of patients diagnosed with DUD compared to GUD (Louw *et al.*, 1993).

4.1.2 Experimental aims

- To determine the density of the bacterium and the pattern of colonisation for patients with DUD, GUD and no ulcer disease.

- To assess the relationship between density of infection and premalignant pathology.

- To assess the relationship between patterns of gastritis and the pattern of *H. pylori* colonisation.

4.2 RESULTS

4.2.1 Determination of *H. pylori* colonisation in the gastric mucosa in patients with differing disease status.

Patterns of gastritis and acid secretion are associated with particular disease profiles, and localisation of the bacterium in the stomach has previously been demonstrated to correlate with disease (Jonkers *et al.*, 1998; Kato *et al.*, 2006; Louw *et al.*, 1993). However, the question of how such patterns of colonisation arise still remains (Kato *et al.*, 2006; Louw *et al.*, 1993).

To further investigate the degree of *H. pylori* colonisation in the gastric mucosa, density was determined by qPCR (as described in Chapter 3) in gastric tissue. Bacterial load is given as *16SrRNA/GAPDH* gene copy numbers unless stated otherwise. Samples were collected from the antrum and corpus of 41 *H. pylori* infected patients. Of these samples, disease status was available for 36 patients, of which 11 presented with duodenal ulcer disease (DUD), 5 with gastric ulcer disease (GUD), and 20 with gastritis alone.

No statistical difference was observed for bacterial load measured in the antrum (gastritis: median= 0.36, n=20, IQR=0.17-0.63; DUD: median=0.37, n=11, IQR=0.21-1.19; GUD: median=0.5, n=5, IQR=0.25-1.38). However, a significantly higher density was observed in the corpus for GUD (median=1.82, n=5, IQR=1.06-6.12) compared to DUD (median=0.14, n=11, IQR=0.07-0.33; p=0.02), and gastritis (median=0.38, n=20, IQR=0.12-1.02; p=0.01) (Figure 4.2). In addition, much lower bacterial loads were measured in the corpus of patients with DUD compared to those with gastritis alone, although not statistically significant. The overall level of *H. pylori* colonisation between all antral (median=0.34, n=41, IQR=0.17-0.71) and corpus biopsies (median=0.25, n=41, IQR=0.08-1.22) of the study population were not statistically significant. Together, these data suggest that the degree of colonisation in the corpus, but not the antrum, may have an impact on the clinical outcome of infection.

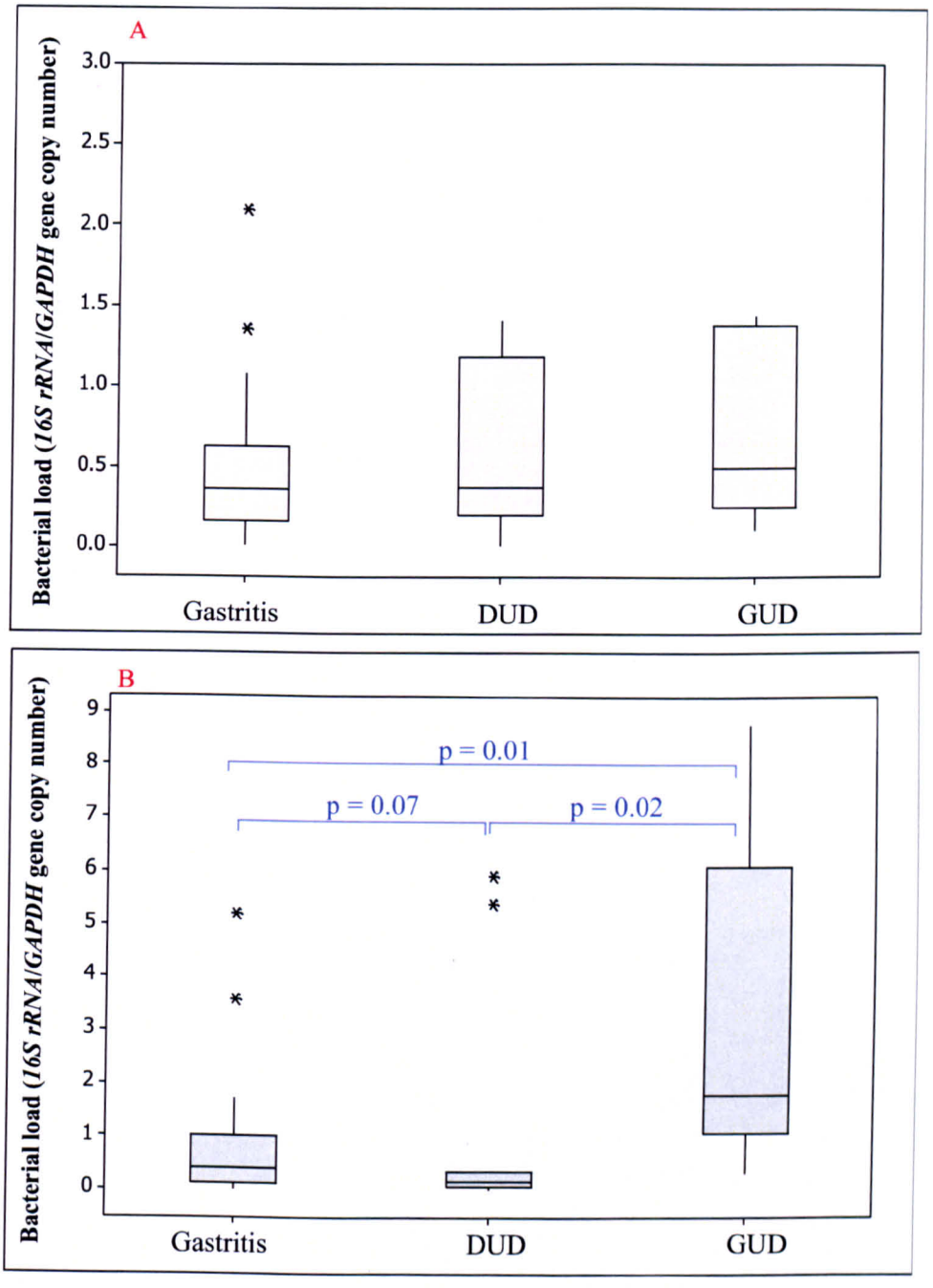


Figure 4.2: The association between bacterial load and disease status.

Bacterial load was measured by qPCR in gastric tissue taken from A- the antrum and B- the corpus of each patient. Colonisation density was stratified according to whether patients presented with gastritis (n=20), DUD (n=11), or GUD (n=5). *H. pylori* genomic DNA copies were normalised for human *GAPDH* gene copy number. * indicates outlier values.

The effect of higher corpus bacterial loads became more apparent when bacterial density was represented as an antrum:corpus ratio. The pattern of colonisation for patients with DUD favoured antral predominance (72% of patients, median=2.10, n=11, IQR=0.99-3.77), whereas a corpus predominant pattern was observed with GUD (100% of patients, median=0.37, n=5, IQR=0.08-0.76). The group of patients with gastritis did not appear to favour a particular distribution, however the median reflected pan-colonisation of the gastric mucosa (median=1.00, n=20, IQR=0.37-2.44) (Figure 4.3).

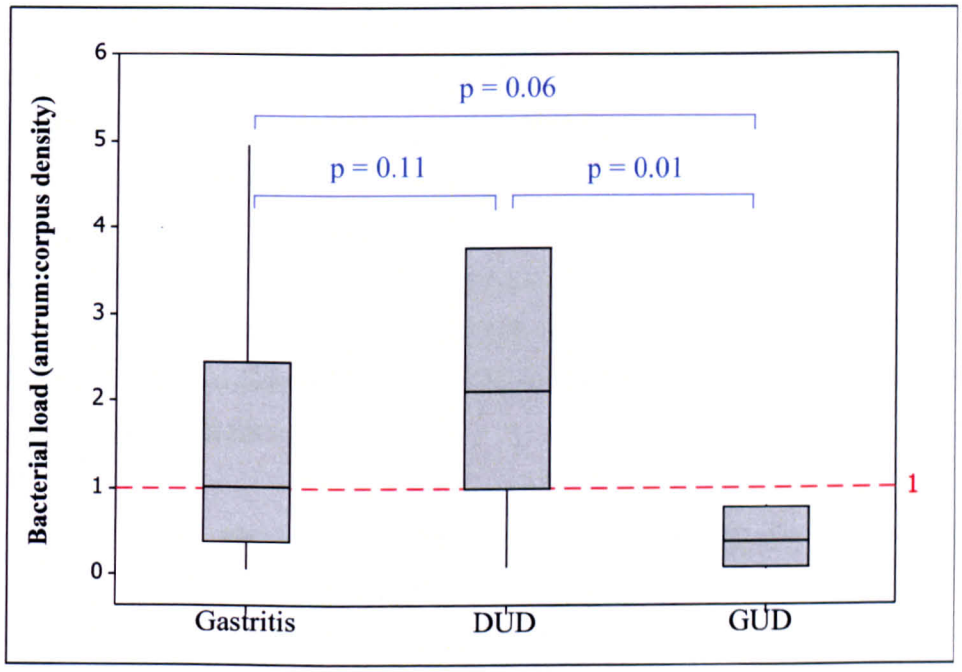


Figure 4.3: The pattern of *H. pylori* colonisation densities observed in the gastric mucosa of patients with gastro-duodenal disease.

Bacterial load was measured in gastric biopsies taken from the antrum and corpus of 20 patients with gastritis, 11 with DUD and 5 with GUD. *H. pylori* genomic DNA copy numbers were normalised for human *GAPDH* gene copy number. The pattern of colonisation was described as the antrum:corpus ratio of bacterial density measured by qPCR. A reference line at 1 on the y-axis indicates equal colonisation in both regions of the stomach. An antral predominant colonisation was described as having a ratio of density greater than 1, and a corpus predominant colonisation described as having a density lower than 1.

4.2.2 The relationship between *H. pylori* colonisation and pre-malignant pathology

To further investigate the relationship between bacterial colonisation and *H. pylori*-associated gastroduodenal disease, the association with premalignant pathology was also assessed as no patients in the current study were found to have gastric cancer. For this, the degree of atrophy and intestinal metaplasia (IM) was measured in antral and corpus tissue sections from individual gastric biopsies using the Sydney scoring system.

Analysis of bacterial loads for each grade of atrophy and IM was not possible as there were too few samples in each group. Instead, the relationship with bacterial load was assessed in the presence (scores 1, 2 and 3) or absence (score 0) of these features. As only 4 patients were found to have atrophy in the corpus, only data on the antrum was assessed. Trends showed higher *H. pylori* density in the antrum with the presence of atrophy (presence of atrophy: median=0.50, n=11, IQR=0.22-0.75; absence of atrophy: median=0.30, n=27, IQR=0.17-0.67) and interestingly with IM too (presence of IM: median=0.45, n=8, IQR=0.13-0.65; absence of IM: median=0.34, n=30, IQR=0.17-0.62). However, these were not statistically significant.

4.2.2.1 The use of serum biomarkers for assessing the relationship between gastric atrophy and bacterial load

Levels of serum gastrin-17 and pepsinogen have been previously used as biomarkers for the degree of gastric atrophy (di Mario & Cavallaro, 2008; Vaananen *et al.*, 2003). G-17 has previously been used to identify atrophic antral gastritis. As G-17 is produced by G cells in the antrum, the loss of glands when atrophy develops correlates with a reduction in G-17 levels (Vaananen *et al.*, 2003). Serum pepsinogen I (sPGI) and pepsinogen II (sPGII) levels have previously been used to assess the degree of atrophy in the corpus (di Mario & Cavallaro, 2008). Pepsinogen is a precursor of the digestive enzyme pepsin, and is secreted in two forms, PGI and PGII. PGI is secreted by chief and mucous neck cells in the corpus. PGII is also produced in these cells, as well as cells in the cardiac, pyloric and duodenal Brunner gland cells, and therefore the distribution

of PGII secretion is widespread through the entire stomach. Serum PGI:PGII levels are often used to assess atrophy in the corpus (Samloff, 1971; Samloff & Liebman, 1973). Reduced levels of serum PGI:PGII are observed with corpus atrophy, which results from a loss of PGI secreting glands.

To assess the degree of atrophy in the antrum in more depth, the level of serum gastrin-17 (G-17) was determined. sG-17 levels were assessed in 60 patients, and atrophy was assessed by histology. sG-17 levels were significantly decreased when atrophy was present (median=7.67, n=25, IQR=4.90-16.0) compared to when atrophy was absent (median=14.7, n=23, IQR=10.5-23.7) (p=0.02) (Figure 4.4). Bacterial loads measured by qPCR were therefore assessed against the level of serum G-17. Data on bacterial load and G-17 levels were available for 16 samples, however an association was not found between the two in the antrum (r= -0.30, p=0.24).

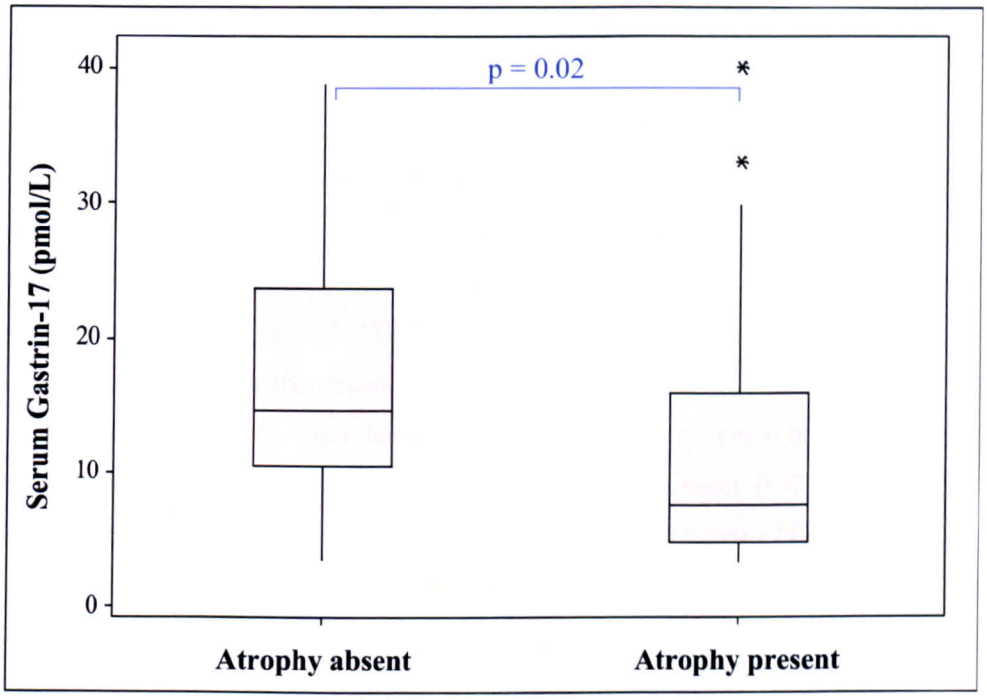


Figure 4.4: Association between serum gastrin-17 levels and presence of atrophy in the antrum.

Serum gastrin-17 levels were measured in 60 patients to assess the relationship with gastric atrophy in the antrum. Atrophy was diagnosed by histopathology, and patients were grouped according to whether atrophy was absent (n=23) or present (n=25). * represents outlier values.

As only 4 patients in the data set examined were found to have atrophy in the corpus, it was not possible to determine if the level of serum pepsinogen was associated with atrophy. The level of sPGI, sPGII and PGI:PGII was determined, and their relationship with bacterial load was assessed.

No correlation was observed between the level of sPGI and bacterial density (density in the antrum: r=0.07, p=0.74; density in the corpus: r=0.37, p=0.09, antral:corpus ratio of colonisation: r=0.05, p=0.81). Similarly, no relationship was observed between levels of sPGII and density (antrum: r= -0.17, p=0.43; corpus: r= -0.04, p=0.84; antrum:corpus ratio: r= -0.09, p=0.70).

Serum PGI/PGII levels were determined in 21 patients for which bacterial density was also available. No correlation was observed in the antrum (r=0.20, p=0.36) or corpus (r=0.16, p=0.45). However, when looking at the pattern of colonisation, a positive correlation was observed with PGI/PGII levels (r=0.51, p=0.02) (Figure 4.5).

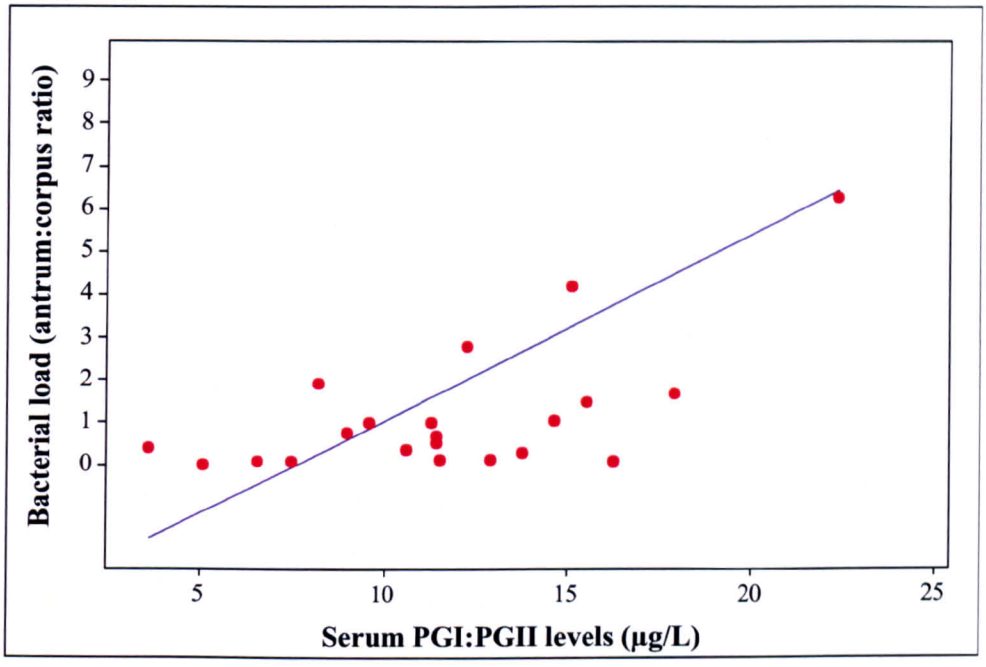


Figure 4.5: Association between patterns of colonisation and serum PGI:PGII levels.

Serum PGI:PGII levels were measures in 21 *H. pylori* infected patients. For these patients, the pattern of *H. pylori* colonisation was determined in gastric biopsies using qPCR. A linear trendline is shown with an r value of 0.51. Apositive correlation between sPGI:PGII and the pattern of colonisation was found (p=0.02).

4.3 DISCUSSION

It is well established that the patterns of gastritis and acid secretion in the stomach are key determinants for the progression of specific disease profiles. Certain factors, both host and bacterial, are known to drive the progression of disease (reviewed by Blaser & Atherton, 2004), but whether colonisation density plays a role is still unclear.

4.3.1 Determination of *H. pylori* colonisation in the gastric mucosa in patients with differing disease status

Colonisation of the gastric mucosa was found to be significantly greater for patients with GUD compared to patients with DUD and gastritis in the corpus, but not in the antrum where bacterial loads were very similar for each patient group. Furthermore, lower bacterial loads were observed in patients with DUD compared to those with gastritis alone. This suggests that disease progression is related to the degree of colonisation in the corpus but not the antrum. Also, antral-predominant colonisation was associated with DUD, whilst corpus-predominant colonisation was found to be associated with GUD. Although a much greater spread in the pattern of colonisation density was observed for patients with gastritis, the median was 1, which represents a pan-colonisation. Notably, this group also represents patients that may be at risk of developing peptic ulcer disease but did not present with ulceration at the time of endoscopy, which this may explain the wide spread of data within the gastritis group.

Previously, an association between the pattern of colonisation and disease status has been reported, and in agreement with Jonkers *et al.*, (1998) the present study demonstrated that corpus colonisation plays a role in determining disease outcome. As known, DUD is linked with an antral-predominant gastritis, which is associated with an increase in acid secretion in the corpus (El-Omar *et al.*, 1995; Graham *et al.*, 1993). This may result in a less hospitable environment and reduced corpus colonisation under such conditions. However, *H. pylori* infection and inflammation in the corpus suppresses acid secretion, and may create a niche capable of supporting bacterial growth. The relationship between patterns of

inflammation and acid secretion may explain the association between corpus colonisation and disease status observed in this study.

Although a relationship was observed between bacterial density and clinical outcome of infection, patients with gastric adenocarcinoma were not represented in this population. Atrophy and IM were assessed however as they represent precancerous changes in the mucosa, and provide an opportunity to investigate host-bacterial relationships prior to the development of cancer. As the development of such pathology alters the gastric environment and no longer supports *H. pylori* colonisation, a negative correlation would be expected with density of infection. Surprisingly, a trend towards a positive association between density and the presence of atrophy and IM was observed, although not statistically significant. It is possible that the level of atrophy and IM was mild, and only when severe, the loss of bacterial cells is seen. Therefore, increased bacterial load could be associated with the development of mild precancerous changes in the gastric mucosa.

Also, histological analysis of IM demonstrates bacterial cells are present except in the vicinity of goblet cells that are characteristic of IM. Unless IM is widespread, the relationship with bacterial load may be masked as bacterial load, when measured by qPCR, used a whole individual tissue biopsy. Assessing the relationship between bacterial load and IM may therefore be more accurately achieved by histology, and the examination of multiple slides from several different tissue samples is necessary for accurate determination of the degree of IM. This was not possible for samples tested in this study as few patients were diagnosed with IM. Of interest may be the use of narrow band imaging, which allows for magnification of the gastric mucosa during endoscopy. This technique assesses the loss of the normal subepithelial capillary network for diagnosis of atrophy, and has been successfully utilised for accurate diagnosis of gastric atrophy (Mannath & Ragunath, 2010; Tahara *et al.*, 2009). With this technique, bacterial density can be assessed in gastric biopsies taken from areas of known atrophy and IM.

Given the association between G-17 levels and antral atrophy, bacterial load was assessed against serum gastrin levels in order to further investigate the relationship with atrophy. However, no association was observed. Serum gastrin levels have previously been shown to negatively correlate with bacterial load in the antrum and corpus (Karttunen *et al.*, 1991), although this was not the case in all studies (Perez-Paramo *et al.*, 1997). Increased sample numbers would be needed in order to investigate this relationship further.

For investigation of corpus atrophy, the levels of serum pepsinogen were assessed. Decreased sPGI:PGII levels would be expected with atrophy. A positive association was observed between sPGI:PGII and the pattern of bacterial colonisation. This may indicate that high corpus colonisation may lead to changes in the corpus, possibly cell damage and loss of cell function, that results in reduced levels of sPGI, and hence sPGI:PGII levels. In contrast, antral-predominant colonisation may induce changes in the antral gastric mucosa that leads to a reduction in sPGII levels, and consequently increased sPGI:PGII levels. Therefore, this data suggests that bacterial colonisation may impact cell secretions in the gastric mucosa, but whether this is a result of cell damage and glandular atrophy is unclear from the data presented in this study.

Overall, patterns of colonisation in the gastric mucosa were found to be associated with disease. However, whether bacterial or host factors play a role in influencing this relationship is not clear.

Chapter 5

5. THE RELATIONSHIP BETWEEN *H. PYLORI* COLONISATION DENSITY AND VIRULENCE DETERMINANTS OF THE INFECTING STRAIN.

5.1 INTRODUCTION

5.1.1 *H. pylori* colonisation density and virulence determinants.

The relationship between *H. pylori* and host responses is complex and bacterial factors have pleiotropic effects (Chapter 1, section 1.6).

It is well known that *cagA* has been associated with the development of peptic ulcer disease and gastric cancer via a number of cellular responses to the cytotoxin and *cag* PAI-encoded type IV secretion system (Blaser *et al.*, 1995; Nomura *et al.*, 2002). For instance, CagA is known to interact with cellular proteins involved in maintaining the integrity of epithelial cell junctions. The adherence of *H. pylori* at cell junctions was associated with the relocalisation of ZO-1 and JAM to the site of bacterial attachment resulting in disruption of the cell junction. Such interactions were found to not only be dependent on CagA, but also related to the EPIYA-repeat region of the gene and not tyrosine phosporylation of the motif (Amieva *et al.*, 2003; Higashi *et al.*, 2005). Loosening of these tight junctions may result in leakage of essential nutrients that may aid in bacterial growth.

Observations of these CagA interactions highlight the close association between *H. pylori cagA* positive strains with the gastric epithelium. *cagA* positive strains have been reported to colonise at the surface of the cell and in the intercellular epithelial spaces more so than in the mucous gel (Camorlinga-Ponce *et al.*, 2004). Such intimate associations between the bacterium and the host cell have been associated with reduced colonisation densities (Twisk *et al.*, 2001), and these

observations might suggest reduced bacterial densities may result from infection with bacteria harbouring the cytotoxin.

The relationship between *cagA* and bacterial load has been studied with varying results. In those studies that observed a correlation, presence of the virulence factor was actually associated with increased colonisation, although this finding was controversial with respect to the localisation in the stomach. Some studies reported such a relationship in the corpus, whilst others found this to be true only for the antrum (Gunn *et al.*, 1998; Umit *et al.*, 2009). An association was also observed between increased density and increasing numbers of EPIYA repeat regions (Basso *et al.*, 2008), and increased bacterial burden with *cagA* positive strains was observed in children (Dzierzanowska-Fangrat *et al.*, 2002; Homan *et al.*, 2009). Importantly, a number of studies have failed to show a significant association (Demirturk *et al.*, 2001; Kaklikkaya *et al.*, 2006). One study however demonstrated reduced density with *cagA* positive strains in comparison to *cagA* negative strains (van Doorn *et al.*, 2000).

cagA positive strains also have a demonstrated function in regulating gastric acidity. Transcription levels of *cagA* were shown to increase at low pH, which suggests a role for the cytotoxin in limiting acid stress (Karita *et al.*, 1996; Karita & Blaser, 1998). Interestingly, *cagA* was found to aid colonisation of the corpus and increase acidity in a gerbil model (Rieder *et al.*, 2005). These observations suggest that *cagA* is important in regulating acid output, permitting increased colonisation of the corpus, and consequently altering the pattern of bacterial colonisation in the stomach. The resulting inflammatory response and mucosal damage that follows in the corpus may provide a means by which this cytotoxin induces the development of gastric adenocarcinoma.

A multitude of functions have been described for VacA which may impact on colonisation of *H. pylori*. The vacuolating activity of this cytotoxin is ascribed to its ability to form selective channels within biological membranes. Pore formation is related to toxicity of VacA, (Atherton *et al.*, 1995; Letley *et al.*, 2003; Rhead *et al.*, 2007). Channel formation is not restricted to internal compartments, and the formation of pores at the surface membrane of the epithelial cell may result in the

release of essential nutrients into the lumen, including pyruvate that has been demonstrated to aid the growth of the bacterium (Testerman *et al.*, 2006; Tombola *et al.*, 1999). With this in mind, strains harbouring the more toxic form of VacA may be associated with increased bacterial density as a result of cell leakage. Urea also becomes available as a result of increased membrane permeability, which aids in neutralising the local acid pH environment and survival of the bacterium. Importantly, neutralising the pH environment may also assist in colonisation of the acid producing corpus, thus having effects on bacterial localisation in the stomach as well as density. Increased corpus colonisation may provide a possible driving force for the development of GUD. VacA association with disease is well established with the more toxic variants, although some questions remain about the impact of moderate toxic forms of VacA, as some are associated with the development of gastric cancer (Basso *et al.*, 1998; Evans *et al.*, 1998). The recent discovery of the polymorphic i-region has been linked to the development of gastric cancer with these strains, and its association with increased vacuolating activity may also implicate this region in bacterial colonisation density (Rhead *et al.*, 2007).

As seen with *cagA*, the observed relationship between bacterial load and polymorphisms of *vacA* is mixed amongst studies. A number of studies have failed to find a relationship with s- and m-type of *vacA* with colonisation density (Atherton *et al.*, 1996; Elitsur *et al.*, 1999; Ko *et al.*, 2008; Soltermann *et al.*, 2007; Umit *et al.*, 2009). However, many studies have demonstrated an association between increased *H. pylori* density and s1 genotypes of the toxin in comparison to the less toxic s2 type (Homan *et al.*, 2009; Nogueira *et al.*, 2001; Tham *et al.*, 2001). Mid-region polymorphisms have been related to increased bacterial burden, where increased load was seen with more virulent forms of the toxin (Han *et al.*, 1999). Conversely, some studies observed increased density with s2 and m2 strains (Nogueira *et al.*, 2001; van Doorn *et al.*, 2000). Again, the question of whether these findings are restricted to either the antrum or the corpus, or both, remains unclear. The relationship between i-region polymorphisms and colonisation density has not previously been investigated.

The recently discovered *dupA* has been described as an independent determinant in the development of DUD and protection against the development of GUD and adenocarcinoma (Lu *et al.*, 2005; Zhang *et al.*, 2008). Little is known about the function of this virulence factor, although induction of an inflammatory response has been demonstrated. Based on previously described associations between an antral-predominant gastritis and colonisation with the development of DUD (Kuipers *et al.*, 1995b), it is possible that the observed association between *dupA* and DUD may result from a preferential colonisation of the antrum.

Overall, it is clear from previous observations that relationships between bacterial density and virulence factor genotype of the colonising strain still remain unclear, with both positive and negative findings reported in different populations.

As previously mentioned, disease progression is widely observed with virulent strains of the bacterium. Although most studies have reported a positive correlation between the presence of both atrophy and IM with *cagA* (Basso *et al.*, 2008; Kaklikkaya *et al.*, 2006; Nogueira *et al.*, 2001; Soltermann *et al.*, 2007; Umit *et al.*, 2009), the relationship between *vacA* subtypes is not as clear, with some studies reporting an association (Basso *et al.*, 2008; Nogueira *et al.*, 2001; Soltermann *et al.*, 2007), whilst others have not (Kaklikkaya *et al.*, 2006; Umit *et al.*, 2009).

5.1.2 Experimental aims

- To investigate the relationship between virulence factors of the bacterium and colonisation, including *dupA*, *cagA*, and *vacA in vivo*.

- To determine the impact of the major virulence determinants CagA and VacA on colonisation of gastric epithelial cells *in vitro*.

- To determine the relationship between the presence of virulence factors and the clinical outcome of infection.

5.2 RESULTS

5.2.1 Associations between bacterial virulence and colonisation density.

Increased virulence properties of the colonising bacterial strain are known risk factors for in the development of gastric disease during *H. pylori* infection (Atherton, 2006).

The major virulence factors CagA and VacA are known to elicit numerous cellular responses that have varying effects. Previous reports investigating the association between these virulence factors and colonisation density conflict (Gunn *et al.*, 1998; Han *et al.*, 1999; Nogueira *et al.*, 2001; Umit *et al.*, 2009; van Doorn *et al.*, 2000). The presence of the *dupA* gene has been linked to the development of DUD (Lu *et al.*, 2005). As antral predominant gastritis and colonisation have been reported as factors contributing to the development of DUD (Kuipers *et al.*, 1995b), the presence of *dupA* may be associated with the localisation of the bacterium in the gastric mucosa. Therefore, the association between bacterial factors and colonisation was investigated.

To determine the virulence status of the colonising strain, bacterial isolates cultured from antral biopsies were genotyped by PCR for the presence of *dupA* and *cagA*, and for *vacA* allelic type. Bacterial loads were measured by real-time PCR. Genotyping data was available for 24 isolated strains for *cagA* status, 23 for *dupA* status, and 26 for *vacA* status. Typing data for some strains was previously obtained by other members of the group using DNA extracted from bacterial cultures. However, not all genes were typed at the same point in time. As strains were often difficult to re-isolate from tissue samples, in cases where bacterial cultures were no longer available, it was not possible to obtain DNA for further typing. Therefore, data for all genes was not available, resulting in genotype numbers differing for each gene.

For the population examined, most strains were *cagA* positive (70.8% of strains). No association between *cagA* status and bacterial density was observed in either the antrum or the corpus. Furthermore, no apparent difference in the pattern of colonisation was observed (Table 5.1).

When assessing *dupA* status, bacterial load was not found to differ significantly between *dupA*+ and *dupA*- bacterial strains in the antrum or the corpus. However, when looking at the distribution of bacteria within the gastric mucosa, patients infected with *dupA*+ strains of *H. pylori* demonstrated a tendency towards an antral predominant colonisation whereas infection with *dupA*- strains preferentially colonised the corpus (Table 5.1). This is represented graphically in Figure 5.1.

To assess the relationship between *vacA* and bacterial load in the gastric mucosa, all 3 polymorphic regions were analysed individually to ascertain if a particular region of the gene was associated with differences in colonisation density. *vacA* was successfully genotyped in 26 strains for all three polymorphic regions. The *vacA* s1 genotype was more common than *vacA* s2 in the population examined (85% of strains). Overall, no difference was observed in bacterial loads between *vacA* allelic types of the colonising strain. A trend towards increased density with m1 type strains in the corpus was found, however this did not achieve statistical significance. Most variability in bacterial density was found in the corpus for the m- and s-region, whilst density measured in the antrum was very similar with all allelic types of each *vacA* region (Table 5.1).

Strains harbouring the more virulent form of the *vacA* m-region, m1, are known to be significantly more likely to also have the s1 and i1 genotype (Rhead *et al.*, 2007). As well as investigating the relationship of the individual regions of *vacA* with bacterial density, the entire polymorphic *vacA* gene was therefore also considered. Strains were classified based on the toxicity of VacA, with s1/i1/m1 type strains considered to be the most toxic, and s2/i2/m2 types the least. Strains with all other combinations of these alleles were referred to as mixed. As only 2 patients in this data set were infected with an s2/i2/m2 type strain, analysis of these data was not possible. A higher degree of colonisation was seen with s1/i1/m1 strains in comparison to mixed strains for the antrum (s1/i1/m1: median= 0.39, n=11, IQR=0.21-0.75; mixed: median=0.22, n=12, IQR=0.13-0.37) and corpus (s1/i1/m1: median=0.32, n=11, IQR=0.11-2.63; mixed: median=0.11, n=12, IQR=0.04-0.98). However, these differences did not quite reach statistical significance (p=0.06). No association was found between the pattern of

colonisation and *vacA* strain type (s1/i1/m1: median=1.01, n=11, IQR=0.11-2.10; mixed: median= 1.00, n=11, IQR=0.07-6.23) suggesting that *vacA* virulence may have an impact on the density of infection but not the pattern of colonisation.

	Bacterial load		
	Antrum	**Corpus**	**Antrum:Corpus**
***cagA*+** median (n)	**0.27** (17)	**0.14** (17)	**1.00** (17)
IQR	0.17-0.62	0.07-1.70	0.24-2.83
***cagA*-** median (n)	**0.21** (7)	**0.20** (7)	**0.67** (7)
IQR	0.01-0.57	0.08-0.85	0.08-2.76
P value	NS	NS	NS
***dupA*+** median (n)	**0.34** (13)	**0.11** (13)	**2.10** (13)
IQR	0.21-0.58	0.07-0.68	0.31-5.21
***dupA*-** median (n)	**0.26** (10)	**0.20** (10)	**0.07** (10)
IQR	0.17-1.04	0.13-1.18	0.07-1.32
P value	NS	NS	**0.03**
***vacA* s1** median (n)	**0.30** (22)	**0.20** (22)	**1.00** (22)
IQR	0.17-0.62	0.08-1.48	0.10-3.87
***vacA* s2** median (n)	**0.39** (4)	**0.13** (4)	**1.46** (4)
IQR	0.21-0.96	0.07-0.85	0.82-2.48
P value	NS	NS	NS
***vacA* m1** median (n)	**0.34** (13)	**0.31** (13)	**1.00** (13)
IQR	0.17-0.75	0.11-2.63	0.24-2.10
***vacA* m2** median (n)	**0.33** (13)	**0.16** (13)	**1.00** (13)
IQR	0.17-0.63	0.07-1.02	0.13-4.18
P value	NS	NS	NS
***vacA* i1** median (n)	**0.31** (14)	**0.28** (14)	0.87 (14)
IQR	0.16-0.69	0.08-1.67	0.10-2.52
***vacA* i2** median (n)	**0.30** (12)	**0.27** (12)	**0.84** (12)
IQR	0.10-0.60	0.08-1.31	0.16-2.48
P value	NS	NS	NS

Table 5.1: The relationship between *H. pylori* virulence determinants and bacterial density.

Bacterial isolates were genotyped for *cagA*, *dupA* and *vacA* status, and median bacterial densities in antral and corpus gastric biopsies are given for each genotype. IQR= inter quartile range.

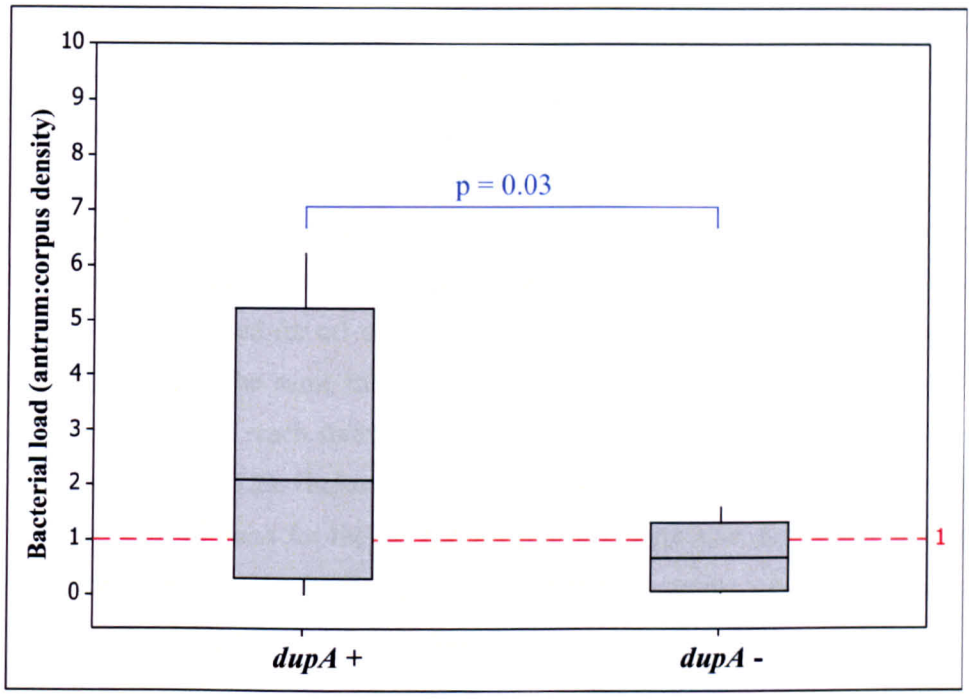


Figure 5.1: *dupA* status of the colonising strain on and the pattern of colonisation in the human gastric mucosa.

The ratio of bacterial load for colonisation with *dupA*+ and *dupA*- strains is shown. A reference line of 1 indicates equal colonisation density in both the antrum and corpus.

As *dupA* status was shown to be linked to antral-predominant patterns of colonisation, data were subdivided into groups controlled for the *dupA* status of the colonising strain. *dupA* status was successfully typed for 29 strains that were also genotyped for *cagA*, and 24 strains that were also typed for *vacA*.

No significant difference was observed between density of infection and *cagA* status when controlled for *dupA*. Within the *dupA*+ group, increased colonisation density was observed for m1 compared to m2-type strains in the corpus (Table 5.2 and Figure 5.2). The same trends were observed with the i-region, however this difference failed to reach statistical significance. Such a trend was not observed in the antrum (Table 5.2). Higher colonisation densities were observed with the more virulent types of *vacA* for the m- and i-region that were also *dupA*-, but this was not statistically significant (Table 5.2). As only 1/12 strains were typed as *vacA* s1, analysis of this region was not possible.

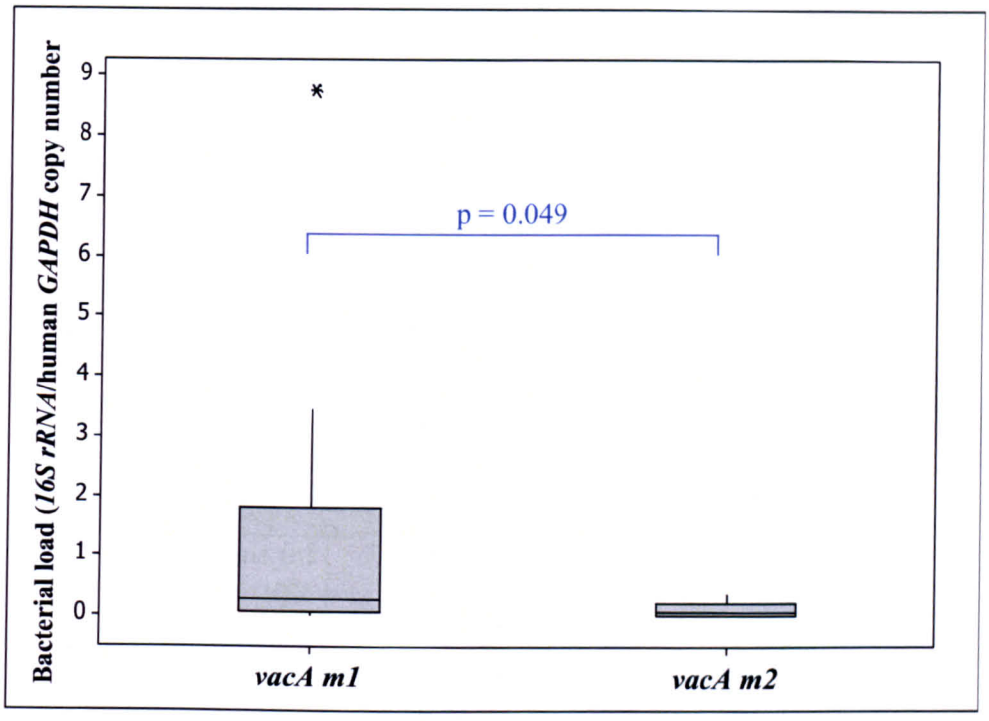


Figure 5.2: Relationship between the polymorphic *vacA* m region and bacterial density in the corpus for *dupA*+ strain infections.

Bacterial colonisation densities were measured in gastric biopsies taken from the corpus that were infected with *dupA*+*vacA* m1 and m2 strain genotypes. * represents outlier values.

	Median bacterial load	
	Antrum	**Corpus**
dupA+vacA:		
m1 (n)	**0.36** (8)	**0.28** (8)*
IQR	0.22-0.62	0.07-1.82
m2 (n)	**0.34** (5)	**0.07** (5)
IQR	0.09-0.83	0.02-0.23
i1 (n)	**0.34** (9)	**0.25** (9)
IQR	0.19-0.39	0.03-1.27
i2 (n)	**0.34** (3)	**0.08** (3)
IQR	0.21-0.38	0.08-0.30
dupA-vacA:		
m1 (n)	**0.74** (4)	**1.00** (4)
IQR	0.05-4.41	0.12-4.50
m2 (n)	**0.26** (8)	**0.50** (8)
IQR	0.18-0.82	0.13-1.10
i1 (n)	**0.74** (4)	**1.00** (4)
IQR	0.05-4.41	0.12-4.50
i2 (n)	**0.22** (7)	**0.50** (8)
IQR	0.15-0.57	0.13-1.10

Table 5.2: Relationship between *vacA* polymorphisms and bacterial load controlled for *dupA* genotype.

Bacterial density was assessed for *vacA* m- and i-region alleic strain types when corrected for *dupA* status. Median bacterial loads in each group are shown. IQR=interquartile range. * p=0.049.

5.2.2 *In vitro* analysis of the relationship between *H. pylori* virulence and density of infection

To assess the relationship between virulence determinants of *H. pylori* and bacterial burden, bacterial density was determined for the *H. pylori* 60190 wild type strain and its *cagA*, *vacA* and *cagE* isogenic deletion mutants. *cagE* mutants render the Cag PAI non-functional. The non-toxic Tx30a (*cagPAI-*, *vacA* s2/i2/m2) was also examined. Strains were cultured with AGS cells in 6 replicate wells, and bacteria were plated after 24hrs in order to determine average CFU counts. Differences in CFU counts between isogenic deletion mutants and wild type strains were not statistically significant (Figure 5.3).

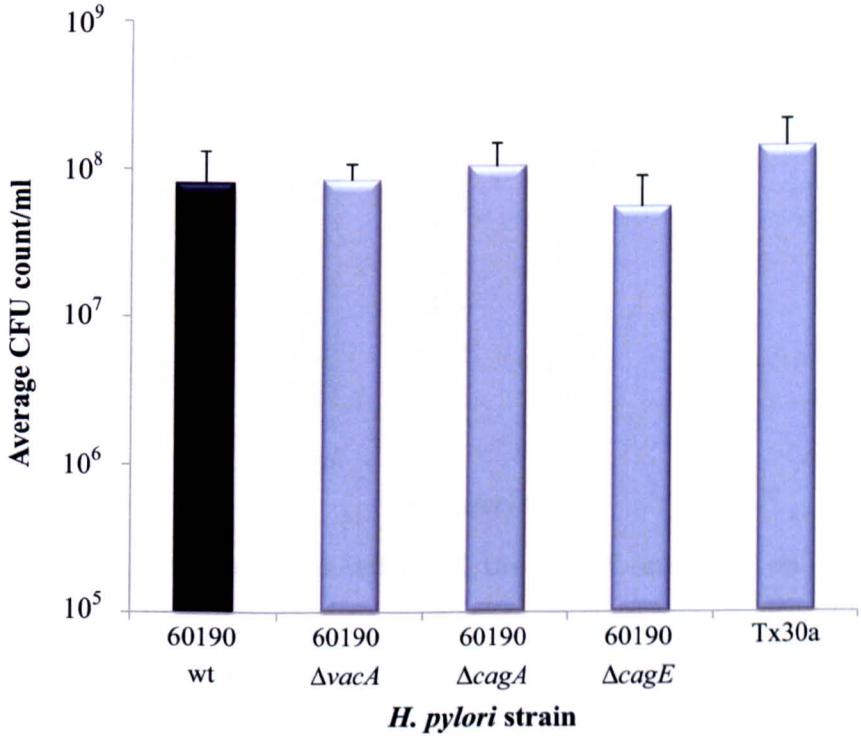


Figure 5.3: *In vitro* analysis of *cagA* as a determinant of bacterial density. AGS cells were cultured with *H. pylori* 60190wt, 60190Δ*vacA*, 60190Δ*cagA*, 60190Δ*cagE*, or Tx30a for 24hrs. Cells were plated onto blood agar plates after at each time point, and CFU counts were enumerated. Each bar is representative of average CFU counts from 6 replicates. Error bars represent standard deviation.

Although statistically non significant, lower CFU counts were observed at 24 hours with *H. pylori* 60190Δ*cagE*. To rule out experimental error, Δ*cagE* mutants in the *H. pylori* 84183 and 11637 strain backgrounds were also cultured along with wild type strains. However, no difference in CFU counts was observed between Δ*cagE* mutant and wild type in any of the three strain backgrounds at 24hrs (Figure 5.4).

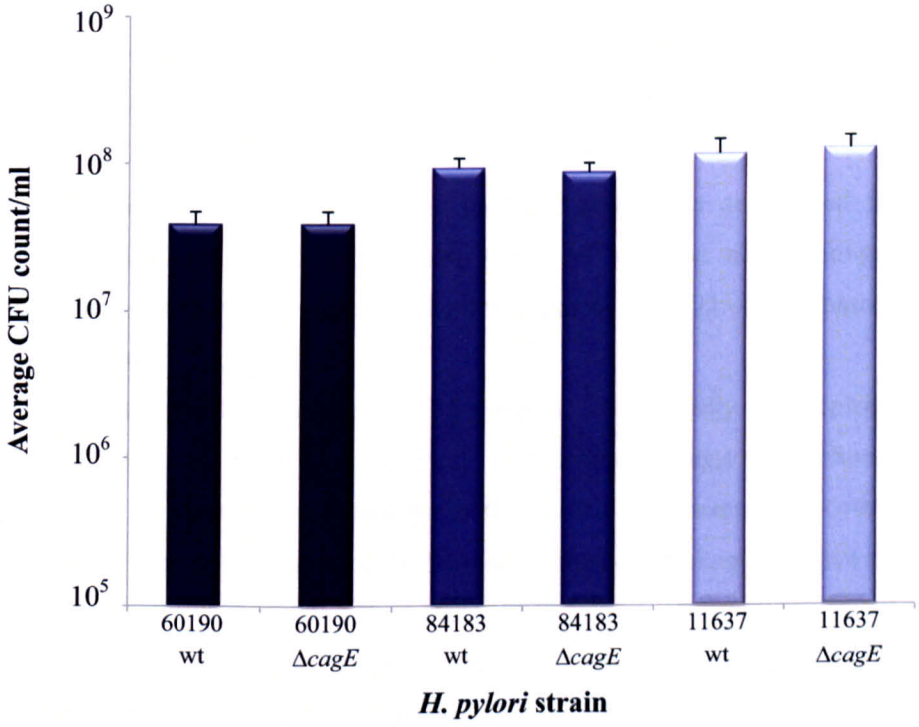


Figure 5.4: Determining the effect of the Δ*cagE* mutation on bacterial density.

AGS cells were cultured with wild type and *cagE* isogenic mutants of *H. pylori* strains 60190, 84183, and 11637. Cells were plated onto blood agar plates after 24hrs, and CFU counts were enumerated. Bars represent average CFU counts in 6 replicates. Error bars represent standard deviation.

5.2.3 The association between virulence determinants and the clinical outcome of infection.

H. pylori virulence factors are a known risk factor for the progression of disease during infection. *cagA* and *vacA* have been linked to the development of peptic ulcer disease and the eventual progression to gastric adenocarcinoma, and *dupA* has been shown to be associated with DUD (Lu *et al.*, 2005; Zhang *et al.*, 2008). To assess the association between disease and virulence determinants of the colonising strain in this population, 56 patients were categorised according to whether they presented with gastritis (25 patients), DUD (26 patients), or GUD (7 patients) during routine endoscopy.

The *dupA* genotype of the colonising bacterial strain was determined for 56 samples. DUD was found to be more prevalent amongst *dupA*+ infections, although not statistically significant. The development of GUD was not associated with *dupA* status (Table 5.3).

cagA was successfully genotyped for 52 samples. The majority of samples were found to be *cagA* positive infections, and amongst *cagA*+ infections, no association was seen between patients with gastritis or patients that developed DUD. Interestingly, all 7 cases of GUD were colonised with *cagA* positive strains (Table 5.3).

Clinical manifestations with *H. pylori* infection were analysed with respect to *vacA* polymorphisms in the m-region (56 samples) and i-region (52 samples). As only 6/55 strains were genotyped with an s2 region, analysis of the s-region was not possible. No association was observed for disease development and the m-region. However, i2-type strains appeared to be related to a decreased occurrence of developing DUD compared to strains carrying i1-type *vacA*, although this was not statistically significant. As with *cagA*, the more virulent alleles of *vacA* were found in strains isolated from patients diagnosed with GUD (Table 5.3).

To further investigate the role of bacterial virulence factors in the progression of disease, premalignant pathology was also considered with respect to the virulence of the colonising strain. Patients were categorised according to whether atrophy

and intestinal metaplasia (IM) were present or absent in gastric biopsies. As only 4 patients were found to present with atrophy and IM in the corpus, only antral data was assessed. Pathology scores were available for 67 patients for which *cagA* status was also determined, 56 with *dupA* status, and 65 with *vacA* status.

No association was observed between the virulence status of the colonising strain and the presence of pre-malignant pathology.

	Gastritis	**DUD**	**GUD**
dupA:			
+	6	10	3
-	17	16	4
cagA:			
+	17	18	7
-	4	5	0
vacA:			
m1	12	14	6
m2	12	11	1
i1	13	15	6
i2	11	6	1

Table 5.3: The association of *H. pylori* virulence determinants with clinical outcome of infection.

H. pylori strain isolates were genotyped for *dupA* (n=56), *cagA* (n=52), *vacA* m-region (n=56), and *vacA* i-region status (n=53). *H. pylori* infected patients were categorised according to whether they presented with gastritis, DUD or GUD. The number of *H. pylori* infected patients that possess *dupA*+, *cagA*+, *vacA* m1, and *vacA* i1 strains for each disease group are shown.

5.3 DISCUSSION

The pathogenicity of bacterial strains varies according to virulence determinants expressed by *H. pylori*, which are known risk factors for in the development of gastric disease during infection (Atherton, 2006). The question of whether density is influenced by the expression of virulence determinants remains unclear.

5.3.1 Association between bacterial virulence and bacterial load

5.3.1.1 cagA

Colonisation density was not found to be associated with *cagA* status when measured by qPCR, as was the case with *in vitro* analysis of infection with *cag*PAI and *cagA* mutants. Although an association with *cagA* and colonisation density was not observed, previous studies in animal models have demonstrated reduced bacterial loads when infected with *H. pylori* strains with non-functional CagPAI (Eaton *et al.*, 2001a; Marchetti & Rappuoli, 2002; Rieder *et al.*, 2005). Interestingly, Eaton *et al.* (2001) demonstrated these trends only at earlier time points, and colonisation densities in comparison to animals infected with wild type *H. pylori* strains were similar at later times point. This suggested that these *cag*PAI mutant strains have delayed growth rates and do not affect final bacterial density. However, numerous studies have demonstrated a positive association between CagA and bacterial load when estimated by histopathology (Atherton *et al.*, 1996; Dzierzanowska-Fangrat *et al.*, 2002; Gunn *et al.*, 1998; Homan *et al.*, 2009), although other studies have failed to show such an association (Basso *et al.*, 2008; Demirturk *et al.*, 2001; Kaklikkaya *et al.*, 2006; Twisk *et al.*, 2001). Importantly, the *cagA* genotype of the colonising strain does not confer functionality of the CagPAI, which is often incomplete or non-functional, which renders the type IV secretion system incapable of delivering CagA into the host cell (Kauser *et al.*, 2004; Matteo *et al.*, 2007). Also of consideration would be the impact of EPIYA-C motifs on density. A previous study which found no association with bacterial load and *cagA* positivity did report increased colonisation densities with multiple EPIYA-C phosphorylation sites (Basso *et al.*, 2008).

5.3.1.2 dupA

Currently, there are no studies that have investigated the association between *dupA* and colonisation density. In this study, *dupA* status was significantly associated with the pattern of colonisation in the stomach, with strains carrying this virulence factor preferentially colonising the antrum. Little is known of DupA function, so the mechanism by which these strains influence bacterial localisation is unclear. Interestingly, Lu *et al.* (2005) observed increased acid tolerance of *dupA*+ strains, suggesting that these strains may be more adapted to survive under high acid conditions in the stomach. Therefore, it is possible that the relationship observed in the current study between bacterial localisation to the antrum and *dupA*+ strains may be linked to an antral-predominant gastritis, which is associated with increased acid load and consequently DUD.

Although *dupA*+ strains are known to be associated with DUD in certain populations (Lu *et al.*, 2005; Zhang *et al.*, 2008), and an antral predominant colonisation is related to development of DUD, this disease profile was not found to correlate with the *dupA* status of the infecting strain. Importantly, almost half of *dupA*- strains were associated with DUD, which suggests that other factors contribute to risk of developing DUD, for example other virulence factors. Lu *et al.* (2005) reported a reduced incidence of GUD with *dupA*+ strains, but this was not apparent in the current data set. Importantly, some studies have failed to observe the association between *dupA* and DUD (Argent *et al.*, 2007; Douraghi *et al.*, 2008). However, such differences may be population based (Schmidt *et al.*, 2009), which may reflect the nature of the plasticity region in which the gene is located. Recently, *dupA* was found to be polymorphic, and a high rate of strain variation within a single host has also been observed (Hussein *et al.*, 2010; Matteo *et al.*, 2010). In the current study, and previous studies investigating *dupA* status, *dupA* genotying was based on the presence or absence of the gene, and did not account for the presence of the more common full length "*dupA1*' allele or the shorter "*dupA2*" type allele (Hussein *et al.*, 2010). However, a strong association in the pattern of colonisation was observed, and it may be possible that all the strains studied in this data set were genotypically the same. In addition, whether polymorphisms in the *dupA* gene confers a phenotypic change is unknown, although Hussein *et al.* (2010) did demonstrate increased levels of IL-12

production in mononuclear cells infected with *H. pylori dupA1* type strains, suggesting that polymorphisms confer a functional difference.

Further investigation of the function and role of *dupA* in colonisation is necessary. As localisation of the bacterium is related to this gene rather than colonisation density, an *in vitro* assay with gastric cell lines would not represent an appropriate model for investigating the effects of *dupA*. However, an *in vivo* analysis using animal models may help to elucidate the nature of infection with strains carrying this gene, and the consequence of infection with *dupA1* and *dupA2* strains. In addition, determining the patterns of colonisation that develop in gastrin knock-out (GAS-OK) mice and hypergastrinemic transgenic (INS-GAS) mice may reveal the possible relationship between acid levels and *dupA* status.

5.3.1.3 vacA

The three polymorphic regions of the *vacA* gene were assessed as possible determinants of colonisation density of *H. pylori*. Only infection with the more virulent m1-type strain in the corpus appeared to associate with increased bacterial loads, but this was not statistically significant. When controlled for *dupA*+ status, increased densities were observed with both m- and i-regions, although not statistically significant with the latter. Interestingly, this trend was observed in the corpus but not the antrum. However, a trend towards increased density with m1 and i1-type strains was observed in both the antrum and corpus among *dupA*- strains. As *dupA*+ strains have a tendency towards antral-predominant colonisation, differences in colonisation density between m1 and m2-type strains may be masked in the antrum, therefore explaining why a trend was not observed in the antrum for strains that were also *dupA*+. Therefore, virulent forms of *vacA* may be associated with increased bacterial density, as found in previous studies (Han *et al.*, 1999; Homan *et al.*, 2009; Nogueira *et al.*, 2001; Tham *et al.*, 2001).

The interaction of the toxin with host cells is known to interfere with tight junction integrity through modulating tyrosine phosphorylation of β-catenin (Adamsky *et al.*, 2003; Meng *et al.*, 2000), which has been implicated in tight junction formation (Rajasekaran *et al.*, 1996). Thus, nutrient "leakage" into the

gastric lumen may be involved in bacterial growth, and may therefore explain the observed relationship in the current study.

Bacterial density was not associated with VacA *in vitro*. However, these growth conditions may not be representative of the human gastric mucosa, and the model utilised may not account for other host factors that may influence growth such as acidity. In this respect, a mouse model may be more beneficial. Furthermore, investigation of earlier time points are important in order to determine whether bacterial overgrowth at later times points may be masking the impact of virulence determinants of bacterial density.

Of consideration is the reported cross-talk between CagA and VacA (Argent *et al.*, 2008; Tegtmeyer *et al.*, 2009). Argent *et al.* (2008) demonstrated increased vacuolation of epithelial cells with Δ*cagA* and Δ*cagE* mutants, whereas the Δ*vacA* mutants significantly increased CagA-induced AGS cell elongation, as compared with wild type strains. Tegtmeyer *et al.* (2009) later found a small proportion of *H. pylori* strains that did not induce cell elongation despite possessing a functional CagPAI, and demonstrated increased vacuolation of gastric cells. Furthermore, this modulation of CagA function was demonstrated to be dependent on VacA. These studies have important implications for the *in vitro* model used in the current study, as *vacA* mutants may have enhanced CagA activity, which may mask any impact on bacterial load, and vice versa. However, the extent of regulation and whether this is restricted to cell elongation and vacuolating activity is unknown. Such cross-talk may therefore make the assessment of individual bacterial factors difficult, although the implication for bacterial density remains unclear.

5.3.2 The association between virulence determinants and the clinical outcome of infection.

An association was not observed between virulence determinants and clinical outcome of infection. As mentioned, *dupA* status was not associated with DUD as shown previously (Lu *et al.*, 2005; Zhang *et al.*, 2008), although the majority of *dupA* positive strains were isolated from DUD patients. No association was found

between disease status and *cagA* and *vacA* strain types. Interestingly, all 7 patients presenting with GUD were infected with *cagA*+ strains, and m1 and i1-types strains were isolated in 6/7 GUD cases, which supports previous reports of increased risk of GUD and gastric cancer with the presence of these virulence factors (Blaser *et al.*, 1995; Kikuchi *et al.*, 1999; Parsonnet *et al.*, 1997). Pathology was also not related to virulence factor expression.

Overall, these data suggest a role of *dupA* in determining patterns of *H. pylori* colonisation in the human stomach, and a possible relationship between bacterial load and *vacA* type.

The presence of other virulence factors, that have not been considered in this study, may also affect colonisation as many have been associated with increased risk of developing severe *H. pylori* related disease. These include *babA2, sabA* and *oipA,* which play a role in bacterial adherence to epithelial cells and encourage bacterial colonisation. *oipA* and *babA2* have in fact previously been associated with increased colonisation density in gastric tissue (Yamaoka *et al.*, 2002a). Furthermore, there is a clear association between these virulence factors and increased inflammatory responses as well as increased risk of developing disease (Rad *et al.*, 2002; Zambon *et al.*, 2003). Importantly, these factors may be linked with gastroduodenal disease because they are associated with the presence of other virulence factors. For instance, *cagA*+ strains are associated with *babA2* expression (Ilver *et al.*, 1998). These close associations make *in vivo* analysis of virulence factor effects in the human host difficult, and illustrates the need for controlled animal studies and *in vitro* models to further investigate the relationship between bacterial colonisation and bacterial factors.

Bacterial factors clearly play a role in modulating colonisation density. However, cytotoxins are known to induce host responses which in turn regulate the local environment and effectively create micro-niches, as well as inducing inflammatory responses that are capable of attacking *H. pylori* in an attempt to clear the infection. With this in mind, it is important to consider the role of the host inflammatory response and its effect on colonisation density.

Chapter 6

6. PRO-INFLAMMATORY HOST RESPONSES TO *H. PYLORI* INFECTION IN THE GASTRIC MUCOSA

6.1 INTRODUCTION

One of the major characteristics of *H. pylori* infection that has drawn much attention is the capacity of the bacterium to maintain a chronic infection in the host (Kusters *et al.*, 2006). *H. pylori* has developed a number of mechanisms that prevent the immune response from clearing the infection. Consequently, a huge number of studies have concentrated on understanding the relationship between the bacterium and host immunity.

During the effector-specific immune response to infection, naive $CD4^+$ T cells differentiate in response to specific cytokine profiles, and polarised responses can either have a Th1, Th2, Th17 or Treg phenotype. Th-cell polarisation is dependent on environmental factors; an IL-4 rich milieu is capable of stimulating Th2 cell differentiation, whereas IL-12 and IFNγ favour the differentiation of Th1 cells (Spellberg & Edwards, 2001). In the presence of TGF-β, naive $CD4^+$ T cell differentiation favours a Treg phenotype (Chatenoud & Bach, 2006). However, differentiation of Th17 cells is dependent on TGF-β, IL-6 and IL-23 (Bettelli *et al.*, 2006).

A large amount of evidence is now available in support of a Th1 polarised response to *H. pylori* infection in the gastric mucosa in humans (Bamford *et al.*, 1998; Karttunen *et al.*, 1995; Mohammadi *et al.*, 1996; Sommer *et al.*, 1998; Tomita *et al.*, 2001). For instance, increased expression of the Th1-defining cytokines IFNγ and IL-12 were found in gastric biopsy samples of *H. pylori*-infected patients (Pellicano *et al.*, 2007). Furthermore, Th2 responses were not found to be up-regulated during *H. pylori* infection, and IL-4 has been detected at low levels, or not at all in some cases, in the gastric mucosa of most *H. pylori*-

infected persons (Karttunen *et al.*, 1995; Lindholm *et al.*, 1998; Sommer *et al.*, 1998). In addition to this evidence, Yamauchi *et al.* examined the role of IL-18, another Th1-related cytokine, which promotes the production of IFNγ by T cells in the presence of IL-12. IL-18 was found to be markedly increased in *H. pylori* infected epithelial cells and monocytes, which was dependent on the CagPAI and OipA (Yamauchi *et al.*, 2008). Other bacterial factors have also been implicated in driving the immune response towards a Th1 phenotype, including HP-NAP, which not only induces an IL-12 rich environment, but also down-regulates the Th2 antibody response resulting in Th1 polarised immunity (Amedei *et al.*, 2006) (Chapter 1, section 1.6.7).

The levels of numerous pro-inflammatory cytokines, including TNFα, IL-1β and IL-8 are up-regulated in the gastric mucosa of *H. pylori* infected individuals compared to uninfected humans, and are key to the immune response against the bacterium (Crabtree *et al.*, 1991a; Crabtree, 1996; Lindholm *et al.*, 1998).

6.1.1 Interleukin-8 (IL-8)

IL-8, also known as CXCL8, is a pro-inflammatory chemokine produced by macrophages, neutrophils and epithelial cells, and is essential for the recruitment of neutrophils to the site of infection. Expression of the *IL8* gene is regulated by NF-κB, although other hormone response elements and NF-IL6 consensus sites have been found in the promoter region of the gene. A number of stimuli can regulate *IL8* expression, including inflammatory signals such as TNFα and IL-1β (Waugh & Wilson, 2008). In addition to its role as a chemoattractant for neutrophils, IL-8 also induces the respiratory burst in neutrophils. Once bound to cell surface receptors, IL-8 also activates signalling pathways involved in cell proliferation and survival (Waugh & Wilson, 2008).

IL-8 production is up-regulated during *H. pylori* infection (Crabtree *et al.*, 1994; Gionchetti *et al.*, 1994; Sharma *et al.*, 1998). A major stimulus for the expression of *IL8* in gastric epithelial cells is the CagPAI, which activates NOD1 through the injection of peptidoglycan into the cell cytoplasm, consequently stimulating *IL8* expression through NF-κB activation (Boughan *et al.*, 2006; Viala *et al.*, 2004). In addition, CagA also induces IL-8 secretion via a mechanism independent of

NOD1 (Brandt *et al.*, 2005) (discussed further in Chapter 1, section 1.6.3.1). In support of this, various studies have observed reduced IL-8 secretion with CagA or CagPAI mutant strains (Brandt *et al.*, 2005; Eaton *et al.*, 2001a). In addition, other *H. pylori*-specific factors have also been associated with up-regulated IL-8 responses in the gastric mucosa, including OipA, BabA (Yamaoka *et al.*, 2002a) and DupA (Lu *et al.*, 2005).

High IL-8 levels may also be associated with an increased risk of developing *H. pylori*-related disease. This is highlighted in studies looking at the effect of the IL-8 -251 polymorphism, which results in high IL-8 production and was found to be associated with increased risk of developing gastric cancer and ulcer disease (Kang *et al.*, 2009; Taguchi *et al.*, 2005). However, this may be population specific as an association was not observed in all populations examined (Canedo *et al.*, 2008). These studies demonstrate the importance of IL-8 during *H. pylori* infection.

6.1.2 Interferon-γ (IFNγ)

IFNγ is a cytokine produced by Th1 cells, $CD8^+$ T cells and natural killer cells, and is essential in the activation of macrophages and suppression of Th2 responses. IFNγ is therefore essential to the activation of the adaptive immune response (Murphy & Reiner, 2002). Other effects of IFNγ include the recruitment of effector cells to the site of inflammation through stimulating the production of chemokines by macrophages, and stimulation of the macrophage oxidative burst via enhanced expression of the enzymes iNOS and NADPH oxidase (Baccala *et al.*, 2005; Borden *et al.*, 2007).

IFNγ production is up-regulated in the gastric mucosa during *H. pylori* infection (Karttunen *et al.*, 1995; Lindholm *et al.*, 1998), and is essential for clearance of the bacterium during immunisation models in mice (Sayi *et al.*, 2009). Furthermore, this cytokine may also play a role in the progression of gastric cancer (Hosseini *et al.*, 2010). In support of this, IFNγ knockout mice infected with *H. pylori* were demonstrated to be protected against the development of gastric atrophy (Houghton *et al.*, 2002; Smythies *et al.*, 2000).

Although polymorphisms in the IFNγ gene have been shown to induce increased cytokine production, an association with *H. pylori* disease has not been shown (Rad *et al.*, 2004; Zambon *et al.*, 2005). However, recent data suggests that a polymorphism in the *IFNGR2* gene (Ex7-128), an important receptor of the IFN-γ pathway, may be linked to the development of gastric cancer. In this study, it was also found that this polymorphism in combination with the TNFα -308 polymorphism (discussed in section 6.1.4) elevated the risk of gastric cancer (Hou *et al.*, 2007). Therefore, IFNγ plays a key role in the immune response to *H. pylori*.

6.1.3 Interleukin 1β (IL-1β)

IL-1β is a pro-inflammatory cytokine produced by many cell types including monocytes, macrophages, neutrophils, and dendritic cells (Curfs *et al.*, 1997). It is produced in response to numerous stimuli and nearly all microbial infections have the capability to induce expression of the cytokine (El-Omar, 2001). IL-1β exerts its biological effects on almost all cell types, and broadly speaking, it functions to induce pro-inflammatory responses, mainly by inducing the expression of other pro-inflammatory cytokines by either initiating transcription, or stabilising their mRNA. These cytokines include TNFα, IL-2, IL-6 and IL-8 (Dinarello, 1996). In addition, it is important in the activation of various cells including T and B cells, and natural killer cells.

IL-1β plays an important role in the pathogenesis of *H. pylori* infection, and is up-regulated during infection with the bacterium (Jung *et al.*, 1997; Noach *et al.*, 1994a). The cytokine is a potent inhibitor of acid secretion in the infected stomach, and does so via a number of different pathways (Beales & Calam, 1998). The importance of IL-1β was demonstrated in a Mongolian gerbil model in which the addition of an IL-1β receptor (IL-1R) antagonist lead to increased gastrin levels and acid output, therefore demonstrating the role of IL-1β in the physiological changes that arise in the stomach (Takashima *et al.*, 2001). Along with inducing hypochlorhydria, IL-1β also modulates the function of several gastric epithelial cells. It stimulates the release of gastrin from G cells, and decreases histamine release and apoptosis in ECL cells (Mahr *et al.*, 2000; Prinz

et al., 1997). Low acid production is strongly associated with gastric cancer (Correa, 1995; El-Omar *et al.*, 2000), and the importance of IL-1β in the development of disease is apparent through studies that have demonstrated gene polymorphisms to be strongly associated with gastric cancer (El-Omar *et al.*, 2000; El-Omar *et al.*, 2003) (discussed in more detail in Chapter 1, section 1.2.2). Furthermore, IL-1β has also been linked to the development of PUD (Hsu *et al.*, 2004; Watanabe *et al.*, 2001), and is therefore fundamental to the pathogenesis of *H. pylori* infection.

6.1.4 Tumour necrosis factor α (TNFα)

TNFα is a pro-inflammatory cytokine that is secreted by a number of cells including macrophages, natural killer cells and T-cells. This cytokine has pleiotropic functions, and is important in promoting inflammatory responses to infection, as well as activating macrophages and neutrophils (Le & Vilcek, 1987). In addition, TNFα induces the expression of adhesion molecules such as VCAM-1, indicating the importance of the cytokine as a chemoattractant for other cells (Curfs *et al.*, 1997). TNFα secretion is induced by a number of stimuli including LPS (Crabtree *et al.*, 1991a). Like IL-1β, TNFα stimulation of cells induces NF-κB signalling pathways, and is therefore important in cell proliferation and survival (Curfs *et al.*, 1997).

TNFα production is upregulated during *H. pylori* infection (Crabtree *et al.*, 1991a; Noach *et al.*, 1994a), and induces the transcription of other pro-inflammatory cytokines and chemokines (Genta, 1997a). An important function of this cytokine is the inhibitory effect on gastric acid secretion (Beales & Calam, 1998). It was found to inhibit somatostatin release from D cells, which negatively regulates the release of gastrin from G cells (Beales *et al.*, 1997). Increased TNFα production has been associated with *H. pylori* specific virulence factors, including TNFα-inducing protein (TIPα) (Suganuma *et al.*, 2005) and JHP940 (Rizwan *et al.*, 2008). A correlation has also been observed between disease severity and TNFα secretion (Lehmann *et al.*, 2002). As discussed earlier (Chapter 1, section 1.2.2), TNFα polymorphisms are associated with an increased risk of developing gastric

cancer (El-Omar *et al.*, 2003), demonstrating the importance of TNFα to *H. pylori*-associated inflammatory responses.

6.1.5 Interleukin 17 (IL-17)

In recent years, the Th1/Th2 paradigm has expanded to include a new subset of T helper effector cells termed Th17 which produce IL-17A and IL-17F. IL-17A is also produced by natural killer cells, neutrophils, eosinophils, and other cells of the innate immune system which are believed to play a role in immune surveillance (Cua & Tato, 2010). Therefore, IL-17 is produced by cells of both the innate and adaptive immune systems, suggesting a role for the cytokine in bridging the two immune responses. IL-17 is a pro-inflammatory cytokine that acts on a broad range of cells (Kolls & Linden, 2004), and functions to induce the expression of numerous cytokines including TNFα, IL-6, IL-1β, and IL-8 (Jovanovic *et al.*, 1998; Laan *et al.*, 1999).

Up-regulation of IL-17A has been demonstrated in the gastric mucosa of *H. pylori* infected compared to uninfected people (Caruso *et al.*, 2008; Luzza *et al.*, 2000). Notably, *IL17* expression was found to be higher in ulcer tissue compared to non-ulcerated tissue in the antrum of infected patients (Mizuno *et al.*, 2005). IL-17 levels have also been found to correlate with neutrophil infiltration in the *H. pylori* infected gastric mucosa (Mizuno *et al.*, 2005), and have been associated with increased secretion of IL-8 (Luzza *et al.*, 2000), suggesting that IL-17 may be crucial to the recruitment of immune cells to the gastric mucosa and the gastritis that develops in response to *H. pylori*.

6.1.6 Interleukin 22 (IL-22)

A novel T helper subset has recently been described which characteristically secretes IL-22, and is thus called Th22 (Duhen *et al.*, 2009; Trifari *et al.*, 2009). IL-22 was later found to be expressed at low levels in natural killer cells, and in activated T cells including Th1 and Th17 cells, with the highest frequency of IL-22 producing cells having the Th22 or Th1 phenotype (Duhen *et al.*, 2009). The functions of IL-22 appear to differ to other cytokines, as it is produced by immune cells but regulates the functions of certain tissue cells. Overall, IL-22 functions to

increase the innate immunity of tissue cells, protects tissue from damage and enhances regeneration of tissue (Duhen *et al.*, 2009). The IL-22 receptor complex is composed of the IL-22R1 and IL-10R2 subunits. As IL10-R1 is ubiquitously expressed, the expression of the IL-22R1 chain determines the cellular targets of IL-22 (Wolk *et al.*, 2004). There is no evidence to date that IL-22 expression is modulated by *H. pylori* infection. However, IL-22R1 chain expression has been demonstrated in the stomach (Wolk *et al.*, 2004).

6.1.7 Evidence for Th1 effects on bacterial load

Various studies in mouse models have demonstrated the detrimental effects of pro-inflammatory responses to *H. pylori* colonisation density. A number of studies have showed that IFNγ-deficient mice develop a mild gastritis post-infection in comparison to wild type strains, and increased bacterial loads were concurrently observed (Eaton *et al.*, 2001b; Sawai *et al.*, 1999; Sayi *et al.*, 2009). Eaton *et al.* (2001) reported the development of more severe gastritis and metaplasia in SCID mice that were given $CD4^+$ splenocytes in comparison to $CD4^+$ depleted recipients. Bacterial loads however were found to be more reduced in mice that exhibited greater degrees of gastritis then in those with mild gastritis. Furthermore, IFNγ-deficient mice infected with *H. pylori* were colonised with 5-fold greater bacterial loads than wild type mice, and gastritis was reduced. IL-10-deficient mice on the other hand exhibited the opposite, with 2-fold reduced bacterial loads compared to wild type strains, and a much more severe gastritis. Notably, Sayi *et al.* (2009) reported increased colonisation densities 1 month post-infection in IFNγ knockout mice, which suggests that the adaptive immune response may play a role in bacterial clearance. In addition to the effect of IFNγ on bacterial load, other pro-inflammatory mediators have also been found to have an inverse relationship with bacterial load, for instance the IL-$12^{-/-}$ (Garhart *et al.*, 2003) and nitric $oxide^{-/-}$ mice (Blanchard *et al.*, 2003) were more severely colonised in comparison to wild type strains. Delayed clearance of *H. pylori* was also observed in neutrophil depleted IL-10 deficient mice, highlighting the importance of these cells in the protection against infection. Importantly, as IL-8 is essential in the recruitment of neutrophils to the gastric mucosa, these data suggest a possible relationship between IL-8 production and bacterial colonisation

density (Ismail *et al.*, 2003). The Th1 response to *Helicobacter* infection was elegantly demonstrated by Fox *et al.* In this study, *H. felis* infected mice were found to express higher levels of pro-inflammatory cytokines IFNγ, TNFα and IL-1β in comparison to mice with a concurrent helminth infection, which characteristically induces a Th2 response. Furthermore, the expression of suppressive cytokines IL-10, IL-4 and TGF-β was found to be higher in these mice compared to those infected with *H. felis* alone (Fox *et al.*, 2000).

Importantly, although a number of different cytokine responses may contribute to the immune response that is responsible for clearing or reducing bacterial load, these studies do suggest that increased pro-inflammatory responses are associated with reduced bacterial load, and limiting this inflammatory response is associated with higher colonisation compared to wild type strains. Therefore, in animal models, an inverse association is observed between inflammation and *H. pylori* colonisation. Although a reciprocal relationship between inflammatory responses and bacterial burden may also be expected in humans, such an association remains to be shown.

6.1.8 Experimental aims

- To evaluate the relationship between *IL8* expression and bacterial burden in the gastric mucosa of infected individuals.

- To investigate the role of pro-inflammatory cytokines stimulation of gastric epithelial cells on *H. pylori* colonisation density *in vitro*.

- To determine the effect of cytokine stimulation on IL-8 secretion in epithelial cells *in vitro*.

- To assess the relationship between *IL8* expression and *H. pylori* strain genotype *in vivo*.

- To investigate the association between *IL8* expression and pathology and disease *in vivo*.

6.2 RESULTS

The inflammatory response to infection plays a key role to the pathogenicity associated with *H. pylori* infection. Whilst inflammation is advantageous with respect to protection against the bacterium, the noxious effects of inflammatory mediators necessary for bacterial clearance also result in mucosal damage, leading to loss of tissue and disease (Robinson *et al.*, 2007). An aggressive inflammatory response would be expected to reduce colonisation density, whereas a higher bacterial load may be seen with reduced inflammation. As mentioned, previous studies in mice suggest that this is the case (Blanchard *et al.*, 2003; Eaton *et al.*, 2001b), however little evidence for this association in the human gastric mucosa is available.

6.2.1 The relationship between *IL8* expression and bacterial load in the *H. pylori* infected gastric mucosa

IL-8 is an important pro-inflammatory cytokine induced by *H. pylori* infection (Sharma *et al.*, 1998). High expression of the cytokine that results from *IL8* gene polymorphisms has been associated with an increased risk of developing *H. pylori* disease (Kang *et al.*, 2009; Taguchi *et al.*, 2005). IL-8 is therefore an important cytokine in the immune response mounted in response to *H. pylori* infection. Previous studies in mice have reported an inverse relationship between bacterial burden and the severity of gastritis in mice (Blanchard *et al.*, 2003; Eaton *et al.*, 2001b; Sawai *et al.*, 1999). However, it is unclear whether *IL8* plays a role in the degree of *H. pylori* colonisation in the gastric mucosa. In order to investigate this, *IL8* expression levels were measured using RNA extracted from 33 gastric tissue samples obtained from the antrum and corpus of infected patients, for which bacterial load was also determined from DNA extracted from the same gastric tissue sample.

A plot of *IL8* expression against bacterial load shows a large group of samples that cluster within a region of low *IL8* expression (appearing below125) and low bacterial load (appearing below 1). It would appear that the highest *IL8* expression levels were observed with lower bacterial loads, and the highest bacterial loads were observed in samples with low *IL8* expression levels (Figure 6.1).

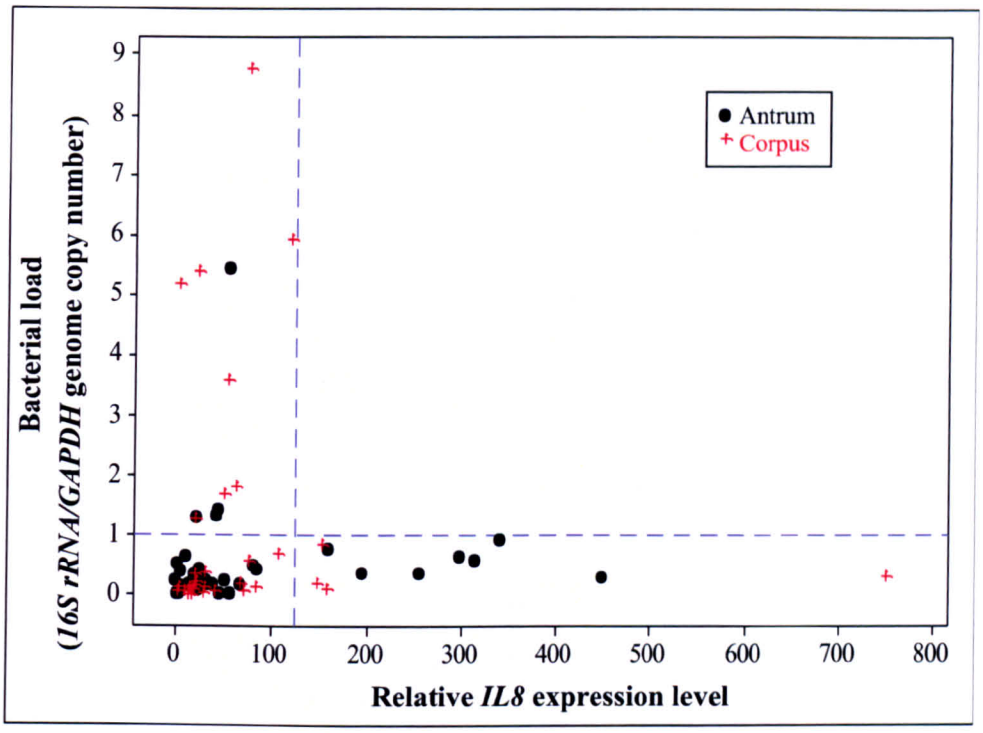


Figure 6.1: The relationship between bacterial load and *IL8* responses in the gastric mucosa.

Bacterial load and *IL8* expression levels were determined in 33 *H. pylori* infected antral gastric tissue biopsies (•), and 33 *H. pylori* corpus gastric biopsies (+). Each data point represents the average *IL8* expression level and bacterial load measured in 3 replicate reactions. To highlight the cluster of samples with low *IL8* expression that also presented with low bacterial density, a reference line for the y-axis was placed at 1, and at 125 for the x-axis.

Based on this data, a simple linear negative relationship was not observed between colonisation density and IL8 expression. To confirm that *IL8* expression is upregulated in the *H. pylori* infected gastric mucosa, *IL8* expression levels were measured in 34 *H. pylori* infected and 11 uninfected biopsies taken from the antrum and corpus. As expected, *IL8* expression levels were elevated in the gastric mucosa of infected patients compared to uninfected patients in the antrum and corpus (Figure 6.2). *IL8* expression between *H. pylori* infected antral and corpus biopsies did not differ (antrum: median=48.7, n=48, IQR=4.49-61.8; corpus: median=29.1; n=34, IQR=15.7-74.8).

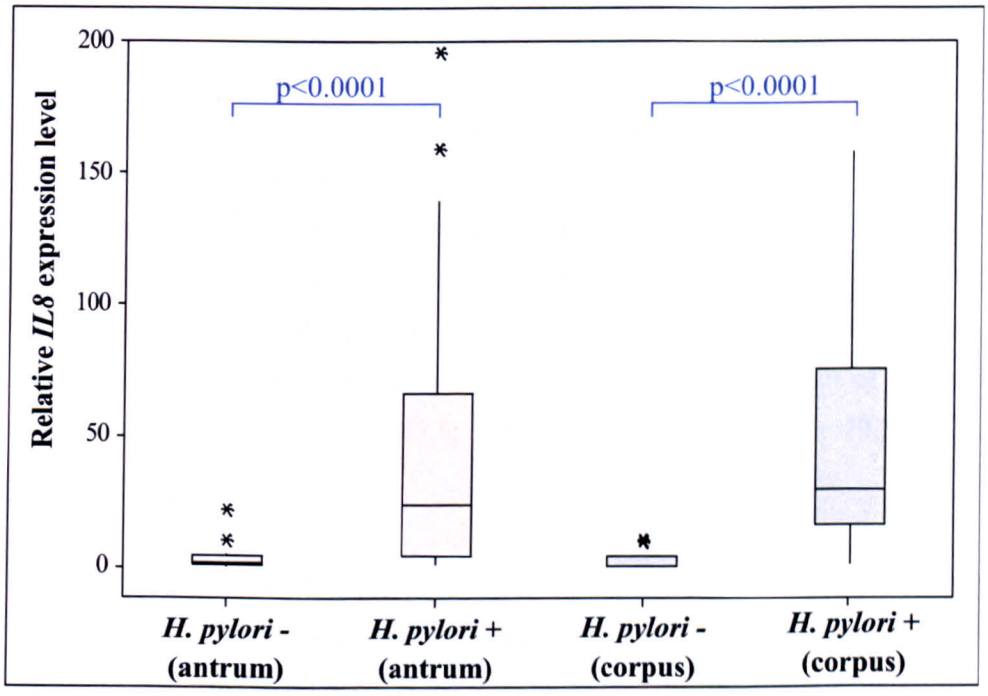


Figure 6.2: *IL8* responses in the gastric mucosa of *H. pylori* infected and uninfected patients.

IL8 expression levels were determined for 34 *H. pylori* infected and 11 uninfected antral and corpus gastric biopsies. Data were normalised relative to *GAPDH* expression, and a sample from an uninfected donor was used as an internal comparator to provide a fold-difference. * represents outlier data points.

6.2.2 The relationship between IL8 expression and inflammation in gastric tissue

As mentioned, IL-8 plays a crucial role in the recruitment of neutrophils to the site of infection, and is hence essential for the induction of the inflammatory response in *H. pylori* infection. The degree of neutrophil infiltration into the lamina propria (described as "activity" by histopathologists) would be expected to reflect the level of *IL8* expression. However, this was not the case in a number of studies (Bayraktaroglu *et al.*, 2004; Camorlinga-Ponce *et al.*, 2003). In order to investigate whether this relationship exists in the gastric mucosa, the grade of activity determined by histology was assessed in comparison to *IL8* expression. Severe grades of activity (Sydney score of 3) were not observed in the data set studied. A small increase in *IL8* expression was observed between mild and moderate degrees of activity in the antrum (for grade 1: median=24.7, n=9, IQR=12.6-55.3; for grade 2: median=36.7, n=17, IQR=4.59-83.9) and corpus (for grade 1: median=17.6, n=9, IQR=12.0-115.5; for grade 2: median=30.5, n=13, IQR=20.8-91.2), but was not statistically significant. Higher *IL8* expression levels were observed in the presence of activity (scores of 1 and 2 combined) compared to samples that showed no activity in the antrum (absence of activity: median=4.47, n=7, IQR=2.61-23.3; presence of activity: median=31.2, n=26, IQR=5.42-80.0; p=0.02), but was not the case in the corpus (absence of activity: median=40.4, n=11, IQR=12.5-67.8; presence of activity: median=29.1, n=22, IQR=18.0-88.9).

The relationship between *IL8* expression and mononuclear cell infiltration (described as "inflammation" by histopathologists) was also investigated, and was positively associated with histological grading of inflammation in both antral (for grade 1: median=3.22, n=8, IQR=2.31-27.0; grade 2: median= 34.2, n=21, IQR=6.06-79.2; grade 3: median=67.8, n=5, IQR=13.6-140.4) and corpus (for grade 1: median=19.8, n=17, IQR=13.1-52.0; grade 2: median= 30.4, n=14, IQR=18.2-83.9; grade 3: median=119.6, n=3, IQR=62.2-158.1) biopsy samples (Figure 6.3A and B). Therefore, *IL8* expression is a good indicator of the degree of mononuclear cell infiltration in the gastric mucosa.

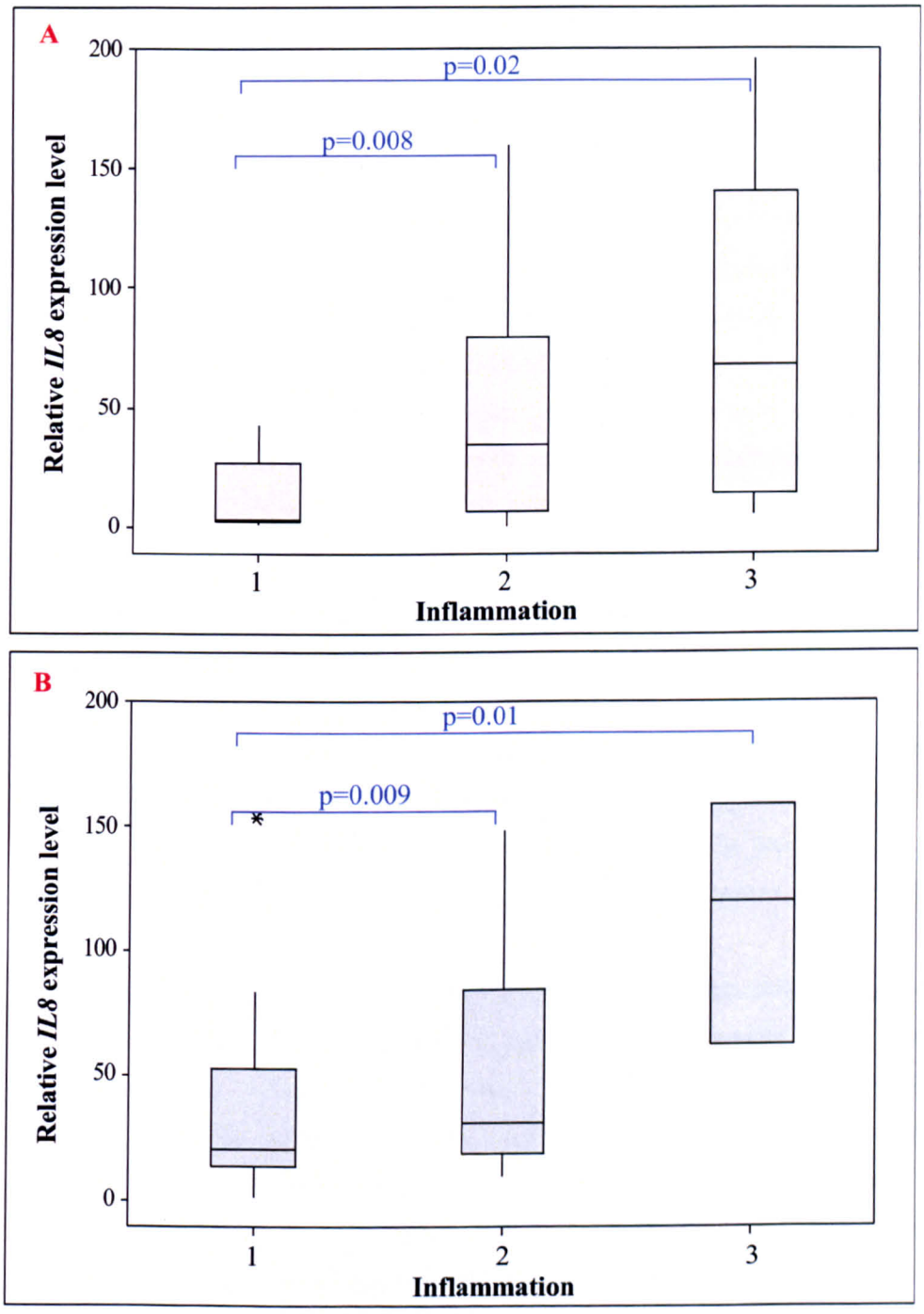


Figure 6.3: Relationship between *IL8* expression and inflammation

Levels of *IL8* mRNA were determined in 34 gastric biopsies taken from **A**- the antrum and **B**- the corpus. Expression levels were categorised by the level of mononuclear cell infiltration (inflammation) as determined by histology. *indicates outlier values.

6.2.3 The effect of pro-inflammatory cytokines on bacterial load *in vitro*

The data presented here have demonstrated that *IL8* mRNA levels are markedly increased in *H. pylori* infected gastric tissue samples in comparison to uninfected samples, but the relationship with bacterial load is complex. However, *IL8* mRNA levels were positively associated with the level of inflammation as determined by histology. Other cytokines are known to modulate *IL8* expression such as IL-1β and TNFα (Crabtree *et al.*, 1994), and IL-17 (Luzza *et al.*, 2000). Therefore, the relationship between these cytokines and bacterial load was assessed.

Previously, our group have demonstrated the effects of IL-10 cytokine stimulation of *H. pylori* infected AGS cells on bacterial CFU counts in co-culture (Robinson *et al.*, 2008). This method was therefore utilised in order to investigate the impact of a panel of inflammatory cytokines on bacterial burden on gastric epithelial cells. *IL1β* and *TNFα* expression is known to be induced in response to *H. pylori*, and as these cytokines are associated with disease progression (Camilleri *et al.*, 2007; Crabtree *et al.*, 1991a; Crabtree *et al.*, 1994), both were chosen for investigation. Recent evidence has highlighted the potential role for IL-17 in *H. pylori*-associated inflammation, suggesting that IL-17 responses may be associated with *H. pylori*-specific disease profiles (Caruso *et al.*, 2008; Mizuno *et al.*, 2005). Th17 skewed responses in mice were found to be associated with reduced bacterial densities, and IL-17 was therefore investigated (Kao *et al.*, 2010).

AGS gastric epithelial cells were infected with *H. pylori* strains 60190 (CagPAI positive) or Tx30a (CagPAI negative), in the presence or absence of cytokine stimulation. Each condition was performed in 6 replicates, on two separate occasions to minimise experimental error. Cells were incubated for 24 hours, after which the number of bacterial CFUs were determined. To assess CFU counts, a serial dilution of the culture suspension was spotted onto blood agar plates. CFU counts were enumerated after incubating the plates for 3-4 days (a detailed description is given in section 2.6). Stimulation with IL-1β, TNFα and IL-17 did not reduce bacterial CFU counts in comparison to the unstimulated control (Table 6.1).

	Average CFU count/ml after 24hrs (SD)		
	IL-1β	**TNFα**	**IL-17**
AGS cells cultured with *H. pylori* 60190			
+ *H. pylori*	9.98×10^7 (1.89×10^7)	1.02×10^8 (2.26×10^7)	3.73×10^7 (1.09×10^7)
+ *H. pylori* + cytokine	1.03×10^8 (5.64×10^7)	1.01×10^8 (2.65×10^7)	4.46×10^7 (8.49×10^6)
P	NS	NS	NS
AGS cells cultured with *H. pylori* Tx30a			
+ *H. pylori*	1.66×10^8 (4.52×10^7)	1.86×10^8 (2.69×10^7)	1.17×10^8 (2.24×10^7)
+ *H. pylori* + cytokine	1.71×10^8 (3.68×10^7)	2.34×10^8 (5.54×10^7)	1.29×10^8 (1.51×10^7)
P	NS	NS	NS

Table 6.1: The effect of cytokine stimulation on bacterial load when cultured with human gastric epithelial cells.

AGS cells were stimulated with 50ng/ml of cytokine and infected with either *H. pylori* strains 60190 or Tx30a (MOI=100). After 24hrs, the average number of colony forming units (CFU/ml) in 6 replicates was determined. Differences in CFU counts between cells cultured in the absence or presence of cytokine were not statistically significant. No *H. pylori* growth was observed in uninfected AGS controls. SD = standard deviation, NS = not significant.

IL-8 responses to *H. pylori* infection and cytokine stimulation were investigated in order to confirm that stimulation with these cytokines induces increased IL-8 production as previously reported (Crabtree *et al.*, 1994; Luzza *et al.*, 2000). AGS cells were cultured as before with *H. pylori* strain 60190 in the presence or absence of cytokine stimulation. An increase in IL-8 secretion was observed in uninfected AGS cells in the presence of IL-1β (54-fold increase compared to AGS control) and TNFα (23-fold increase). IL-8 responses were also induced with unstimulated *H. pylori* infected cells (34-fold increase). However, the greatest response was observed with infected cells that were stimulated by IL-1β and TNFα. Interestingly, although IL-17 stimulation of AGS cells did not induce IL-8 secretion, IL-17 stimulation of *H. pylori* infected AGS cells further induced IL-8 responses in comparison to untreated *H. pylori* infected cells (Figure 6.4).

Overall, *H. pylori* infected AGS cells were in fact responsive to cytokine treatment, but these responses were not associated with a reduction in bacterial numbers.

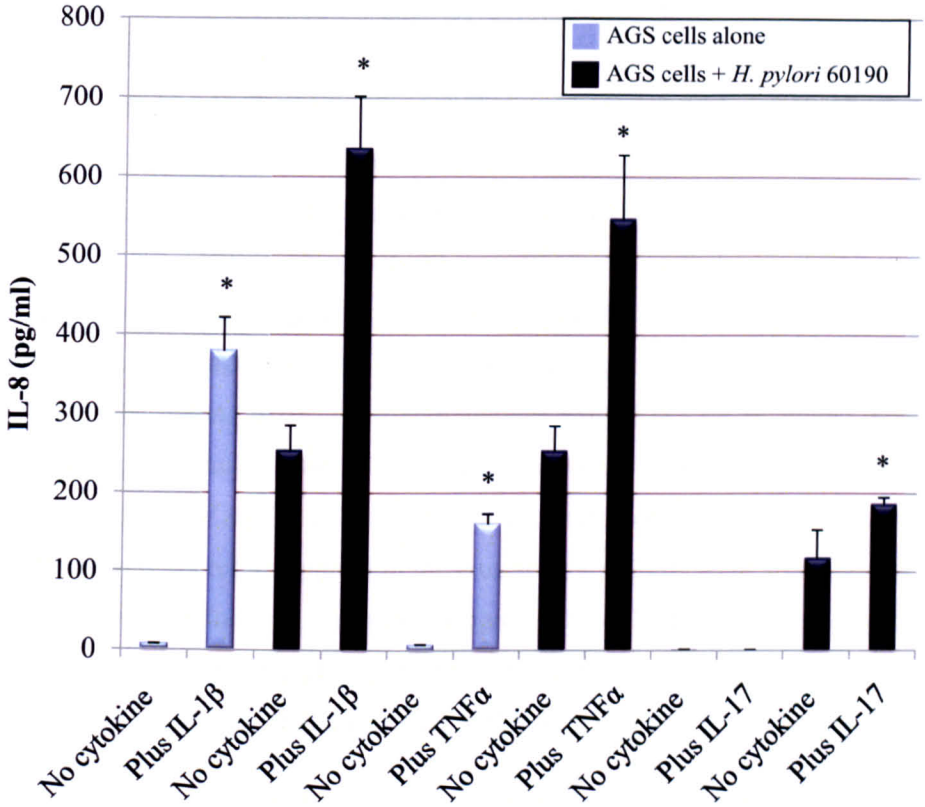


Figure 6.4: IL-8 secretion levels in response to cytokine stimulation of gastric epithelial cells infected with *H. pylori*.

AGS cells were cultured with *Helicobacter pylori* strain 60190 (MOI=100) in the presence or absence of cytokine (50ng/ml). IL-8 responses to IL-1β, TNFα and IL-17 stimulation were measured by ELISA after 24 hours in 6 replicates. Unstimulated and stimulated AGS uninfected controls were included for each experiment. * represents a significantly higher IL-8 secretion after cytokine stimulation in comparison to unstimulated controls ($p<0.005$).

The role of IL-17 in the defence against Gram negative bacteria was demonstrated with *Klebsiella pneumonia* infection, where stimulation of bronchial cell lines with IL-17 induced the expression of IL-6, and the addition of IL-22 further increased expression levels of IL-6 (Aujla *et al.*, 2008). Furthermore, bacterial burden in IL-$17^{-/-}$ mice was found to be elevated in comparison to wild type strains, and the addition of anti-IL-22 was associated with higher CFU counts compared to controls, suggesting that functions of IL-17 are enhanced by IL-22. Given the recent evidence of IL-22 receptor expression in the stomach (Wolk *et al.*, 2004), the effects of IL-22 in combination with IL-17 on *H. pylori* density were investigated. Aujla *et al.* (2008) demonstrated increased cytokine expression after the addition of IL-22 at 2-fold higher concentrations than IL-17. The same conditions were therefore applied in the current study. Cells were stimulated with IL-22 at concentrations ranging from 20ng/ml-80ng/ml, and IL-17 ranging from 20-10ng/ml. However, a significant reduction in bacterial CFU counts was not observed. This was also the case when cells were stimulated with IL-22 alone (Table 6.2A and B).

A

	Average CFU count/ml after 24hrs
Minus cytokine	1.97×10^9
Plus IL-22 (50ng/ml)	2.29×10^9

B

Plus IL-22:IL-17 at concentrations:	Average CFU count/ml after 24hrs
80ng:40ng	9.20×10^7
40ng:20ng	8.85×10^7
20ng:10ng	9.65×10^7
Minus cytokines	7.00×10^7

Table 6.2: IL-22 mediated effects on bacterial load when cultured with human gastric epithelial cells.

AGS cells were infected with *H. pylori* strains 60190 (MOI=100), and stimulated with A- IL-22 alone (50ng/ml). B- cultures stimulated with a combination of IL-22 and IL-17 at IL-22:IL-17 concentrations of 80ng:40ng, 40ng:20ng, and 20ng:10ng per ml were also examined. After 24hrs, the average number of colony forming units (CFU/ml) was determined in 6 replicates. Differences in CFU counts between cells cultured in the absence or presence of cytokine were not statistically significant.

As IL-22 has previously been demonstrated to work synergistically with IL-17 for the induction of IL-6 in bronchial epithelial cells (Aujla *et al.*, 2008), the effects of this cytokine combination were investigated in order to determine if such a relationship exists for the secretion of IL-8 in *H. pylori* infected gastric cells.

AGS cells infected with *H. pylori* strain 60190 were stimulated with IL-22 and IL-17 as previously described (Table 6.2). Stimulation of *H. pylori* infected cells with IL-22 at various concentrations did not significantly induce IL-8 production. As before, IL-17 stimulation was associated with increased levels of IL-8 secretion, although this was not a dose dependent effect. Furthermore, IL-8 responses to IL-17 treatment were not affected by the addition of IL-22 (Figure 6.5).

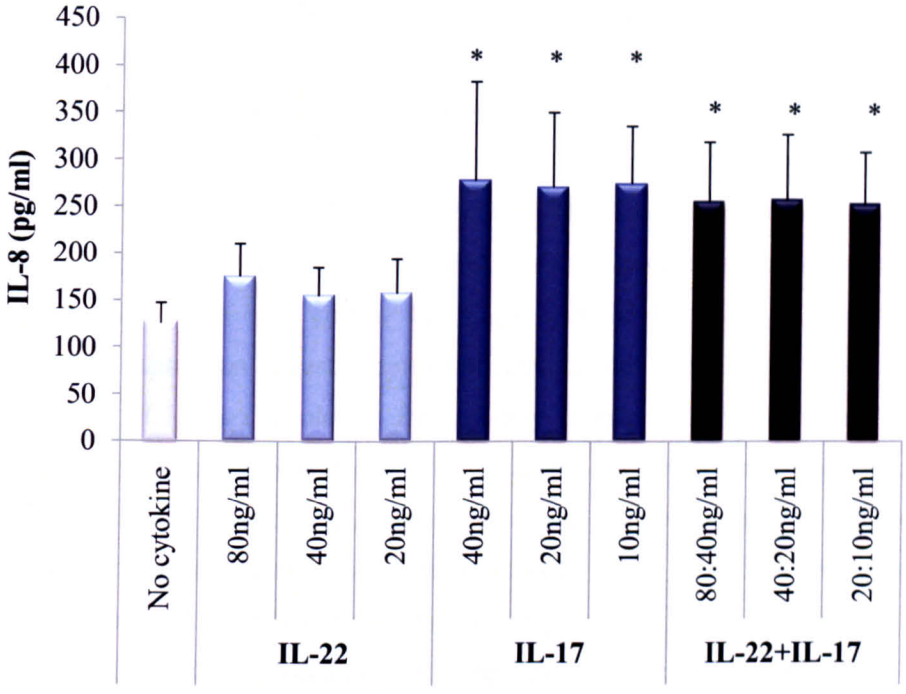


Figure 6.5: IL-8 response to IL-22 and IL-17 cytokine stimulation of *H. pylori* infected gastric cells.

AGS cells were cultured with *Helicobacter pylori* strain 60190 (MOI=100). Cells were stimulated with IL-22 (at concentrations of 80ng/ml, 40ng/ml, and 20ng/ml), IL-17 (40ng/ml, 20ng/ml, and 10ng/ml), or a combination of IL-22 and IL-17 (80:40ng/ml, 40:20ng/ml, and 20:10ng/ml). IL-8 responses were measured by ELISA after 24 hours. A *H. pylori* infected no cytokine control was included. The level of IL-8 was also measured in *H. pylori* negative AGS cells. A level of 13.3ng/ml was measured (data not shown in the above graoh). Error bars represent the standard deviation between 4 replicates. * represents a significantly higher IL-8 secretion after cytokine stimulation in comparison to unstimulated controls ($p<0.03$).

6.2.4 The relationship between *IL8* expression levels and bacterial virulence factors

Recent evidence suggests that VacA may have the capacity to induce NF-κB activation in T cells, and therefore presents a possible route for induction of *IL8* expression (Takeshima *et al.*, 2009). In addition, DupA was found to induce IL-8 secretion in gastric cell lines (Lu *et al.*, 2005) and the gastric mucosa (Hussein *et al.*, 2010). Also, it is well known that CagA and the CagPAI are capable of inducing *IL8* expression in gastric epithelial cells (Brandt *et al.*, 2005). These data suggest that *H. pylori* virulence factors may induce pro-inflammatory host responses, in particular *IL8* expression, which in turn may impact bacterial load.

To investigate the relationship between bacterial virulence factors and host inflammatory responses, *H. pylori* strains isolated from gastric biopsies were genotyped for *cagA* (n=20), *vacA* s-, m-,and i-region (n=23), and the presence of *dupA* (n=22), and *IL8* expression was compared (summarised in Table 6.3). In antral biopsies, *cagA* positive strains were associated with numerically almost 5-fold greater *IL8* levels compared to those determined in *cagA* negative infected biopsies, although this difference was not statistically significant. The opposite trend was observed in the corpus, but again was not statistically significant.

Expression levels did not differ between *dupA* positive and negative isolated in the antrum or corpus. As CagA and CagPAI induces *IL8* expression (Brandt *et al.*, 2005), samples were corrected for *cagA* positivity. Consequently, *IL8* expression was found to be numerically higher for *dupA+cagA+* strains in comparison to *dupA-cagA+* strains in both the antrum and corpus but this was not statistically significant.

In the corpus, high *IL8* expression was observed in biopsies infected with the most virulent isoforms of *vacA*, but this was not statistically significant. Antral samples demonstrated opposite trends with exception to the s-region. Correcting for *cagA* positivity did not alter the trends. As too few strains were genotyped as *vacA* S2-type, correcting for *cagA* status was not possible.

Overall, the levels of *IL8* expression between different *H. pylori* strain genotypes were not significantly different.

	Antrum		Corpus	
	Median (n)	IQR	Median (n)	IQR
cagA				
+	22.4 (16)	4.47-61.7	52.0 (16)	15.1-80.9
-	4.54 (4)	1.23-49.1	83.7 (4)	5.81-152.3
dupA				
+	28.3 (11)	4.20-99.4	30.3 (11)	13.3-74.2
-	42.3 (11)	10.8-62.0	30.5 (11)	17.6-83.2
+ (*cagA*+)	34.2 (8)	4.77-79.2	50.5 (8)	14.0-98.0
- (*cagA*+)	23.3 (4)	6.41-62.0	42.2 (4)	18.7-75.8
vacA				
s1	32.6 (19)	4.72-63.2	40.4 (19)	19.0-74.2
s2	11.9 (4)	0.51-241.2	12.0 (4)	3.25-118.9
m1	14.7 (12)	3.02-56.2	66.5 (12)	18.2-126.7
m2	31.1 (11)	4.77-195.9	19.6 (11)	13.2-37.9
i1	22.4 (12)	4.54-61.7	66.5 (12)	19.7-137.4
i2	31.1 (11)	4.77-63.1	30.3 (11)	9.27-44.6
m1 (*cagA*+)	14.9 (9)	3.81-70.5	70.7 (9)	23.2-132.0
m2 (*cagA*+)	27.3 (7)	4.61-59.1	30.3 (7)	13.3-53.9
i1 (*cagA*+)	22.4 (9)	6.06-79.2	70.7 (9)	23.2-132.0
i2 (*cagA*+)	31.1 (5)	4.77-67.8	50.2 (5)	22.5-68.5

Table 6.3: *IL8* responses in relation to *H. pylori* strain virulence.

H. pylori strains colonising gastric biopsies were genotyped for *cagA*, *dupA*, and *vacA* s-, m- and i-region. *IL8* expression levels in the antrum and corpus were determined for each strain genotype. As CagA is a potent inducer of *IL8* expression, data for *dupA* and *vacA* were also corrected for *cagA* to rule out a bias. Sample sizes (n) are indicated in brackets.

6.2.5 IL8 responses in relation to pathology and disease.

Severe inflammatory responses are associated with damage to the gastric mucosa, which may induce the development of disease, and *IL8* expression has previously been associated with increased risk of developing disease. (Kang *et al.*, 2009; Taguchi *et al.*, 2005) This was not found for the data set investigated in the present study, for which there was no significant difference in *IL8* expression between patients diagnosed with gastritis, although a trend towards increased *IL8* expression in the antrum was observed in patients with gastritis (antrum: median=23.3, n=17, IQR=12.0-122.3; corpus: median=29.1, n=18, IQR=15.7-59.5) and those diagnosed with DUD (antrum: median=48.1, n=12, IQR=26.3-48.1; corpus: median=20.8, n=12, IQR=14.4-80.1). As only 3 patients in this data set were diagnosed with GUD, they were not considered for analysis.

The relationship between *IL8* expression and premalignant pathology, i.e. atrophy and IM, was also considered. As mentioned earlier, too few samples were available with either atrophy or IM to assess each grade of pathology. Therefore, samples were categorised according to whether pathology was present or absent. A statistical difference was not observed for atrophy in the antrum (absent: median=43.7, n=23, IQR=19.1-80.8; present: median=22.4, n=11, IQR=2.4-159.7) or IM in the antrum (absent: median=39.2, n=27, IQR=14.9-80.8; present: median=34.2, n=7, IQR=14.6-298.9). Too few patients were found to have atrophic gastritis or signs of IM in the corpus, and were therefore not assessed further.

6.3 DISCUSSION

Variations in the host immune response and the resulting inflammation are key to the mucosal damage that is associated with *H. pylori*-associated disease progression (Robinson *et al.*, 2007).

H. pylori infection characteristically induces a Th1 immune response, with a significant upregulation of inflammatory mediators (section 6.1.1 to 6.1.6). Mouse studies have demonstrated the importance of these inflammatory mediators to the development of gastritis, and the resulting impact on bacterial density (Blanchard *et al.*, 2003; Eaton *et al.*, 2001b; Garhart *et al.*, 2003) (discussed in section 6.1.7). This chapter investigated the role of the inflammatory response in regulating bacterial density in co-cultures with gastric epithelial cells, and the relationship in the gastric mucosa.

6.3.1 *IL8* expression in the *H. pylori* infected gastric mucosa and the relationship with bacterial density

Numerous cytokines are involved in the host inflammatory response to *H. pylori* (Lindholm *et al.*, 1998). As IL-8 is an important proinflammatory cytokine expressed in the response to *H. pylori* infection, expression levels of this cytokine in the gastric mucosa were estimated in order to determine if an inverse relationship existed between bacterial load and inflammation. This hypothesis was based on the observation of previous studies that reported an inverse relationship exists between gastritis and bacterial load in mice studies (Blanchard *et al.*, 2003; Eaton *et al.*, 2001b; Sawai *et al.*, 1999).

The majority of biopsies in the data set were found to have low bacterial colonisation and low *IL8* expression. High *IL8* expression levels were found for a number of samples and interestingly, these samples were concurrently colonised with low bacterial densities. In contrast, tissue colonised with high levels of *H. pylori* demonstrated low *IL8* expression. These data suggest that in the majority of cases, a balance exists between colonisation and *IL8* expression resulting in relatively low *IL8* expression and low colonisation densities. However, when this balance is disrupted, a negative association appears to exist and demonstrates the inverse relationship between inflammation and bacterial burden. Of note, and in

support of this hypothesis, is the absence of samples that exhibited high *H. pylori* colonisation and high *IL8* expression.

Few studies have characterised *IL8* expression levels with respect to bacterial density, as most reports which have attempted to determine the relationship between inflammation and bacterial density have instead used IFNγ as a marker for inflammation (Bamford *et al.*, 1998; Karttunen *et al.*, 1995; Sommer *et al.*, 1998). Yamaoka *et al.* (1999) did however demonstrate a positive relationship between bacterial load and IL-8 production. Although the less sensitive methods of quantitative culture and ELISA were used in comparison to the current study, their data set was much larger. This positive correlation was also demonstrated between bacterial density and the degree of mononuclear cell and polymorphonuclear cell infiltration. Moreover, they investigated the relationship in *cagA* positive strains only, and divided the groups according to disease profiles (Yamaoka *et al.*, 1999). This data does highlight the need to correct for such parameters, which was not possible in the current study as sample sizes were too small. In contrast, a study of *H. pylori* infection in children, observed few cases where IL-8 staining co-localised with bacterial density measured by immunofluorescence, and in many cases no *H. pylori* colonisation was evident in areas stained for IL-8 (Camorlinga-Ponce *et al.*, 2003). Therefore supporting the hypothesis that an inverse relationship may exist between inflammation and bacterial density.

Although quantitative real time PCR is an accurate tool for determination of cytokine expression and bacterial colonisation, the use of immunostaining may be beneficial in assessing this relationship in more detail as demonstrated by Camorlinga-Ponce *et al.* (2003), and would allow us to confirm if indeed low levels of IL-8 are observed within these areas of high *H. pylori* colonisation (or vice versa). Of further interest would be the relationship between the number of inflammatory cells in the gastric mucosa and bacterial load. Histology is useful in determination of the extent of the inflammatory infiltrate in the gastric mucosa. However, mononuclear cells observed by histology encompass all T helper subsets, including suppressive T helper cells, and is therefore not truly representative of the inflammatory infiltrate. Bearing this in mind, the use of FACs for determination of the number of Th1 or Th17 cells in gastric tissue may

be more appropriate for investigation of the relationship between *H. pylori* density and the number of inflammatory cells. Previously, IFNγ has been extensively used as a marker for Th1 cells (Bamford *et al.*, 1998; Sommer *et al.*, 1998). A clear limitation of this approach is the need for different sets of gastric biopsies for FACs analysis and for bacterial quantification. This was not the case for the method applied in the current study as RNA and DNA was extracted from the same biopsy for quantification of *IL8* and bacterial load.

IL-8 is an important cytokine expressed in response to *H. pylori*, however it is not representative of pro-inflammatory responses as a whole, and other cytokines must also be investigated to determine if inflammation is related to bacterial density. As mentioned, IFNγ has been shown to be inversely associated with bacterial density in mouse models (Bamford *et al.*, 1998; Karttunen *et al.*, 1995; Sommer *et al.*, 1998). Furthermore, IFNγ has been associated with atrophy, inflammation and hyperplasia, demonstrating the importance of the cytokine to *H. pylori*-associated disease (Sayi *et al.*, 2009), and supporting the need to investigate the role of IFNγ in determining bacterial density in humans.

Although a simple negative correlation was not observed between *IL8* expression levels and bacterial load, *IL8* levels were upregulated in the gastric mucosa of *H. pylori* infected compared to uninfected samples. Importantly, *IL8* expression was found to be associated with the level of mononuclear cell infiltration in the gastric mucosa, thus demonstrating that IL-8 is a key cytokine expressed during an inflammatory response to *H. pylori* infection.

IL-8 is a chemoattractant factor for neutrophils, and as would be expected, a positive association has been previously observed between *IL8* and neutrophil infiltration in the gastric mucosa (Mizuno *et al.*, 2005; Suzuki *et al.*, 2004b). In the current study, severe neutrophil infiltration (an activity score of 3) was not observed in the data set examined. A trend towards higher *IL8* expression was observed in both the antrum and corpus between mild and moderate, although not statistically significant, which may be a consequence of sample size. Increased *IL8* expression was observed where activity scores were greater than 0 compared to samples with activity scores of 0.

6.3.2 The effect of cytokine stimulation on bacterial load

In vitro culture assays were used to determine if pro-inflammatory cytokines, which are known to induce IL-8 production, have an effect on bacterial density. Cytokine stimulation of gastric AGS cells in the presence of *H. pylori* strain 60190 or Tx30a had no impact on bacterial CFU counts. This was surprising, particularly as both IL-1β and TNFα have been shown to induce the expression of human β-defensin 2 in AGS cells, which has potent bactericidal activity against *H. pylori* (O'Neil *et al.*, 2000). One experimental factor that may have suppressed the response to cytokine stimulation is the use of serum rich media. In this study, cells were maintained and cultured in FBS-supplemented F-12 ham medium. Both have been shown to support higher growth rates of *H. pylori* (Douraghi *et al.*, 2010; Sainsus *et al.*, 2008), therefore the culture conditions may have enhanced the rate of *H. pylori* growth and masked the epithelial cell response to cytokine stimulation. The choice of incubation time may also be of consideration. Although Douraghi *et al.* found bacterial viability to be maximal at 24 hours in comparison to later time point, the effect of growth supplements at earlier time points were not investigated (Douraghi *et al.*, 2010). In addition, hβD2 expression was previously shown to be highest at 4 hours post infection in comparison to 24 hours (O'Neil *et al.*, 2000). These factors merit the investigation of cytokine stimulation of gastric epithelial cells and their effect on bacterial density at earlier time points.

IL-8 secretion levels in AGS cells in response to cytokine stimulation were also investigated. Increased IL-8 secretion was observed when uninfected AGS cells were stimulated with IL-1β (*H. pylori*-, IL-1β+ AGS cells), and also with unstimulated *H. pylori* infected AGS cells. The highest level of IL-8 was observed with *H. pylori* infected AGS cells that were stimulated with IL-1β. Interestingly, the levels detected for IL-1β+ uninfected cells plus that measured in unstimulated *H. pylori* infected cells would be similar to that of AGS cells that were infected with *H. pylori* and treated with IL-1β, suggesting an additive effect. The same was observed for TNFα stimulation.

Of interest was the level of IL-8 detected in response to IL-17 stimulation. In this study, IL-17 did not induce IL-8 secretion in AGS cells alone. However, when *H. pylori* infected AGS cells were stimulated with IL-17, an increase in IL-8

secretion was observed. This could be due to direct effects of bacterial infections or as a result of the induction of other cytokines. It is also possible that *H. pylori* infection stimulates the expression of IL-17 receptor. Of note is a study by Liang *et al.* who reported increased *hBD2* expression after IL-17 stimulation of human keratinocytes (Liang *et al.*, 2006). If this also applies to gastric epithelial cells, then it suggests that with increased epithelial cell responsiveness to IL-17 during infection, this cytokine may be essential as an inflammatory mediator in response to *H. pylori* infection. In contrast, previous studies have demonstrated increased IL-8 secretion in IL-17 stimulated, uninfected AGS cells. However, different culture conditions were used to the current study, which may induce secretion of IL-8. Also, studies have shown a further increase in IL-8 secretion after infection with *H. pylori* in comparison to uninfected cells, supporting that found in the current study (Mizuno *et al.*, 2005; Zhou *et al.*, 2007).

As mentioned, IL-17 and IL-22 cooperatively enhance defence against *Klebsiella pneumonia* (Aujla et al., 2008). This cooperative relationship was also investigated in *H. pylori* infection, but was not found to influence bacterial CFU counts in AGS cultures. Furthermore, stimulation of AGS cells with IL-22 alone, or in combination with IL-17, did not induce IL-8 secretion in the presence or absence of *H. pylori*. However, whether AGS cells express the IL-22 receptor must be established before it can be concluded that IL-22 responses are not targeted towards increasing IL-8 secretion. Little is known of the signalling pathways that are associated with IL-22 receptor activation, although most studies to date have demonstrated the activation of JAK/STAT pathways, particularly the activation of STAT3 (Wolk *et al.*, 2010). As *IL8* expression is regulated by NFκB, IL-22 is unlikely to activate IL-8 production. The fact that receptor expression has previously been found in the stomach, and IL-22 reportedly functions to upregulate AMP secretion (Wolk *et al.*, 2010) suggests a potential for IL-22 based cell responses during *H. pylori* infection (Wolk *et al.*, 2004).

6.3.3 Characterisation of the relationship between *IL8* expression levels with bacterial virulence factors and disease.

H. pylori virulence factors are commonly associated with gastroduodenal disease induced by infection with the bacterium. Many of these are thought to enhance disease risk through the induction of pro-inflammatory cytokine release from epithelial cells (Robinson *et al.*, 2007). The relationship between *H. pylori* virulence factors and the degree of *IL8* expression was determined in order to investigate this relationship in the population examined for the current study.

Overall, *IL8* expression levels were not associated with strain genotype. Although CagPAI activity is associated with increased *IL8* expression (Brandt *et al.*, 2005; Eaton *et al.*, 2001a), an association was not observed between *cagA* status and *IL8* mRNA levels. However, as only 4 isolated strains were genotyped as *cagA* negative, statistical analysis was difficult. Furthermore, *cagA* genotyping does not account for CagPAI functionality, which may introduce error into the analysis.

Little is known about the function of DupA, however recent studies have demonstrated the association between this virulence factor and *IL8* expression (Hussein *et al.*, 2010; Lu *et al.*, 2005). Recent work in our group found an association between *dupA* and *IL8* expression in gastric tissue samples donated by *H. pylori* infected patients (Hussein *et al.*, 2010). This result was not observed, however, when a larger data set was examined, as demonstrated in the present study. Furthermore, our group demonstrated that like *vacA*, gene variability is seen within the *dupA* gene, giving rise to the extended *dupA1* type and the shorter *dupA2*. Strain genotyping of *H. pylori* isolates in the current study did not investigate the effect of *dupA* gene polymorphism, which may have a significant impact on cytokine expression.

As mentioned, increased risk of developing *H. pylori*-associated disease is principally a result of an aggressive pro-inflammatory response. In the present study, increased *IL8* expression was observed in the antrum with DUD in comparison to gastritis, although not statistically significant, and an association was not observed with pathology.

Overall, specific inflammatory responses did not correlate with bacterial load *in vitro*, and the *IL8* expression levels may be inversely related to bacterial load when the equilibrium established between the bacterium and host is disrupted. However, other markers of inflammation must be investigated in order to determine if pro-inflammatory responses are related to bacterial density. Evidence now suggests that the T cell response to infection is mixed, with a regulatory T cell response also induced during *H. pylori* infection (Goll *et al.*, 2007). Whether such suppressive cells are capable of influencing bacterial colonisation must also be investigated.

Chapter 7

7. ANTI-INFLAMMATORY HOST RESPONSES TO *H. PYLORI* INFECTION IN THE GASTRIC MUCOSA

7.1 INTRODUCTION

The immune response has evolved to protect the host from a wide range of potentially pathogenic microorganisms. In parallel, mechanisms to control excessive responses and prevent reactivity to self antigens are required to limit host damage. Regulatory T-cells are essential for such control.

As mentioned previously (Chapter 1, section 1.7.2.4), regulatory T cells are key to the modulation of inflammation, and one mechanism by which they exert such regulation is through the secretion of suppressive cytokines such as IL-10 and TGF-β, both of which have been shown to be important in the host response to *H. pylori* infection (Chen *et al.*, 2001; Kao *et al.*, 2010; Li & Li, 2006; Maloy *et al.*, 2003).

7.1.1 Interleukin 10 (IL-10)

IL-10 is an anti-inflammatory cytokine that plays a crucial role in preventing inflammatory and autoimmune pathologies (Moore *et al.*, 2001). This cytokine was initially thought to be a product of Th2 cells that inhibit Th1 inflammatory responses. Since the realisation that the repertoire of T helper cell types is more expansive than the Th1/Th2 paradigm, IL-10 production has been associated with regulatory T cells as well as Th1, Th17, $CD8^+$ T cells and B cells (Maynard & Weaver, 2008; Moore *et al.*, 2001; Trinchieri, 2007). In addition, the production of IL-10 has been reported in cells of the innate immune response such as DCs, macrophages, mast cells, eosinophils and neutrophils (Moore *et al.*, 2001).

The IL-10 receptor is composed of the IL-10Rα chain, which is unique to IL-10 signalling and is expressed on hematopoietic cells, and the IL-10Rβ chain which is ubiquitously expressed and is also involved in IL-22 signalling (Moore *et al.*,

2001). Stimulation of the receptor activates Jak1 signalling pathways (O'Shea & Murray, 2008), and it is believed that Stat3 is required for the inhibitory functions of IL-10. In addition, other signalling pathways play a role in the anti-inflammatory function of IL-10 (Calado *et al.*, 2006). A particularly pertinent mechanism for *H. pylori* infection is the ability of IL-10 to prevent NF-κB activation, and thus suppress pro-inflammatory gene expression including IL-8 (Robinson *et al.*, 2008).

IL-10 appears to indirectly affect T cell and NK cell function by inhibiting MHC class II and B7-1/B7-2 expression on monocytes and macrophages, and limiting the production of pro-inflammatory cytokines such as IL-1β, TNFα, IL-8 and RANTES (Moore *et al.*, 2001). Furthermore, IL-10 signalling in DCs has been shown to inhibit chemokine production and prevent the trafficking of these cells to lymph nodes, resulting in a disruption of Th1 differentiation and recruitment (Demangel *et al.*, 2002). Direct effects of IL-10 on $CD4^+$ T cells have also been reported through inhibition of T cell proliferation and the production of pro-inflammatory cytokines (Joss *et al.*, 2000; Moore *et al.*, 2001; Taylor *et al.*, 2007).

The importance of IL-10 in the regulation of inflammatory responses was first suggested by Kuhn *et al.* who demonstrated IL-10 to be essential in the modulation of gut inflammation (Kuhn *et al.*, 1993). During infection with *H. pylori*, increased *IL10* expression has been observed in the gastric mucosa (Goll *et al.*, 2007; Robinson *et al.*, 2008). In *H. pylori* infected $IL\text{-}10^{-/-}$ mice, severe inflammation coupled with reductions in colonisation density has been demonstrated. This suggests an essential role for this cytokine in modulating inflammatory responses, which favours persistence of *H. pylori* infection (Chen *et al.*, 2001). Interestingly, Berg *et al.* also found a greatly exacerbated inflammation and pre-malignant pathology in the gastric mucosa of *H. felis* infected $IL\text{-}10^{-/-}$ mice, suggesting a role for this cytokine in limiting disease progression (Berg *et al.*, 1998). In addition, our group have shown low levels of IL-10 secreting T-cells in the gastric mucosa of *H. pylori* infected patients with peptic ulcer disease (Robinson *et al.*, 2008). Therefore, IL-10 appears to be important for the modulation of inflammatory responses elicited in response to *H. pylori*.

As mentioned previously, polymorphisms in the *IL10* gene have been reported (Chapter 1, section 1.2.2), which alter the amount of IL-10 secreted, and therefore have an impact on the inflammatory response to infection and risk of developing disease.

7.1.2 Transforming growth factor β (TGF-β)

Transforming growth factor β (TGF-β) belongs to a family of cytokines with pleiotropic functions, having potent regulatory and inflammatory activity. Its effects on numerous immune functions are dependent on the cellular and environmental context within which it is expressed (Li *et al.*, 2006b). Unlike most other cytokines, TGF-β is produced by many immune and non-immune cells, and virtually all cell types are responsive to the cytokine (Li *et al.*, 2006b).

There are three forms of TGF-β in mammals (TGF-β1, TGF-β2, and TGF-β3), of which TGF-β1 is the major form expressed in the immune system. The active form of the cytokine binds to the TGF-β receptors (TGF-βRI and TGF-βRII), which are widely expressed on hematopoietic and nonhematopoietic cells (Li *et al.*, 2006b). Binding of TGFβ triggers the kinase activity of the receptor cytoplasmic domain, which ultimately leads to nuclear translocation of Smad molecules, and the transcription of target genes (Derynck & Zhang, 2003; Shi & Massague, 2003).

TGF-β is important for the regulation of immune responses and the induction of peripheral tolerance. One mechanism by which TGF-β maintains peripheral tolerance is through the maintenance and survival of naturally occurring Treg cells (Li *et al.*, 2006a). In the periphery, TGF-β is necessary for the survival of naive T cells (Li *et al.*, 2006b), and in the presence of IL-2 and retinoic acid (RA), it promotes the differentiation of induced Treg cells (iTreg) (Coombes *et al.*, 2007; Davidson *et al.*, 2007) (Figure 7.1). TGF-β also plays an important role in suppressing the responses of innate immune cells (Laouar *et al.*, 2005; Laouar *et al.*, 2008; Town *et al.*, 2008).

Furthermore, TGF-β is also known to be involved in the induction of pro-inflammatory responses. In the presence of IL-6, TGF-β stimulates the

differentiation of T helper 17 (Th17) cells, which can promote further inflammation in the presence of IL-23 (Bettelli *et al.*, 2006) (Figure 7.1).

Increased levels of TGF-β have been demonstrated in the gastric mucosa of *H. pylori* individuals in comparison to uninfected patients (Li & Li, 2006; Robinson *et al.*, 2008). Similar to IL-10, expression of the cytokine tended to be negatively associated with the development of peptic ulcer disease, although this trend for TGF-β did not reach statistical significance (Robinson *et al.*, 2008). The importance of TGF-β during *H. pylori* infection was also demonstrated in mouse studies reporting that neutralisation of the cytokine was associated with an increased severity of gastritis (Kao *et al.*, 2010). Interestingly, the expression of TGF-β by *H. pylori* infected gastric cell lines appears to be controversial. Some studies have observed increased expression in numerous gastric cell lines, including AGS cells (Wu *et al.*, 2007). However, another study failed to show an effect on cytokine levels on infection with the bacterium in AGS cells, as well as other gastric cell lines (Nakachi *et al.*, 2000).

Single nucleotide polymorphisms in the *TGFB1* gene have been observed at positions -509, -869 and -915, and an insertion in the 5'-untranslated region (5'-UTR) (at position +72) has also been identified. These mutations appear to be associated with a reduced risk of developing duodenal ulcers (Polonikov *et al.*, 2007), and an association with gastric cancer has not been reported (Garcia-Gonzalez *et al.*, 2007; Seno *et al.*, 2007). However, a recent study reported a relationship between the -509 polymorphism and an increased risk of developing premalignant lesions (Achyut *et al.*, 2009).

Overall, these studies demonstrate the importance of TGF-β during infection with *H. pylori* and the progression of disease.

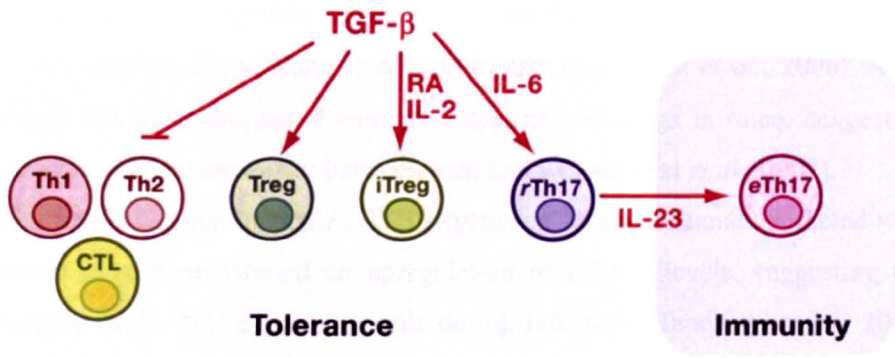


Figure 7.1: Role of TGF-β in the regulation of T cell differentiation.
TGF-β controls T cell tolerance through direct inhibition of T helper 1 (Th1), Th2, and cytotoxic T lymphocyte (CTL) differentiation. TGF-β is involved in the maintenance of regulatory T (Treg) cells, and also promotes the differentiation of induced Treg (iTreg) cells, which also requires the presence of IL-2 and retinoid acid (RA). However, when IL-6 is present, TGF-β drives the differentiation of Th17 cells and maintains these cells in a regulatory state (rTh17). IL-23 stimulation of rTh17 cells in the absence of TGF-β induces their differentiation into effector Th17 cells (eTh17) (Taken from Li & Flavell, 2008).

7.1.3 FOXP3

As mentioned, *FOXP3* is a transcription factor that is expressed in subsets of T cells, and has key functions in the suppression of immune responses (Chapter 1, section 1.7.2.4). In mice, *foxp3* expressing cells also express CD25, and *foxp3* mutant or deficient mice develop severe autoimmune disease, demonstrating that Foxp3 is a key regulator of Treg cell development and function in mice (reviewed by Buckner & Ziegler, 2008). However, the Treg population of cells in humans is heterogeneous, and the role of FOXP3 in human Tregs is less straightforward. Although FOXP3 is required for human Treg cell development and function, stable and high expression of the transcription factor is required to maintain regulatory function (reviewed in Sakaguchi *et al.*, 2010). Furthermore, *FOXP3* expression is not sufficient for regulatory function, as a proportion of *FOXP3* expressing cells do not possess suppressive activity (Allan *et al.*, 2007; Morgan *et al.*, 2005; Wang *et al.*, 2007). In addition, a significant proportion of T cells with suppressive activity do not express FOXP3, for example Tr1 cells (O'Garra &

Vieira, 2007). The expression of *FOXP3* has been shown to be induced by cytokines such as IFNγ (Wang *et al.*, 2006) and IL-2 (Zorn *et al.*, 2006). Also, oestrogen has been associated with the expansion of Tregs in mice, suggesting that Treg responses may differ between men and women (Tai *et al.*, 2008).

Studies that have investigated *FOXP3* expression levels in humans infected with *H. pylori* have demonstrated an upregulation of mRNA levels, suggesting the importance of *FOXP3* expressing cells during infection (Kandulski *et al.*, 2008; Robinson *et al.*, 2008).

7.1.4 Regulatory T cell responses in *H. pylori* infection

The importance of Tregs for the modulation of the inflammatory response and persistence of *Helicobacter* infections has been demonstrated widely in mouse models. In a study by Rad *et al.*, depletion of $CD25^+$ T cells in C57BL/6 mice infected with *H. pylori* resulted in the development of severe gastritis with increased infiltration of mucosal T cells and macrophages and a reduction in bacterial load (Rad *et al.*, 2006). In support of this data, Maloy *et al.* (2003) demonstrated the suppression of immune responses when $RAG^{-/-}$ mice infected with *H. hepaticus* were reconstituted with $CD4^+CD25^+$ T cells, a suppression dependent on IL-10 and TGF-β. Furthermore, a study by Lee *et al.* (2007) using *H. pylori* infected $RAG^{-/-}$ mice showed decreased bacterial loads after transfer of effector T cells. Following the introduction of Tregs however, increased CFU counts were recovered and a reduction in the expression of inflammatory cytokines IFN-γ and TNF-α was observed. These studies demonstrate the role of regulatory T-cells in the persistence of infection and the attenuation of deleterious host responses associated with *H. pylori* infection in mouse models.

Studies in humans have reported increased expression of the Treg markers FOXP3, CTLA-4, IL-10 and TGF-β in gastric tissue of *H. pylori* infected patients in comparison to uninfected controls (Lindholm *et al.*, 1998; Lundgren *et al.*, 2005b; Robinson *et al.*, 2008). The association between Treg responses and colonisation density in humans is particularly poorly studied.

7.1.5 *H. pylori* virulence factors and immunomodulatory responses

Whether *H. pylori* virulence determinants are associated with the modulation of suppressive immune responses is unknown. Antigens present in the lamina propria as a result of virulence factor-induced mucosal damage and leakage (described in Chapter 1, section 1.6) are capable of activating Treg cells. As the degree of virulence of the colonising strain determines the extent of mucosal damage, it is feasible to hypothesise that strain virulence may be associated with Treg responses (reviewed and discussed in Raghavan & Holmgren, 2005). In support of this hypothesis is the recent suggestion that the Treg response to *H. pylori* infection may be implicated in the protection against atopic disease such as asthma, with large epidemiological studies reporting a negative association between the disease and the incidence of *H. pylori* infection (Blaser *et al.*, 2008; Chen & Blaser, 2007), an association that was found to be strongest with *cagA* positive strain infections (Chen & Blaser, 2008).

Recently, the neutrophil activating protein of *H. pylori* (HP-NAP) was shown to strongly inhibit bronchial inflammation and to reduce Th2 cytokine production when administered to ovalbumin sensitised mice, suggesting this virulence factor may be an important candidate in the prevention of asthma during *H. pylori* infection and may therefore play a role in induction of suppressive responses (Codolo *et al.*, 2008).

Therefore, a relationship may exist between *H. pylori* virulence determinants and Tregs responses, and hence *H. pylori*-associated disease.

7.1.6 Tregs and cancer

As well as modulating gastric inflammation during *H. pylori* infection (Chen *et al.*, 2001; Kuhn *et al.*, 1993), Tregs may encourage cancer development through inducing tolerance to tumours (Yamaguchi & Sakaguchi, 2006). Activated tumour-specific immune cells have been found in cancer patients; however, the immune response fails to prevent tumour growth, suggesting a mechanism of escape or suppression is induced.

Tumour antigens are derived from the host, and many are also self-antigens, therefore promoting tumour tolerance through the activation of Tregs, which protect against immunity to self (Pardoll, 2003). In support of this hypothesis,

murine tumour models have shown that transfer of $CD4^+CD25^+$ Tregs can inhibit tumour-specific $CD8^+$ cell-mediated immunity (Antony *et al.*, 2005), whereas depletion of $CD25^+$ Tregs significantly enhanced tumour rejection (Onizuka *et al.*, 1999; Shimizu *et al.*, 1999). Furthermore, an increased frequency of $CD4^+CD25^+FOXP3^+$ Tregs have been found in patients with various types of cancers (Zou, 2006), and increased $CD4^+CD25^{hi}$ cells were found in patients with gastric cancer (Kawaida *et al.*, 2005; Kono *et al.*, 2006; Sasada *et al.*, 2003). Treg responses are therefore associated with poor prognosis. In addition, the tumour environment has been shown to promote Treg responses through secretion of tumour-specific antigens capable of activating Tregs (Nishikawa *et al.*, 2005). Furthermore, the tumour cell environment induces the secretion of TGF-β from immature myeloid DCs, which in turn promotes Treg proliferation (Ghiringhelli *et al.*, 2005).

We have previously found that low-level mucosal expression of Treg-associated genes were associated with the presence of PUD, but whether a similar putative protective association existed with the development of gastric adenocarcinoma is not known. However, as Treg responses are elevated during cancer (Kuhn *et al.*, 1993; Lundin *et al.*, 2007), it is difficult to assess whether Treg responses are beneficial in preventing carcinogenesis. It may be more appropriate to investigate this relationship at stages of pre-cancerous pathology.

7.1.7 Experimental aims

- To investigate the association between anti-inflammatory responses and bacterial load.

- To investigate the relationship between bacterial virulence determinants and the anti-inflammatory response.

- To determine the relationship between inflammation and anti-inflammatory responses.

- To assess the degree of anti-inflammatory responses in *H. pylori* associated disease and pathology.

7.2 RESULTS

Numerous studies have demonstrated that a regulatory T cell response is induced during *H. pylori* infection in the human gastric mucosa. These responses have been characterised by quantifying expression levels of IL-10, FOXP3 and TGF-β, and increased numbers of Tregs (Lindholm *et al.*, 1998; Lundgren *et al.*, 2005b; Robinson *et al.*, 2008).

7.2.1 The relationship between Treg-gene expression and *H. pylori* density in human gastric mucosal tissue

As mentioned, Treg responses have previously been linked to the density of *H. pylori* infection in mouse models, where the depletion of Tregs or neutralisation of suppressive cytokines has been associated with reduced bacterial loads (Kao *et al.*, 2010; Rad *et al.*, 2006). Conversely, adoptive transfer of Tregs was found to increase bacterial burden (Lee *et al.*, 2007a). In order to determine if such a relationship exists in the human stomach, the density of infection and the level of the Treg response and cytokine gene expression was investigated in human gastric mucosal biopsies.

IL10 expression levels and bacterial colonisation densities were determined in 33 antral gastric biopsies. An association was not observed ($r=0.21$, $p=0.24$). No association was observed between expression levels of *FOXP3* and *TGFB1* and bacterial load measured in 21 antral gastric biopsies (Figure 7.2).

Overall, although we have previously demonstrated that treatment of *H. pylori* infected cells with recombinant IL-10 *in vitro* induced increased bacterial numbers (Robinson *et al.*, 2008), suppressive responses *in vivo* were not associated with bacterial density.

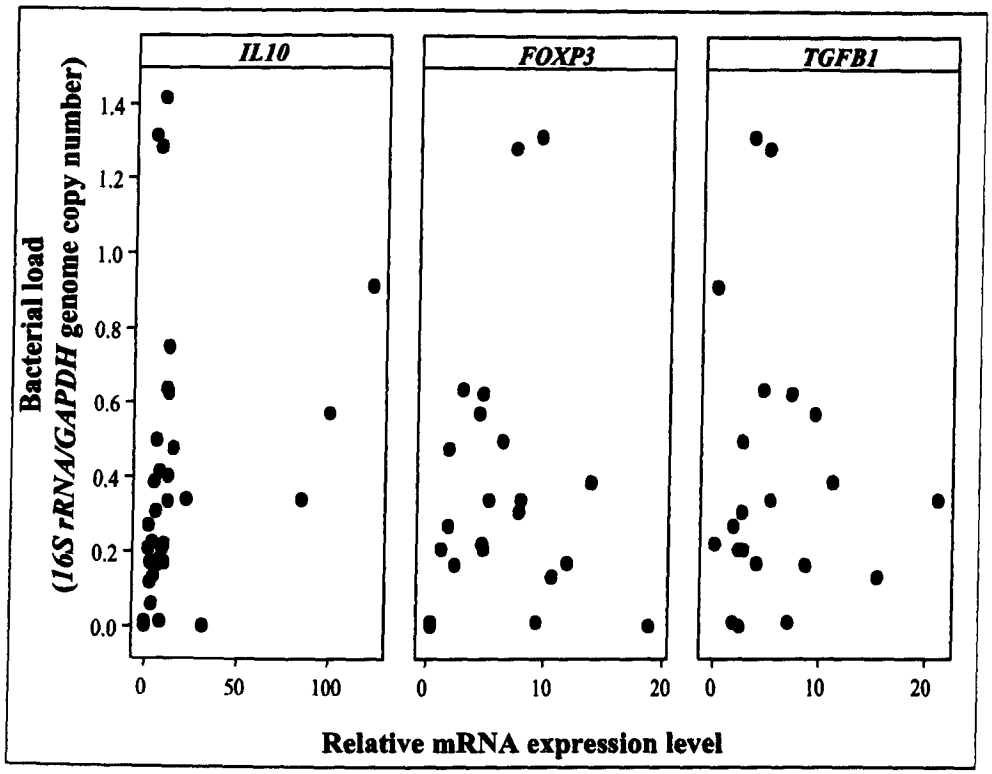


Figure 7.2: The relationship between bacterial load and Treg-associated gene expression levels in the gastric mucosa.

Bacterial load and *IL10* mRNA expression levels were determined in 33 *H. pylori* infected antral gastric tissue biopsies. *FOXP3* and *TGFB1* expression levels were determined in 21 antral biopsies for which bacterial load was also quantified.

To confirm that suppressive immune responses are upregulated in the *H. pylori* infected gastric mucosa, the expression level of *IL10*, *FOXP3* and *TGFB1* were determined for 50 antral gastric biopsies. Cytokine expression was also assessed in 14 uninfected antral biopsies. Increased levels of *IL10*, *FOXP3* and *TGFB1* were found in *H. pylori* infected compared to uninfected individuals (Figure 7.3). Therefore, although expression levels were not associated with bacterial load, increased expression was found in comparison to uninfected individuals, as previously demonstrated (Lindholm *et al.*, 1998; Lundgren *et al.*, 2005b; Robinson *et al.*, 2008).

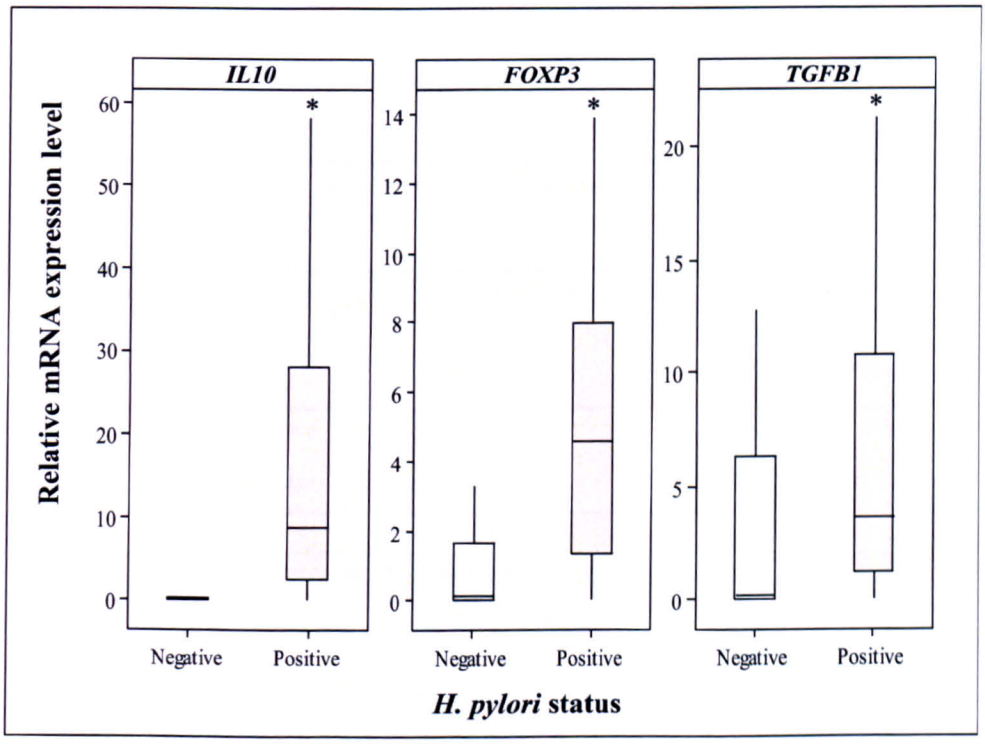


Figure 7.3: Treg associated gene expression in the gastric mucosa of *H. pylori* infected and uninfected patients.

IL10, FOXP3, and *TGFB1* expression levels were determined for 50 *H. pylori* infected and 14 uninfected antral gastric biopsies. Data were normalised relative to *GAPDH* expression, and a sample from an uninfected donor was used as an internal comparator to provide a fold-difference. * $p<0.03$.

7.2.2 The effects of anti-inflammatory cytokines on bacterial density *in vitro*

As we have previously demonstrated increased bacterial CFU counts in response to IL-10 *in vitro* (Robinson *et al.*, 2008), the effects of TGF-β on bacterial colonisation were assessed here, and the effects of IL-10 were repeated. AGS cells were infected with *H. pylori* strains 60190 and Tx30a at an MOI of 100, and incubated for 24 hours before viable counts in 6 replicates were determined. The presence of 50ng/ml IL-10 resulted in an increase in CFU counts as previously found (2-fold increase with 60190, 1.4-fold with Tx30a), however, this only achieved statistical significance with Tx30a. The treatment of *H. pylori* 60190 infected AGS cells with TGF-β resulted in only a small increase in CFU counts (1.2-fold increase), but this was not the case when infected with Tx30a (Table 7.1).

	Average CFU count/ml after 24hrs (SD)	
	IL-10	**TGFβ**
AGS cells cultured with *H. pylori* 60190		
+ *H. pylori*	3.55×10^7 (1.72×10^7)	6.44×10^8 (9.01×10^6)
+ *H. pylori* + cytokine	5.74×10^7 (2.51×10^7)	7.63×10^8 (5.72×10^6)
P	0.06	0.02
AGS cells cultured with *H. pylori* Tx30a		
+ *H. pylori*	1.34×10^8 (3.55×10^7)	1.08×10^8 (1.01×10^7)
+ *H. pylori* + cytokine	1.89×10^8 (4.99×10^7)	1.06×10^8 (1.25×10^7)
P	0.03	NS

Table 7.1: The effect of anti-inflammatory cytokine stimulation on bacterial load when cultured with human gastric epithelial cells.

AGS cells were treated with 50ng/ml of cytokine and infected with either *H. pylori* strains 60190 or Tx30a (MOI=100). After 24hrs, the average number of colony forming units (CFU/ml) in 6 replicates was determined. Standard deviation (SD) is shown. NS = not significant.

7.2.3 The association between Treg gene expression levels and virulence determinants of *H. pylori*

Although a Treg response is induced during *H. pylori* infection, whether bacterial factors are associated with driving such a response is still unknown. Previously, increased levels of *IL10* mRNA were found in the corpus of patients infected with *cagA*+ strains compared to *cagA*- infections. This trend was also observed in the antrum, but was not statistically significant (Hida *et al.*, 1999). However, numerous other studies have failed to show an association between bacterial virulence and Treg responses (Goll *et al.*, 2007; Obonyo *et al.*, 2006; Wu *et al.*, 2007). As strain genotype information was available for the data set examined here, the relationship between Treg-associated gene expression levels and virulence of the colonising strain was assessed.

IL10, FOXP3 and *TGFB1* mRNA expression levels were determined in 39 gastric biopsy samples for which *cagA* status was also available, in 30 samples with known *dupA* status, and 35 with known *vacA* genotype. As only 4 samples in the data set were genotyped as s2-type strains, the s-region was not considered for further analysis. Expression levels of Treg associated genes stratified according to strain virulence are presented in Table 7.2.

A trend towards increased levels of *IL10* was observed for *cagA*+ strains, although not statistically significant. This trend was not observed with *FOXP3* and *TGFB1* expression levels.

A trend towards increased *FOXP3* expression was observed with *dupA*+ strains, although not statistically significant. This was not observed with *IL10* and *TGFB1* expression. As *cagA* has previously been shown to be associated with increased *IL10* mRNA levels, *dupA* status was controlled for the *cagA* status of the colonising strain. When looking at *dupA*+*cagA*+ strains, increased *FOXP3* expression was found in comparison to *dupA*-*cagA*+ ($p=0.04$). A similar trend was also seen with *IL10* expression, but did not achieve statistical significance. Such a trend was not observed for *TGFB1* expression levels. As only 9/39 strains were found to be *cagA*-, it was not possible to assess Treg-associated gene expression when correcting for *cagA*-status.

Treg-associated gene expression levels did not differ with *vacA* m-region allelic type, even when controlled for *cagA*+ status. Increased expression of *TGFB1* was observed with i2-type strains compared to i1-type strains ($p=0.02$). This trend was

still present when controlled for *cagA*+ status, but was not statistically significant. No difference in *IL10* and *FOXP3* expression was found between *vacA* i-region alleic types.

	Treg gene expression level					
	IL10		*FOXP3*		*TGFB1*	
	Median (n)	**IQR**	**Median (n)**	**IQR**	**Median (n)**	**IQR**
cagA +	6.06 (31)	2.77-22.5	4.70 (31)	1.36-8.12	2.79 (30)	1.12-8.47
cagA -	1.93 (9)	0.00-17.5	4.40 (11)	1.05-9.37	2.79 (9)	2.14-8.34
P	NS		NS		NS	
dupA +	7.63 (13)	3.11-27.0	6.83 (10)	4.00-9.05	2.64 (12)	1.72-9.75
dupA -	6.06 (20)	1.40-28.9	3.84 (22)	0.51-10.0	2.79 (22)	0.84-7.40
P	NS		NS		NS	
dupA+ (*cagA*+)	8.21 (10)	5.25-24.7	7.26 (9)	5.58-9.97	2.60 (9)	1.75-16.3
dupA- (*cagA*+)	3.71 (15)	0.28-22.9	2.57 (15)	0.23-9.39	2.71 (15)	0.16-4.05
P	NS		**0.04**		NS	
vacA m1	5.15 (24)	2.08-22.8	4.80 (25)	1.30-8.75	2.55 (23)	1.13-8.43
vacA m2	7.21 (20)	0.93-11.9	4.69 (18)	1.90-10.5	4.57 (18)	1.82-7.06
P	NS		NS		NS	
vacA m1 (*cagA*+)	8.79 (19)	2.77-24.1	4.85 (21)	2.18-8.75	2.45 (20)	1.08-11.8
vacA m2 (*cagA*+)	6.06 (20)	0.00-10.5	3.52 (10)	0.41-8.91	4.26 (10)	1.82-7.61
P	NS		NS		NS	
vacA i1	5.57 (21)	1.06-22.7	4.80 (25)	1.30-8.75	2.31 (19)	1.07-4.05
vacA i2	7.42 (16)	3.35-22.6	4.53 (18)	2.56-7.41	6.31 (16)	2.57-9.27
P	NS		NS		**0.02**	
vacA i1 (*cagA*+)	7.63 (19)	2.53-22.9	4.85 (17)	0.96-10.6	2.60 (17)	1.10-4.33
vacA i2 (*cagA*+)	5.18 (8)	3.35-11.5	4.65 (7)	2.38-6.40	5.59 (8)	1.96-10.6
P	NS		NS		NS	

Table 7.2: The association between Treg gene expression levels and *H. pylori* strain virulence.

H. pylori strains colonising gastric biopsies were genotyped for *cagA*, *dupA*, and *vacA* m- and i-region. *IL10, FOXP3* and *TGFB1* expression levels in antral gastric tissue were determined and stratified according to strain genotype. Data for *dupA* and *vacA* were also corrected for *cagA*+ status. Sample sizes (n) and interquartile ranges (IQR) are given. NS=not significant.

7.2.4 The relationship between suppressive host responses and the degree of inflammation of the gastric mucosa

As Treg responses play a role in dampening inflammation, a negative correlation between the degree of inflammation and the number of Tregs in the gastric mucosa would be expected. This has previously been demonstrated in a mouse study, where infection of $RAG2^{-/-}$ mice with *H. hepaticus* induced inflammation, but mice reconstituted with purified $CD4^{+}CD25^{+}$ cells developed reduced inflammation (Maloy *et al.*, 2003). To investigate the relationship between Treg responses and the level of inflammation in the human gastric mucosa, the expression levels of *IL10, FOXP3* and *TGFB1* were assessed in 50 antral gastric biopsies of *H. pylori* infected patients, and were stratified according to the level of inflammation as determined by histology.

The levels of *IL10* expression was significantly decreased in samples with inflammation scores greater than 1 (for grade 1: median=24.1, n=15, IQR=3.23-151.1; for grade 2: median=5.10, n=30, IQR=0.00-15.2; for grade 3: median=22.5, n=7, IQR=5.18-43.0). This was also the case with the level of *FOXP3* expression (for grade 1: median=6.7, n=13, IQR=3.04-19.5; for grade 2: median=4.09, n=30, IQR=1.2-6.83; for grade 3: median=2.38, n=7, IQR=1.78-7.89) (Figure 7.4). Levels of *TGFβ1* mRNA were not significantly different (for grade 1: median=2.88, n=16, IQR=0.10-32.0; for grade 2: median=2.71, n=30, IQR=1.18-7.28; for grade 3: median=9.93, n=4, IQR=6.13-18.8).

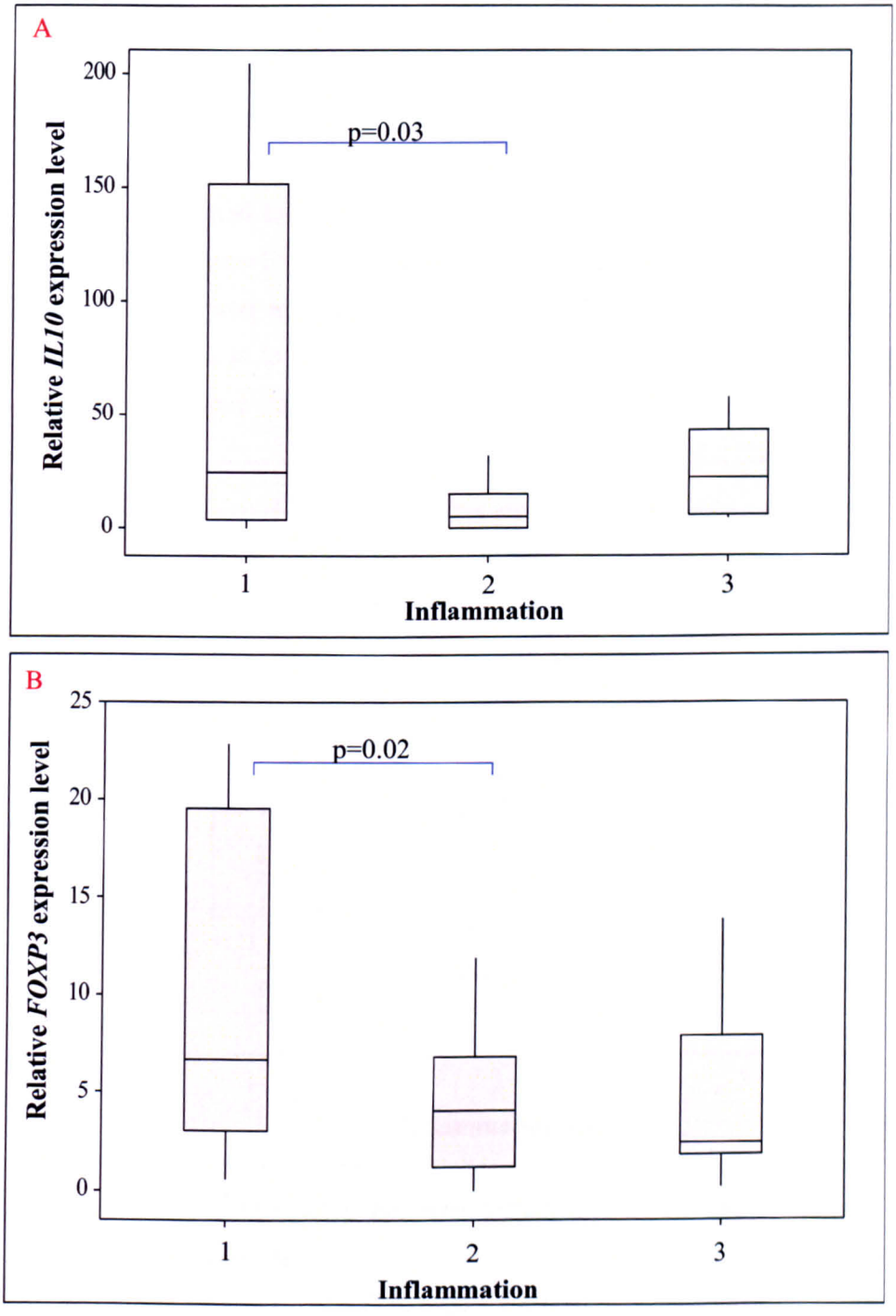


Figure 7.4: The relationship between Treg associated gene expression and inflammation in antral gastric tissue.

Expression levels of A- *IL10* and B- *FOXP3* were measured in 50 antral gastric biopsies of *H. pylori* infected patients, and stratified according to the degree of inflammation determined by histopathological scoring (grade 1=mild, grade 2=moderate, grade 3=severe).

The degree of inflammation was also assessed in comparison to the frequency of Treg cells in the gastric mucosa. The number of $CD4^+CD25^{hi}IL\text{-}10^+$ cells was assessed in pooled cells isolated from 6 antral gastric biopsies, and FACs analysis was completed by a colleague (Darren Letley). The numbers of $CD4^+CD25^{hi}IL\text{-}10^+$ were also found to negatively correlate with the severity of inflammation. With moderate (score 2) and severe (score 3) inflammation, fewer Treg cells were found in comparison to mild inflammation (p=0.006 and p=0.02 respectively) (Figure 7.5). This is in agreement with the negative association observed with Treg-associated gene expression levels (Figure 7.4).

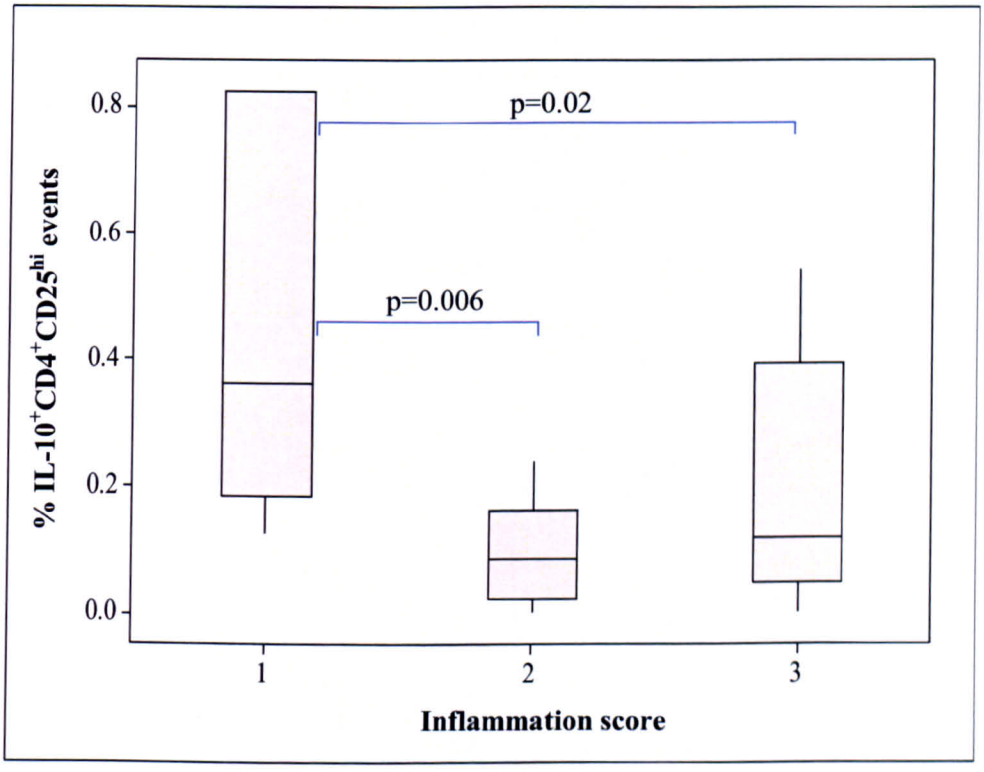


Figure 7.5: The relationship between inflammation and the frequency of Treg cells in the antrum.

The percentage of $IL10^+$ events amongst $CD4^+CD25^{hi}$ events were measured in antral gastric biopsies of 30 *H. pylori* infected patients, and stratified according to the degree of inflammation determined by histopathology. 1=mild inflammation (n=6); 2=moderate inflammation (n=18); 3=severe inflammation (n=6).

7.2.5 The relationship between suppressive immune responses and pre-malignant pathology in the gastric mucosa

We have previously demonstrated that increased Treg gene expression is associated with reduced occurrence of peptic ulcer disease (Robinson *et al.*, 2008). The investigation of Treg responses to cancer is complicated by the induction of cancer specific Tregs (Chattopadhyay *et al.*, 2005; Kosmaczewska *et al.*, 2008). In order to fully understand the relationship between *H. pylori* related Tregs and cancer progression, their association with precancerous changes in the gastric mucosa such as atrophy and metaplasia must be investigated. To assess this relationship, expression levels of *IL10, FOXP3* and *TGFB1* were measured in 50 antral gastric biopsies and were stratified according to the presence or absence of atrophy and IM. Of the 50 patients included, 21 patients had gastric atrophy and 12 had intestinal metaplasia in the stomach. Significantly lower expression levels of all 3 genes were observed in the presence of atrophy (Figure 7.6A). Where IM was present (scores 1-3), significantly lower levels of *FOXP3* and *IL10* mRNA were observed (Figure 7.6B). *TGFB1* mRNA levels were lower when IM was present but the difference did not reach statistical significance.

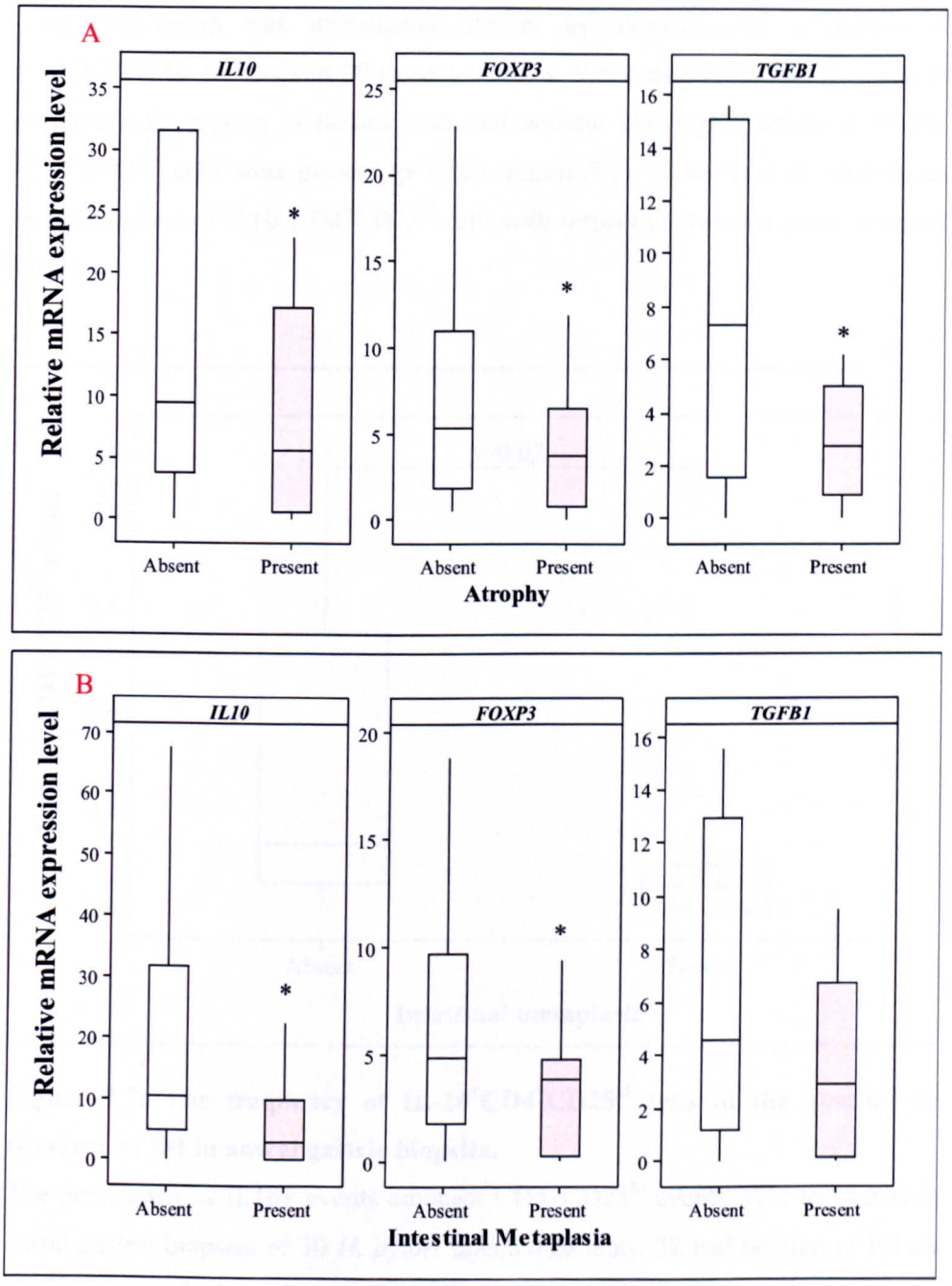


Figure 7.6: Investigating the relationship between Treg gene expression levels and premalignant pathology.

Expression levels of *FOXP3, IL10* and *TGFB1* were measured in 50 antral gastric biopsies of *H. pylori* infected patients, and stratified according to the presence or absence of A- atrophy or B- IM. * significantly reduced expression ($p<0.05$).

This relationship was investigated further by assessing the frequency of $CD4^+CD25^{hi}IL\text{-}10^+$ cells in 30 patient samples. When examining the frequencies of Treg cells present in tissues with and without IM, higher levels of $IL\text{-}10^+$ $CD4^+CD25^{hi}$ cells were present (p=0.02) (Figure 7.7). There were no differences in the numbers of $IL10^+CD4^+CD25^{hi}$ cells with respect to presence and absence of atrophy.

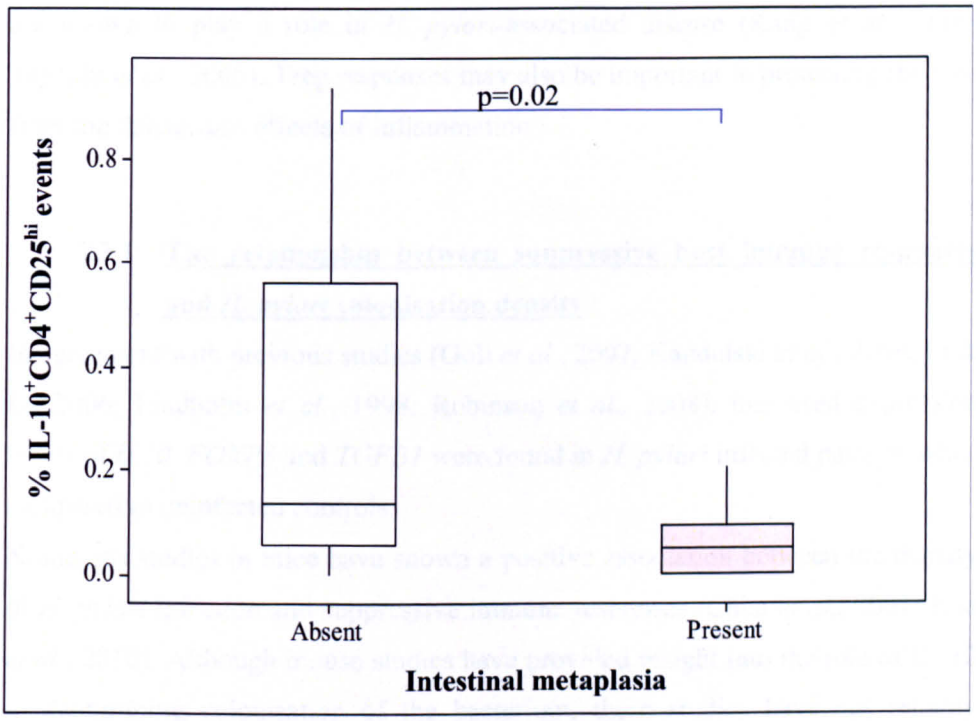


Figure 7.7: The frequency of $IL\text{-}10^+CD4^+CD25^{hi}$ cells in the absence or presence of IM in antral gastric biopsies.

The percentage of IL10+ events amongst $CD4+CD25^{hi}$ events were measured in antral gastric biopsies of 30 *H. pylori* infected patients. 22 had no sign of IM on histological analysis, and 8 had IM.

7.3 DISCUSSION

As well as a Th1 polarised immune response (Bamford *et al.*, 1998; Karttunen *et al.*, 1995; Mohammadi *et al.*, 1996), it is now known that a potent regulatory T cell response is also induced during infection with *H. pylori*. The suppression of inflammatory responses may play a role in the persistence of infection. Treg responses have been negatively associated with the level of inflammation and gastritis in mice (Kao *et al.*, 2010; Maloy *et al.*, 2003). As inflammatory responses are known to play a role in *H. pylori*-associated disease (Kang *et al.*, 2009; Taguchi *et al.*, 2005), Treg responses may also be important in protecting the host from the deleterious effects of inflammation.

7.3.1 The relationship between suppressive host immune responses and *H. pylori* colonisation density

In agreement with previous studies (Goll *et al.*, 2007; Kandulski *et al.*, 2008; Li & Li, 2006; Lindholm *et al.*, 1998; Robinson *et al.*, 2008), increased expression levels of *IL10, FOXP3* and *TGFB1* were found in *H. pylori* infected patients when compared to uninfected controls.

Numerous studies in mice have shown a positive association between the density of *H. pylori* infection and suppressive immune responses (Chen *et al.*, 2001; Kao *et al.*, 2010). Although mouse studies have provided insight into the role of IL-10 in determining colonisation of the bacterium, these studies have not reliably shown whether this association also occurs in humans. Most studies in mice have used $IL10^{-/-}$ mice, in which IL-10 production is completely abrogated (Berg *et al.*, 1998; Chen *et al.*, 2001). It is not possible to gain any information on the physiological threshold level of this suppressive cytokine required for reducing bacterial load.

In the current study, no association was observed between Treg-associated gene expression levels and bacterial load *in vivo*. This is in agreement with a previous study of 126 *H. pylori* infected patients in Korea, which reported no significant difference in the proportion of Tregs between different grades of *H. pylori* density (Jang, 2010). In addition, a study of Treg responses in children and adults reported significantly increased numbers of $FOXP3^{+}$ cells in children, but no difference in

colonisation was observed (Harris *et al.*, 2008). However, a positive association has been observed in a German population, although a smaller sample set of 17 antral biopsies were assessed. Furthermore, it appeared that samples with a density grade of 0 were included in the statistical analysis conducted, which may have skewed the data (Kandulski *et al.*, 2008). In support of Treg association with bacterial density, Kao *et al.* (2010) demonstrated reduced *H. pylori* CFU counts recovered from mice treated with anti-CD25 monoclonal antibody, and adoptive transfer of Tregs resulted in higher bacterial recovery (Lee *et al.*, 2007a).

It is important to note that mRNA expression levels may not always reflect the suppressive state in the gastric mucosa. As mentioned, cells expressing *IL10* and *FOXP3* may not necessarily have suppressive functions (Allan *et al.*, 2007; Couper *et al.*, 2008), highlighting the importance of assessing protein levels. In this context, the use of FACs to assess these responses may be more informative. However, this method may not benefit the investigation of TGF-β as it is believed that this cytokine is stored in cells ready for release when needed and detected mRNA levels may not reflect secretion of the cytokine.

In contrast to that observed *in vivo*, treatment of *H. pylori* infected AGS cells with recombinant IL-10 resulted in an increased number of CFUs. It is likely that IL-10 regulates inflammatory responses in the gastric epithelium resulting in persistent colonisation. In support of this hypothesis, our group have previously shown a dose dependent reduction of IL-8 secretion when *H. pylori* infected AGS cells were stimulated with IL-10 (Robinson *et al.*, 2008).
AGS cells treated with TGF-β were associated with higher CFU counts when infected with *H. pylori* 60190. As this was not observed when cells were infected with the non-toxic Tx30a strain, this response may be dependent on strain virulence, and suggests that TGF-β may interfere with signalling pathways that are activated in response to such bacterial factors. However, the major virulence factors, including CagA and VacA, were not previously found to be associated with TGF-β levels *in vitro* (Wu et al., 2007), Interestingly, Wu *et al.* (2007) also demonstrated a relationship between the multiplicity of infection and TGF-β production in AGS cells, with increasing levels detected as MOI increased. Whether this is a direct effect is unknown. However, a recent study showed

decreased detection of TGF-β secretion in *H. pylori* infected SNU-16 gastric cells (Jo *et al.*, 2010). The observed TGF-β response may therefore be cell specific, and highlights the need to repeat *in vitro* studies completed in the current study with various cell lines in order to confirm the relationship observed. It is also important to note that only a small increase in CFU counts were observed when cultures were stimulated with TGFβ, and requires repeating to rule out experimental error. A further limitation in the current study is the use of AGS cells when investigating the effects of TGF-β as the function of TGF-β secreted by tumour cells may be different to normal cells (Siegel & Massague, 2003). Therefore, use of primary gastric epithelial cells may be more appropriate for such investigations.

It is interesting that suppressive cytokines were associated with bacterial colonisation *in vitro*, but not in the human gastric mucosa. The high concentrations of recombinant cytokine may have induced a response in AGS cells that influences *H. pylori* density, and such levels may not represent physiological thresholds in the gastric mucosa. Also, the *in vitro* study did not account for the influence of immune cells which are present in the lamina propria. It has been demonstrated that cytokines alter suppressive functions of Tregs and make T effector cells resistant to Treg function, which has been demonstrated with the pro-inflammatory cytokines IL-15 (Ben Ahmed *et al.*, 2009) and IL-21 (Peluso *et al.*, 2007), which have both been found to be upregulated in the gastric mucosa of *H. pylori* infected patients (Caruso *et al.*, 2007; Luzza *et al.*, 1999). However, the mechanism by which these cytokines exert their action is unknown. Therefore, in the gastric mucosa, even if high levels of suppressive cytokine are present, which are associated with reduced gastric inflammation, they do not appear to be directly associated with bacterial colonisation.

7.3.2 The association between Treg gene expression levels and virulence determinants of *H. pylori*

Treg-associated gene expression levels were stratified according to the virulence status of the colonising strain in order to determine if bacterial factors may be involved in driving the induction of Treg responses. *IL10* expression levels tended to be greater with *cagA* positive strains, although not statistically significant. This

was not the case for *FOXP3* and *TGFB1* levels, suggesting a possible role of CagA in regulating the IL-10 response. Further investigation of this relationship is required as few samples in the data set were genotyped as *cagA* negative, which may affect the statistical test. Also, CagA functionality must also be determined for each strain.

Interestingly, higher expression levels of *IL10* and *FOXP3* were found with *dupA* positive strains, and when corrected for the *cagA* status of the bacterial strain, a significantly higher level of *FOXP3* expression was observed. This is in support of previous data reported in our group, which suggest that *dupA* does not affect gastric epithelial cells, but exerts its effects on the underlying immune cells (Hussein *et al.*, 2010). The data presented in the current study suggests that Tregs may be an additional target for this virulence factor. Again, *dupA* positivity by PCR may not reflect the presence of the functional protein. *TGFB1* levels were not found to be linked with *cagA* or *dupA* status.

Overall, *vacA* alleic type did not correlate with *IL-10* and *FOXP3* expression levels. Interestingly, a recent study did demonstrate a role of VacA and γ-glutamyl transpeptidase (GGT) in the upregulation of microRNA (miR-155) (Fassi Fehri *et al.*, 2010), which is known to be upregulated during *H. pylori* infection (Xiao *et al.*, 2009). miR-155 was shown to be needed for the induction of *FOXP3* on human T cells, and suggests a possible relationship between VacA and *FOXP3* expression levels. However, induction of *FOXP3* may not confer suppressive function.

Interestingly, increased *TGFB1* responses were observed with i2-type strains. The function of the i-region is yet to be determined, but it has been shown that i1 strains are associated with the development of gastric ulceration and cancer (Hussein *et al.*, 2008; Rhead *et al.*, 2007). It would be of interest to investigate whether strains carrying the *vacA* i1-type strain drive reduced expression of *TGFB1* which may induce a disease-prone state in the gastric mucosa.

7.3.3 The relationship between suppressive host responses and the degree of inflammation of the gastric mucosa

Tregs are known to modulate immune responses through numerous mechanisms including the secretion of anti-inflammatory cytokines, and direct binding through CTLA-4 which inhibits DC maturation. Previous studies in mice show an inverse relationship between the degree of gastritis that develops when Treg responses are abrogated (Chen *et al.*, 2001; Maloy *et al.*, 2003). Here, we show that expression levels of the Treg marker *IL10* and *FOXP3*, but not *TGFB1*, are negatively associated with inflammation in humans.

In contrast, Kandulski *et al.* (2008) reported a positive association between inflammation and the *β-actin*-normalised *FOXP3* mRNA levels. However, this was observed in a smaller number of samples. Also, some *H. pylori* infected gastric biopsies had an inflammatory score of zero and the maximum score was two. In the current study, gastritis was present in all infected samples and a maximum score of three was observed in this data set. Quantification of mRNA and histopathology analyses was carried out on different biopsy specimens here. It is possible that this may introduce error in association data, which may explain why the negative relationship observed in this study between inflammation and suppressive immune responses was in contrast to that reported by Kandulski *et al.* (2008). However, associations observed with RT-PCR data presented in this study were confirmed with flow cytometry analysis of gastric mucosal lymphocytes, providing strong evidence of a negative association. However, Jang *et al.* (2010) also reported a positive association between inflammation and the total number of $FOXP3^+$ cells using immunohistochemistry. Higher inflammation scores are indicative of increased numbers of immune and inflammatory cells, and these contain variable proportions of Tregs. In the current study, the proportion of $CD25^{hi}$ Tregs was measured amongst the infiltrating lymphocytes, not all of which were $FOXP3^+$.

The data presented here suggest that suppressive responses are associated with reduced inflammation, but this suppression may not be sufficient to impact bacterial density. Whether this association is dependent on the action of suppressive cytokines or a mechanism involving direct cell contact, or both, is unknown.

7.3.4 The relationship between suppressive immune responses and pre-malignant pathology in the gastric mucosa

We have previously shown that Treg responses are reduced in patients with peptic ulcer disease, suggesting that modulation of inflammation may play a role in protecting the host from mucosal damage and disease (Robinson *et al.*, 2008). Whether Tregs are also associated with protection from gastric cancer is unknown. Importantly, no patients in the current study had gastric cancer, and as Treg expression is different in cancer, it was important to look at stages of disease progression before the development of adenocarcinoma. Here, it has been shown that decreased levels of Treg specific gene expression were associated with the progression of disease to atrophy. Furthermore, lower expression levels of *IL10* and *FOXP3* were associated with the presence of IM, although *TGFB1* was not. In support of the association between *IL10* expression and the presence of IM, the number of $IL10^{+}CD4^{+}CD25^{hi}$ cells was also associated with the presence of IM, suggesting that IL10 producing Tregs are important in protection against the development of *H. pylori*-associated disease. The number of $IL10^{+}CD4^{+}CD25^{hi}$ cells did not correlate with atrophy, however the grading of IM via presence of goblet cells is more straightforward. *FOXP3* expression levels were also associated with premalignant pathology. To further investigate this relationship, the number of $FOXP3^{+}$ Tregs in the gastric mucosa of patients with such pathology must also be investigated.

Interestingly, decreased *TGFB1* expression was associated with atrophy but not IM. Based on this, it may be possible that TGF-β plays a smaller role in the modulation of inflammation and hence mucosal damage that leads to disease, but this is cannot be concluded from the data presented here.

Previously, IL-10 has been shown to also play a protective role in the development of IBD and cancer in $Rag2^{-/-}$ mice (Erdman *et al.*, 2003a). Interestingly, another study demonstrated Treg intervention in mice infected with *H. hepaticus* with early stages of cancer development reduced disease symptoms. However, with late stage cancer, Treg intervention was not enough to reduce disease development. Importantly, this study also found that IL-10 was required to interrupt the progression to IBD and cancer (Erdman *et al.*, 2003b). Also, a recent study by Arnold *et al.* found little if any atrophy and IM in *H. pylori* infected

neonate mice compared to infected adults at 1, 2 and 4 months post infection. When neonate mice were depleted of Tregs, severe pathology was observed, supporting the role of Tregs in protecting against the development of disease (Arnold *et al.*, 2010).

Overall, suppressive immune responses have been shown to be associated with decreased inflammation and may play a role in protecting the host against damage leading to the development of pre-cancerous changes in the gastric mucosa. Whether such responses are capable of modulating bacterial density is still not clear, and suppressive functions may not extend to modulating the antimicrobial cell responses required to directly combat infection.

Chapter 8

8. ANTIMICROBIAL PEPTIDES AND *HELICOBACTER PYLORI* INFECTION

8.1 INTRODUCTION

Antimicrobial peptides (AMPs) are essential components of the innate immune response to infection. Numerous AMPs have been described to have bactericidal activity against *H. pylori*, including members of the defensin family and the cathelicidin LL-37 (George *et al.*, 2003; Hase *et al.*, 2003; Otte *et al.*, 2009). However, mechanisms involved in the regulation of gene expression remains unclear in many cases. AMPs have also been reported to act as immunomodulators of the host response, and may have other roles in the immune response to *H. pylori* infection.

8.1.1 AMPs and *H. pylori* infection

8.1.1.1 α-defensins

Little is known of the antimicrobial activity of α-defensins against *H. pylori*, although a few studies have observed increased release of both neutrophilic and enteric α-defensins during infection (Isomoto *et al.*, 2004; Kocsis *et al.*, 2009b; Shen *et al.*, 2005). Release of these AMPs from NK cells induced by other bacterial factors such as OmpA and flagellin has been demonstrated (Chalifour *et al.*, 2004). Expression of α-defensin, specifically *hD5*, has been observed in gastric tissue in the presence of IM only, a likely consequence of intestinal paneth cell differentiation associated with this condition (Shen *et al.*, 2005). Strong bactericidal activity of recombinant h-D5 and h-D6 against *H. pylori* observed *in vitro* (Tanabe et al., 2008) would suggest that enteric α-defensin expression may be important in defence against the bacterium during more progressive stages of *H. pylori* associated disease.

8.1.1.2 β-defensins

β-defensins are abundantly expressed during *H. pylori* infection, and have been shown to have antimicrobial activity against the bacterium (George *et al.*, 2003; Kawauchi *et al.*, 2006; Otte *et al.*, 2009).

Much work surrounding β-defensin activity against *H. pylori* has concentrated on hβD2, for which gene expression is inducible. Regulation pathways involved in defensin expression seem to be best understood with hβD2, which is modulated in an NF-κB-dependent manner (Boughan *et al.*, 2006). This was found true in numerous cell types both *in vivo* (Taha et al., 2005) and *in vitro*, including keratinocytes, intestinal cells, and AGS, MKN45 and MKN7 cell lines (Bajaj-Elliott *et al.*, 2002; O'Neil *et al.*, 2000; Wada *et al.*, 1999; Wada *et al.*, 2001). Interestingly, numerous other signalling pathways have also been implicated in hβD2 modulation based on studies in different cell lines including JNK and MAPK (Boughan *et al.*, 2006). Transcription factor STAT3 has been associated with regulation of gene transcription (Wehkamp *et al.*, 2004). Other potential promoter binding domains have been recognised including AP-1 and NF-IL6, indicating other pathways that may be involved in regulation of *hβD2* expression (Wehkamp *et al.*, 2004).

Of importance is hβD3, which was not only found to be induced during *H. pylori* infection, but was also observed to have greater bactericidal activity against the bacterium compared to hβD1 and hβD2 (George *et al.*, 2003). Regulation of *hβD3* is controlled by an NF-κB and *cag*PAI-independent pathway, although IL-1β stimulation was observed to increase expression (Boughan *et al.*, 2006). The ERK pathway has been implicated in regulation of this defensin, suggesting a possible role for EGFR, a major receptor upstream to the ERK pathway, in gene modulation (Boughan *et al.*, 2006). Involvement of TLR4 has also been suggested, based on downregulation of *hβD3* when the receptor is blocked (Kawauchi *et al.*, 2006) and the observed increase in TLR4 expression during *H. pylori* infection (Su *et al.*, 2003).

The more recently identified hβD4 is also known to be upregulated during *H. pylori* infection by a *cag*PAI-dependent mechanism. However, unlike with hβD2, this was not NF-κB-dependent (Otte *et al.*, 2009), consistent with the lack of NF-

κB binding sites observed in the promoter sequence of the gene (Garcia *et al.*, 2001). Furthermore, stimulation with IL-1β, TNF-α, and IFNγ failed to induce hβD4 expression (Otte *et al.*, 2009). Pathways involved in the modulation of the inducible *hβD4* gene remain unclear, although sequence analysis has determined several AP-1 binding sites (Kawahara *et al.*, 2001).

Unlike β-defensins-2, -3, and -4, *hβD1* expression is known to be constitutive in many cell types. However, expression of the gene was observed to increase with *H. pylori* infection *in vivo* (Kocsis *et al.*, 2009a) and *in vitro*, and expression was augmented with IL-1β stimulus. However, this was observed in MKN7 cells but not in AGS and MKN45 cell lines (Bajaj-Elliott *et al.*, 2002), and this apparent regulation of *hβD1* gene expression was not found in other studies (Liu *et al.*, 1997; O'Neil *et al.*, 2000). Interestingly, decreased expression of hβD1 has been reported in *H. pylori* infected patients (Taha *et al.*, 2005). These studies, although contradictory, suggest that regulatory pathways may exist for the *hβD1* gene. Consistent with these findings are the observed binding motifs for NF-IL6 and IFNγ in the promoter sequence of the gene (Liu *et al.*, 1997). Sequence analysis of the *DEFB1* gene indicated CCAAT/enhancer binding protein-β (C/EBPβ) (Zhu *et al.*, 2003) adjacent to an NF-κB binding site (Prado-Montes de Oca *et al.*, 2009), which implies the potential for regulation of transcription.

The downregulation of *hβD1* has been observed in other bacterial infections including enteric pathogens *Vibrio cholerae*, *Escherichia coli* (ETEC) and *shigella dysenteriae* (Chakraborty *et al.*, 2008; Islam *et al.*, 2001). Importantly, the downregulation observed was ascribed to bacterial toxins, which were found to induce cAMP accumulation by several intracellular signalling pathways including protein kinase A (PKA) and ERK/MAPK (Chakraborty *et al.*, 2008). Clearly, the role of *hβD1* during *H. pylori* infection is unclear, and based on previous reports, modulation of *hβD1* expression by both host and bacterial factors may be possible.

8.1.1.3 Cathelicidins

The other class of AMPs produced in humans are the cathelicidins encoded by the *camp* gene, of which only one example has been found in humans named LL-37. To date, only two studies have investigated the role of LL-37 during *H. pylori* infection, which found that mRNA expression of this AMP is increased during infection by a mechanism that may be dependent of the *cag*PAI, and also showed antimicrobial activity of LL-37 against the bacterium (Hase *et al.*, 2003; Leszczynska *et al.*, 2009). Like *hβD1*, *LL37* expression appears to be tissue specific as inflammatory mediators were shown to induce *LL37* expression in keratinocytes (Frohm *et al.*, 1997), but not gastric and colonic cell lines (Hase *et al.*, 2002; Hase *et al.*, 2003). Response to bacterial infection also seems to vary, with some studies reporting downregulation of *LL37* expression (Chakraborty *et al.*, 2008), whist others have reported upregulation (Hase *et al.*, 2002; Hase *et al.*, 2003).

A number of external stimuli have been reported to modulate *LL37* expression, including the bacterial metabolite butyrate, which enhances histone acylation at the promoter site thus enabling AP1 binding and augmented gene expression. Lithocholic acid (LCA) has also been implicated in modulation of *LL37*; this binds nuclear receptors that cause recruitment of PU1, a transcription factor important in *camp* gene transcription. (Kida *et al.*, 2006; Raqib *et al.*, 2006; Schauber *et al.*, 2004; Zasloff, 2006). The promoter sequence of *LL37* was found to include binding sites for NF-κB, as well as NF-IL6 and IFNγ response elements (Brown & Hancock, 2006), and both IL-1α and IL-6 stimuli were found to regulate expression in keratinocytes (Erdag & Morgan, 2002). *LL37* expression was also found to be induced in gastrointestinal cells by histone-deacetylase inhibitors (Schauber *et al.*, 2004), and modulated by short fatty acids in colonocytes (Schauber *et al.*, 2003). Conversely, *LL37* expression can be reduced by bacterial proteases or through interference with *hCAP-18* expression, although the mechanisms involved remain unclear (Belas *et al.*, 2004; Bergman *et al.*, 2005; Islam *et al.*, 2001). Importantly, a number of truncated forms of the AMP have been isolated from the skin which exhibit antimicrobial activity, but have reduced immunomodulatory functions (Murakami *et al.*, 2004).

Due to the tissue specific nature of *LL37* modulation and the many potential stimuli that may modulate gene expression, it is difficult to hypothesise how the

gene is regulated during *H. pylori* infection, and clearly further investigation is needed.

8.1.2 Immunomodulatory functions of AMPs.

More recent findings have established a much broader range of biological activities for these AMPs other than their direct killing effects, including their roles as modulators of the immune response. Many AMPs have been associated with chemotactic activity, working together to direct immune effector cells to the site of infection. α-defensins are important in recruiting neutrophils, phagocytic cells and monocytes, as are hβD3 and hβD4. Importantly, hβD1, hβD2 and hβD3 are associated with recruiting immature dendritic cells and memory T cells through CC-chemokine receptor-6 (CCR6), hence representing a bridge between the innate and adaptive immune response (Yang *et al.*, 2002). Cathelicidins were found to be involved in the recruitment of neutrophils, in addition to circulating and tissue-derived monocytes (De *et al.*, 2000). AMPs therefore act to induce pro-inflammatory immune responses, in some cases inducing immune mediators that further induce the expression of these AMPs, effectively creating a positive feedback loop. Importantly, considerably higher concentrations of AMPs are needed for chemoattraction of leukocytes, limiting their activity to the local environment (Zasloff, 2007).

AMPs may also act to enhance the immune response. For instance, LL-37 was found to induce the differentiation of monocyte-derived dendritic cells, and mouse βD2 was observed to stimulate dendritic cell maturation and upregulation of MHC II (Biragyn *et al.*, 2002).

Furthermore, studies have shown a role for AMPs in immune suppression as well as pro-inflammatory responses that effectively promote bacterial clearance. LL-37 was observed to bind and neutralise LPS, protecting mice from LPS-induced inflammation (Rosenfeld *et al.*, 2006). LL-37 also exhibits suppressive functions towards LPS-induced translocation of NF-κB into the nucleus of human monocytes and epithelial cells, and reduced expression levels of pro-inflammatory cytokines TNFα and IL-6 (Mookherjee *et al.*, 2006). Defensins have also been associated with immune-suppressive properties, with murine βD2 mediating dendritic cell death through activation of TLR4, therefore suggesting a role for this AMP in inducing the immune response to infection through their chemotactic

ability, as well as terminating the immune response through elimination of activated APCs (Biragyn *et al.*, 2008).

Thus, AMPs are important modulators of the immune response, protecting the host from potentially harmful microbes directly through their antimicrobial activity, and indirectly through recruiting immune effector cells and protecting the host from the harmful effects of an active inflammatory response.

The chemotactic properties of β-defensins towards dendritic cells may also implicate a potential function for AMPs in tumour suppression. Dendritic cells are the most powerful APCs and have the capability to prime naive T-cells that induce anti-tumour responses (Thomas-Kaskel *et al.*, 2007). However, tumours have developed mechanisms to impair dendritic cell function or numbers. Downregulation of hβD1 is observed in a number of cancers, including pancreatic cancer, as well as oral squamous cell carcinomas (Bose *et al.*, 2009; Wenghoefer *et al.*, 2008), and reduced *hβD1* levels has been related to binding of the Pax2 oncoprotein, for which expression is upregulated during the development of prostate cancer (Bose *et al.*, 2009).

8.1.3 Experimental aims

- To investigate the expression of *hβD1* and *LL37* in *H. pylori* infection both *in vivo* and *in vitro*.
- To determine the relationship between *hβD1* and *LL37* expression levels and colonisation density *in vivo* and *in vitro*.
- To assess the role of bacterial virulence factors in regulation of *hβD1* and *LL37*.
- To determine possible host factors that may be involved in the regulation of *hβD1* and *LL37* expression.

8.2 RESULTS

8.2.1 The relationship between *hβD1* and *H. pylori* infection

hβD1 is thought to be constitutively expressed in many cell types, with a number of reports demonstrating this to be true for *H. pylori* infection as well (Liu *et al.*, 1997; O'Neil *et al.*, 1999). However, a number of studies have described regulation of *hβD1* expression, although such data is not always consistent (Bajaj-Elliott *et al.*, 2002; Kocsis *et al.*, 2009a; Taha *et al.*, 2005). These studies, along with the presence of consensus binding regions for regulatory modulators in the promoter region of the *hβD1* gene, would suggest a potential for regulation of expression during *H. pylori* infection.

In order to investigate the possible regulation of *hβD1*, expression levels were quantified using real-time RT-PCR in uninfected and *H. pylori* infected antral gastric biopsies. A 3-fold decrease in *hβD1* expression was observed for infected tissue compared to uninfected controls ($p=0.003$ by Mann-Whitney U-test) (Figure 8.1).

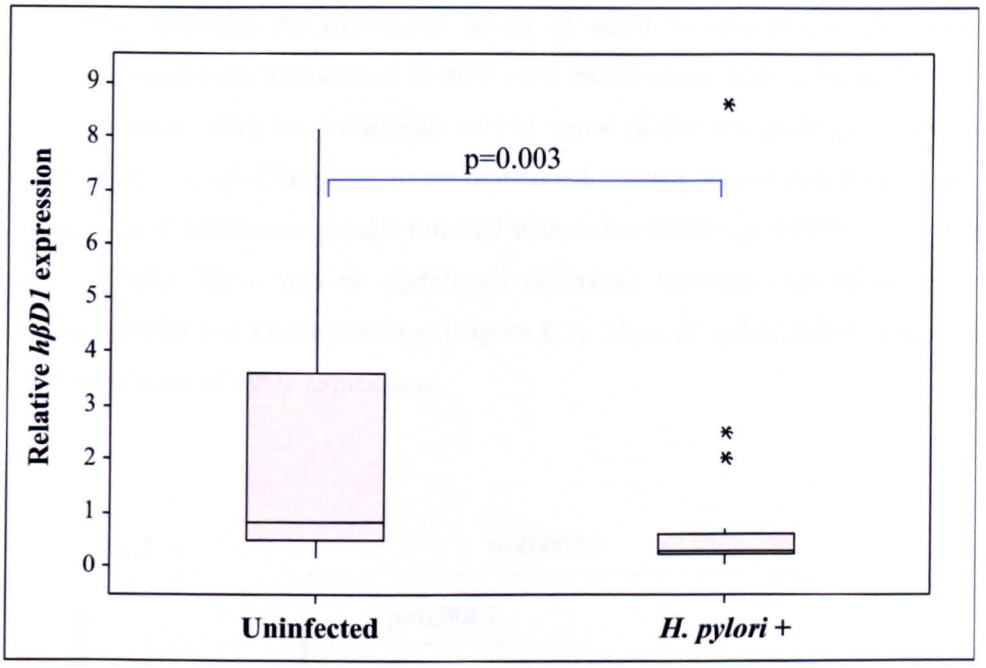


Figure 8.1: *hβD1* expression during *H. pylori* infection.

hβD1 expression levels were measured in uninfected (n=15) and *H. pylori* infected (n=26) antral gastric tissues samples, and normalised against *GAPDH* expression levels. * represents outlier values.

To further examine the expression levels of *hβD1* in response to *H. pylori* infection, expression was studied in an *in vitro* model using AGS cells infected for 24 hours with either the pathogenic 60190 strain or the non-pathogenic Tx30a strain of *H. pylori*. Compared to an uninfected control, expression levels were found to be 9-fold lower in cells infected with either 60190 (p=0.0003) or Tx30a wt (p=0.005). There was no significant difference between expression levels between 60190 and Tx30a infection (Figure 8.2). Thus, *H. pylori* infection causes downregulation of *hβD1* expression.

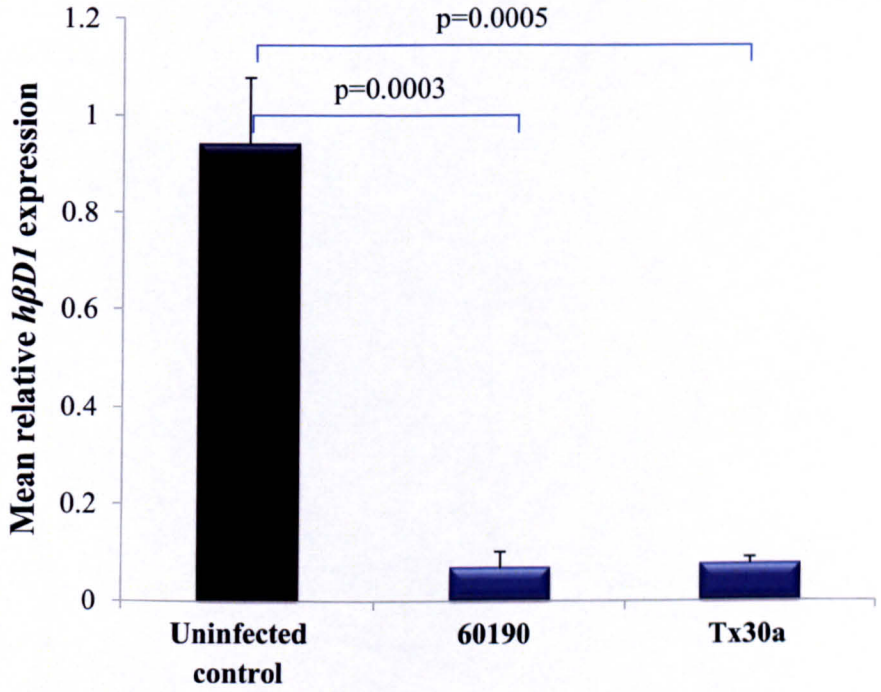


Figure 8.2: *In vitro* analysis of *hβD1* expression with *H. pylori* infection.
AGS cells were incubated with 24 hour cultures of *H. pylori* strains 60190 and Tx30a at an MOI of 100. AGS cells alone were used as an uninfected control. Cells were harvested after 24 hours and *hβD1* expression was measured using quantitative RT-PCR. Mean *hβD1* expression of 6 replicates is shown with bars representing standard deviation.

In vitro analysis of *hβD1* expression in gastric epithelial cell lines was previously found to vary in a time dependent manner with *H. pylori* infection (Bajaj-Elliott *et al.*, 2002; Kocsis *et al.*, 2009a). To assess the variability in expression over time, *hβD1* levels were measured in cultured cells at an earlier time point of 8 hours. Mean *hβD1* levels in infected cultures were the same as the uninfected controls, suggesting mechanisms involved in down-regulating *hβD1* expression are activated late during infection (Figure 8.3).

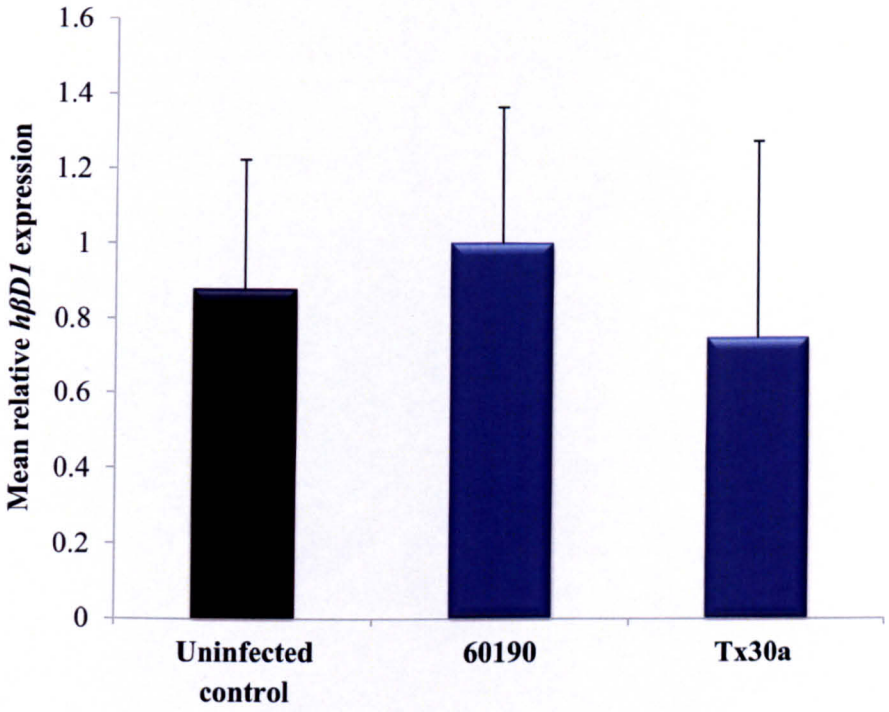


Figure 8.3: *In vitro* analysis of *hβD1* expression after 8 hours *H. pylori* infection.

AGS cells were incubated with *H. pylori* strains 60190 or Tx30a at an MOI of 100. AGS cells alone were used as an uninfected control. Cells were harvested after 8 hours incubation and *hβD1* mRNA transcript levels were measured by real-time RT-PCR. Expression levels were normalised against *GAPDH* expression levels. Mean *hβD1* expression of 6 replicates is shown with bars representing standard deviation.

Antimicrobial activity of hβD1 has been previously observed against *H. pylori* (George *et al.*, 2003). However a relationship between *H. pylori* density and *hβD1* expression *in vivo* has not yet been shown. Bacterial load and *hβD1* levels were determined in gastric antral tissue samples from *H. pylori* infected patients. Colonisation density measured by qPCR did not correlate with *hβD1* expression in the antrum (n=25, r= -0.42, p=0.42 by Pearson correlation test). However, using histological grading of density, a 2-fold higher *hβD1* expression level was observed for mild *H. pylori* colonisation compared to severe *H. pylori* colonisation density (Figure 8.4). No significant difference was observed with grade 2 colonisation density, but there were only 4 samples in this category (median=0.46, n=4, IQR=0.13-1.84).

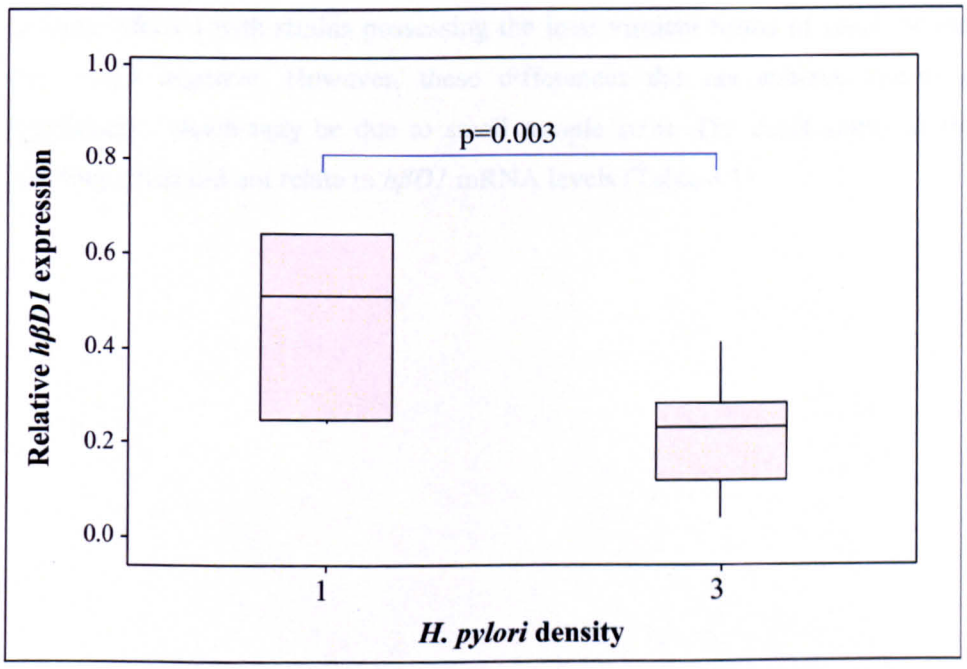


Figure 8.4: The relationship between *hβD1* expression and bacterial density. *HβD1* expression levels were evaluated for grades of *H. pylori* density that were determined by histopathology in antral gastric biopsies. Median expression levels for mild (score of 1, n=11) and severe colonisation (score of 3, n=11) are shown. The relative level of *hβD1* expression meaursed in uninfected patients was 0.82.

8.2.1.1 The effect of known H. pylori *virulence determinants on* hβD1 *expression*

Regulation of the *hβD1* gene remains unclear as the few studies that have investigated expression of this AMP with respect to *H. pylori* infection have provided conflicting results. However, the observed downregulation of *hβD1* reported here has also been noted in other bacterial infections, and bacterial derived factors have been implicated as modulators of gene expression (Chakraborty *et al.*, 2008). To address the possible involvement of *H. pylori* virulence determinants in *hβD1* regulation, *hβD1* expression data for antral gastric biopsies was stratified according to the virulence of the colonising strain with respect to the three major virulence determinants *cagA*, *vacA* and *dupA*. As only 1 isolated strain carried the *vacA* s2 genotype, *hβD1* expression levels for the s-region were not assessed. An increase in antral *hβD1* expression was observed in patients infected with strains possessing the least virulent forms of *vacA*, or that were *cagA* negative. However, these differences did not achieve statistical significance, which may be due to small sample sizes. The *dupA* status of the infecting strain did not relate to *hβD1* mRNA levels (Table 8.1).

	***hβD1* expression**	
	Median (n)	**IQR**
H. pylori negative	0.82 (15)	0.49-3.59
cagA		
+	0.28 (10)	0.13-0.41
-	0.53 (4)	0.12-0.67
p	NS	
dupA		
+	0.33 (9)	0.18-0.61
-	0.28 (5)	0.15-0.46
p	NS	
vacA		
m1	0.16 (6)	0.13-0.58
m2	0.38 (8)	0.27-0.56
p	NS	
i1	0.14 (6)	0.13-0.46
i2	0.28 (8)	0.23-0.60
p	NS	

Table 8.1: The association between *hβD1* expression and virulence determinants of the colonising strain.

Median *hβD1* expression levels were measured in antral tissue samples and stratified according to the virulence factor status of the colonising strain. Inter quartile ranges are given (IQR). Expression levels were not statistically different (NS). The expression levels determined in *H. pylori* negative patients are given for comparison.

To investigate this relationship further, *hβD1* expression was assessed with an *in vitro* model using AGS cells cultured with a series of *H. pylori* strains of different virulence status. These included the non-virulent Tx30a strain (*cagPAI/cagA-*, *vacA* s2/i2/m2), and the virulent strains 11637 and 60190. To assess the impact of individual virulence factors on *hβD1* expression levels, *cagA*, *cagE* and *vacA* null mutants of the 60190 strain were used. For all strains tested, an 8-fold decrease in *hβD1* expression was observed compared to the uninfected control. No significant difference in expression levels between 60190wt and *cagA* or *vacA* mutant strains was observed. A significantly higher levels of *hβD1* expression was observed for the 60190Δ*cag*PAI strain compared to the wild type (p=0.03), although this difference was less than 2-fold. A second virulent strain, 11627, and the non-virulent Tx30a both reduced *hβD1* expression to the same degree as 60190, suggesting that the factors responsible for downregulating *hβD1* expression are present in virulent and non-virulent strains (Figure 8.5).

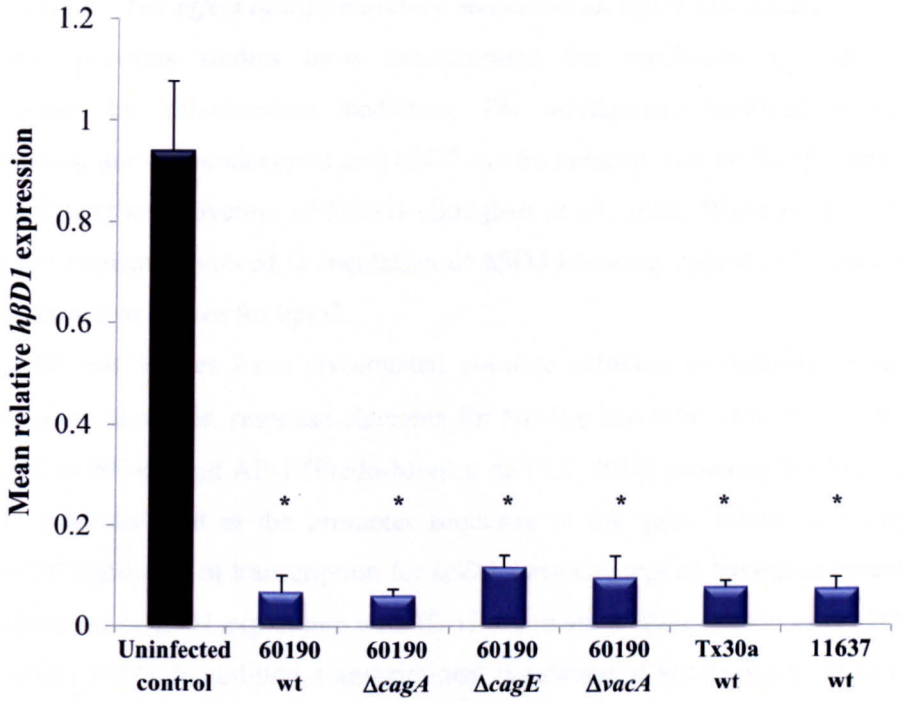


Figure 8.5: *In vitro* analysis of the relationship between *hβD1* expression and *H. pylori* virulence determinants.

AGS cells were incubated with *H. pylori* strains 60190wt, 60190Δ*cagA*, 60190Δ*cagE*, 60190*vacA*, Tx30a and 11637wt at an MOI of 100. AGS cells alone were used as an uninfected control. *hβD1* expression was measured in cells after 24 hours. Mean *hβD1* expression levels of 6 replicates are shown with bars representing standard deviation. * represents a significant difference in expression compared to the uninfected control ($p<0.005$).

8.2.1.2 The effect of inflammatory mediators on hβD1 *expression*

Several previous studies have demonstrated the regulation of β-defensin expression by inflammatory mediators. The mechanisms involved in *hβD2* expression are well understood and *hβD2* can be induced with an IL-1β stimulus, as well as other activators of NF-κB (Boughan *et al.*, 2006; Wada *et al.*, 1999). The mechanisms involved in regulation of hβD3 however, appear to be different from those that known for hβD2.

To date, few studies have investigated possible cytokine modulators of *hβD1* expression. However, response elements for NF-IL6 and IFNγ (Liu *et al.*, 1997), as well as NF-κB and AP-1 (Prado-Montes de Oca, 2010) promoter binding sites have been observed in the promoter sequence of the gene, which may imply potential regulation of transcription for *hβD1*. Previous reports have demonstrated upregulation of *hβD1* expression with IL-1β stimulation (Bajaj-Elliott *et al.*, 2002; Liu *et al.*, 1997). In addition, transcriptional regulation of *hβD1* may be mediated through signalling pathways involving ERK and MAPK (Chakraborty *et al.*, 2008). As TNFα is an important activator of ERK/MAPK signalling pathways, it may also play a role in the regulation of *hβD1*. Although there is no evidence for an IL-17-dependent modulation of *hβD1*, IL-17 has been shown to regulate *hβD2* expression via a JAK and NF-κB-dependent pathway. With the known existence of NF-κB response elements within the *hβD1* gene, IL-17 may also play a role in the regulation of *hβD1* (Kao et al., 2004).

In order to investigate the potential role of cytokine regulation of *hβD1*, expression levels were assessed in AGS cells when stimulated with IL-1β, TNFα or IL-17. The effects of IL-10 and TGF-β were also investigated to determine if anti-inflammatory responses, which are upregulated in the gastric mucosa during *H. pylori* infection (Robinson *et al.*, 2008), have an impact on *hβD1* expression. AGS cells infected with *H. pylori* 60190 were also included as a control to demonstrate modulation of *hβD1* expression, and cytokine stimulation of infected cells was also assessed to determine if immune modulators could further modulate gene expression.

As observed before, *hβD1* expression levels were markedly reduced when AGS cells were infected with *H. pylori* in all experiments conducted ($p<0.02$). Stimulation with IL-1β, IL-17, IL-10 and TGF-β showed no effect on *hβD1* levels (Table 8.2). However, TNFα stimulation of AGS cells did result in a reduction in the expression level of *hβD1*. Expression levels did not differ for *H. pylori* infected cells in the presence or absence of TNFα (Figure 8.6).

	Mean *hβD1* expression level (SD)	
	AGS cells	**AGS cells + *H. pylori* 60190**
Pro-inflammatory cytokines		
No cytokine	0.83 (0.14)	0.50 (0.10)
Plus IL-1β	0.95 (0.28)	0.38 (0.35)
p	NS	NS
No cytokine	1.17 (0.33)	0.21 (0.11)
Plus IL-17	1.12 (0.19)	0.20 (0.20)
p	NS	NS
No cytokine	1.06 (0.12)	0.12 (0.05)
Plus TNF-α	0.73 (0.25)	0.19 (0.15)
p	0.04	NS
Anti-inflammatory cytokines		
No cytokine	0.81 (0.02)	0.36 (0.17)
Plus TGF-β	0.98 (0.19)	0.39 (0.13)
p	NS	NS
No cytokine	0.87 (0.10)	0.21 (0.11)
Plus IL-10	1.01 (0.21)	0.29 (0.19)
p	0.04	NS

Table 8.2: *hβD1* expression levels in response to cytokine stimulation in AGS cells.

AGS cells were incubated for 24 hours in the presence or absence of cytokine stimulation. AGS cultures infected with *H. pylori* 60190wt at an MOI of 100 were included as a positive control for the suppression of *hβD1* expression. The mean *hβD1* expression level and standard deviation (SD) are given for 4 replicates. Levels of *hβD1* were significantly lower in *H. pylori* infected cultures compared to the uninfected culture ($p<0.02$). NS=not significant.

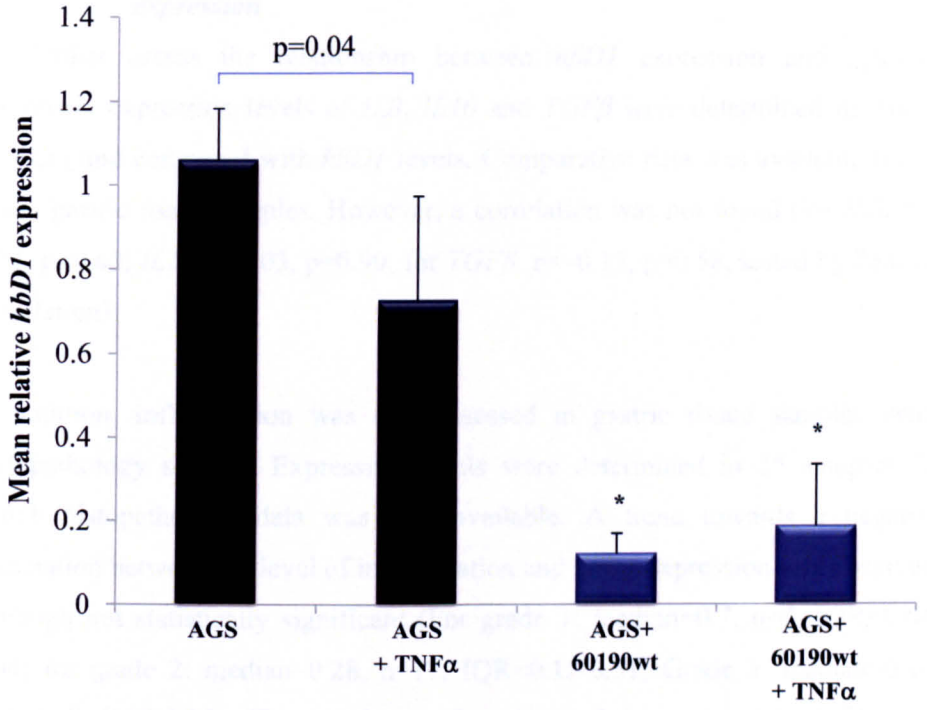


Figure 8.6: The effect of TNFα on *hβD1* expression.

AGS cells were infected with either *H. pylori* strain 60190wt wt at an MOI of 100, which were incubated in the presence or absence of TNFα for 24 hours. The mean *hβD1* expression in 4 replicates is shown, with bars representing standard deviation. * represents a significant difference in expression compared to the uninfected control ($p<0.001$).

8.2.1.3 In vivo relationship between host immune responses and hβD1 *expression*

To further assess the relationship between *hβD1* expression and cytokine responses, expression levels of *IL8*, *IL10* and *TGFβ* were determined in gastric biopsies and compared with *hβD1* levels. Comparative data was available for 15 antral gastric tissue samples. However, a correlation was not found (for *IL8*: r= -0.11, p=0.63; *IL10*: r=0.03, p=0.90; for *TGFβ*: r= -0.15, p=0.58, tested by Pearson correlation).

In addition, inflammation was also assessed in gastric tissue samples using histopathology scoring. Expression levels were determined in 25 samples for which histopathology data was also available. A trend towards a negative association between the level of inflammation and *hβD1* expression was observed, although not statistically significant (For grade 1: median=0.7, n=3, IQR=0.64-2.04; for grade 2: median=0.28, n=17, IQR=0.15-0.51; Grade 3: median=0.24, n=5, IQR=0.18-0.26). This trend was also observed for neutrophilic infiltration (For grade 0: median=0.7, n=3, IQR=0.24-2.04; for grade 1: median=0.41, n=5, IQR=0.25-0.61; for grade 2: median=0.24, n=17, IQR=0.14-0.41. No samples were found to have grade 3 neutrophilic infiltration).

8.2.1.4 The relationship between hβD1 *expression and pathology*

hβD1 has also been proposed to have a tumour suppressor function based on the absence or downregulation of transcription in numerous tumours, as well as having the capability to induce apoptosis in cancerous cells (Bullard *et al.*, 2008; Sun *et al.*, 2006; Wenghoefer *et al.*, 2008). Given the association of *H. pylori* infection with gastric cancer, a possible role for hβD1 may exist in cancer protection within the gastric mucosa.

To address the possible relationship between defensin expression and the development of gastric cancer, *hβD1* expression levels were compared in the presence or absence of atrophy and IM in 25 antral gastric biopsy samples. The presence of such pathology was not associated with *hβD1* expression (Atrophy present: median=0.34, n=9, IQR=0.19-0.61; absence of atrophy: median=0.28,

n=17, IQR=0.20-0.59; IM present: median= 0.24, n=5, IQR=0.20-0.59; absence of IM: median=0.31, n=20, IQR=0.23-0.63).

8.2.2 Relationship between *LL37* expression and *H. pylori* infection

The expression of *LL37* is inducible in a number of cell types. However, it is unclear as to which mechanisms are involved in the regulation of the gene. Studies have demonstrated increased expression of *LL37* during *H. pylori* infection (Hase *et al.*, 2003; Leszczynska *et al.*, 2009). To confirm this finding, *LL37* expression levels were measured in antral gastric tissue biopsies from both uninfected and *H. pylori* infected patients. Expression levels were found to be 5-fold greater in infected tissue (uninfected: median=0.62, n=15, IQR=0.23-0.87; *H. pylori* positive: median=3.02, n=17, IQR=1.35-8.86) (p=0.0003) (Figure 8.7).

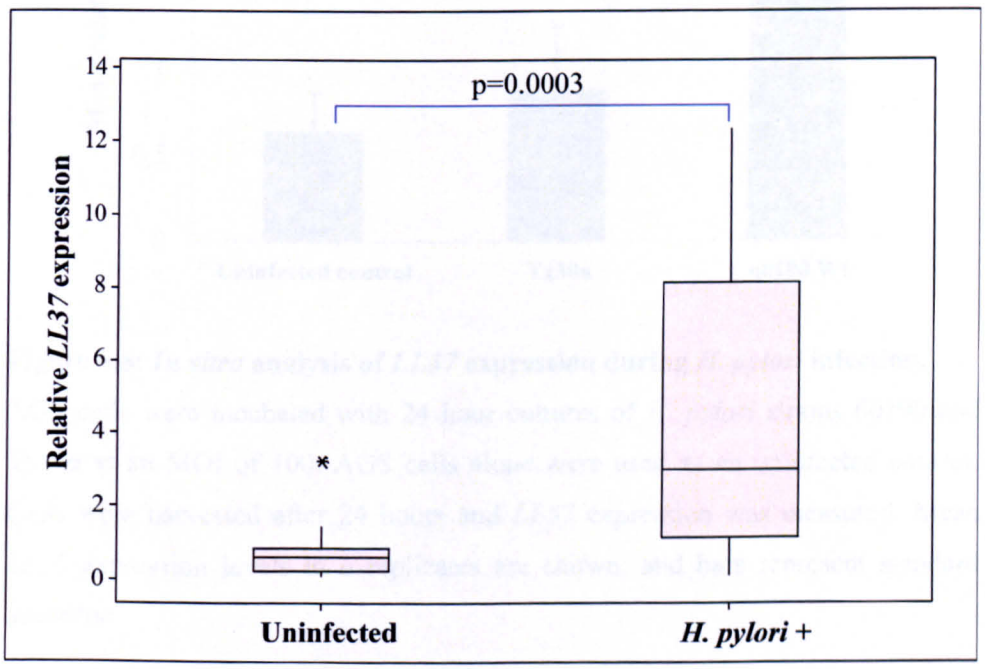


Figure 8.7: *LL37* expression and *H. pylori* infection.

LL37 expression levels were measured in uninfected (n=15) and *H. pylori* infected (n=17) antral gastric tissues samples. * represents outlier value.

This was confirmed with an *in vitro* AGS culture model, which demonstrated upregulation of *LL37* gene expression upon infection with a pathogenic strain of *H. pylori* (p=0.0003), but not with the non-pathogenic Tx30a strain (Figure 8.8). Therefore, suggesting that expression may be modulated by *H. pylori* virulence determinants.

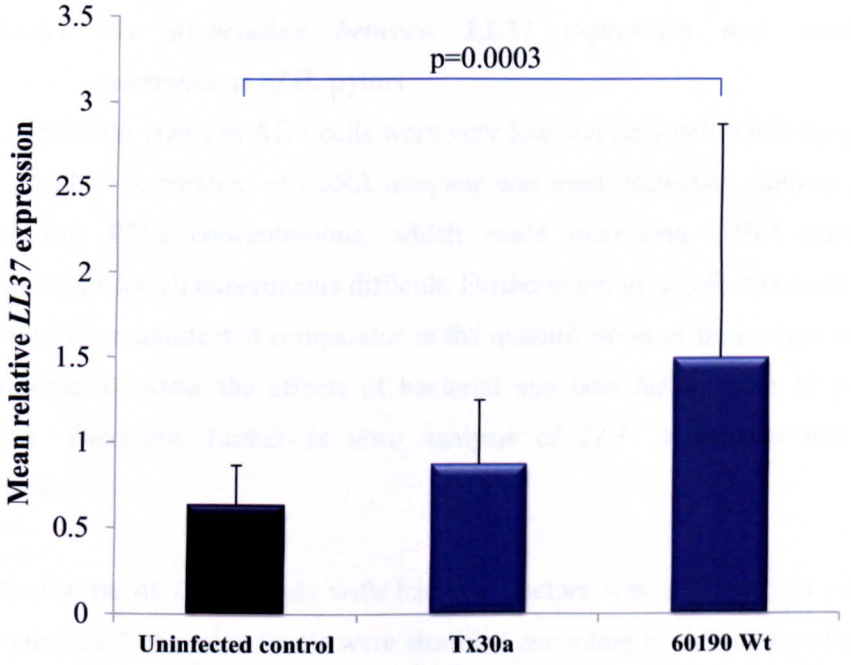


Figure 8.8: *In vitro* analysis of *LL37* expression during *H. pylori* infection. AGS cells were incubated with 24 hour cultures of *H. pylori* strains 60190 and Tx30a at an MOI of 100. AGS cells alone were used as an uninfected control. Cells were harvested after 24 hours and *LL37* expression was measured. Mean *LL37* expression levels in 6 replicates are shown, and bars represent standard deviation.

LL-37 has broad-spectrum antimicrobial activity, and has previously been demonstrated to have bactericidal activity against *H. pylori* (Hase et al., 2003; Leszczynska et al., 2009). To confirm this association *in vivo*, bacterial density was assessed with *LL37* expression levels in antral gastric tissue. Density of infection measured by qPCR did not correlate with *LL37* expression (r= -0.24, p=0.25).

8.2.2.1 The association between LL37 *expression and virulence determinants of* H. pylori

LL37 expression levels in AGS cells were very low and only detectable by qPCR when a high concentration of cDNA template was used. However, cultures often yielded low RNA concentrations, which made increasing cDNA template concentrations for all experiments difficult. Furthermore, as uninfected AGS cells were used as an uninfected comparator in the quantification of transcripts, it was not possible to assess the effects of bacterial and host factors with *H. pylori* infection. Therefore, further *in vitro* analysis of *LL37* expression was not conducted.

The association of *LL37* levels with bacterial factors was addressed in gastric tissue samples. Expression levels were stratified according to the *cagA* and *dupA* status and *vacA* alleic type. *LL37* levels were determined for 15 antral biopsy samples for which strain genotype data was also available. As only 1 sample was found to carry the s2 alleic form of *vacA*, the s-region was not assessed. *LL37* expression was not associated with *cagA* or *vacA* alleic strain type. Increased levels of *LL37* were observed for samples infected with *dupA*- strains compared to *dupA*+, however this was not statistically significant (Table 8.3).

	LL37 expression	
	Median (n)	**IQR**
H. pylori negative	0.82 (15)	0.49-3.59
cagA		
+	3.02 (10)	1.13-4.32
-	4.86 (4)	0.30-9.59
p	NS	
dupA		
+	1.92 (9)	0.95-6.11
-	4.32 (5)	2.03-8.88
p	NS	
vacA		
m1	3.02 (5)	0.76-8.11
m2	4.22 (9)	1.11-8.57
p	NS	
i1	3.02 (5)	1.01-8.38
i2	4.15 (9)	1.14-7.31
p	NS	

Table 8.3: The association between bacterial virulence factors and *LL37* expression *in vivo*.

Median *LL37* expression levels were measured in antral tissue samples and stratified according to the virulence factor status of the colonising strain. Inter quartile ranges are given (IQR). Expression levels were not statistically different (NS).

8.2.2.2 The relationship between LL37 *expression and pathology*

There is growing evidence suggesting a role for LL-37 in tumour suppression, with decreased detection of the peptide in gastric adenocarcinoma (Hase *et al.*, 2003). Furthermore, LL-37 has recently been shown to activate signalling pathways involved in cell-cycle arrest, inhibiting cell proliferation (Wu *et al.*, 2010). To assess the relationship between this AMP and gastric cancer, *LL37* expression levels were measured in the presence of premalignant pathology in 25 gastric antral biopsies. *LL37* expression was not significantly related to the presence of IM (Presence: median=8.11, n=5, IQR=2.26-9.82; absence of IM: median=3.97, n=20, IQR=1.29-9.07). Reduced expression was observed in the presence of atrophy (median=1.92, n=9, IQR=0.63-6.27) compared to non-atrophic tissue samples (median=6.42, n=16, IQR=2.62-10.54) (p=0.037) (Figure 8.9).

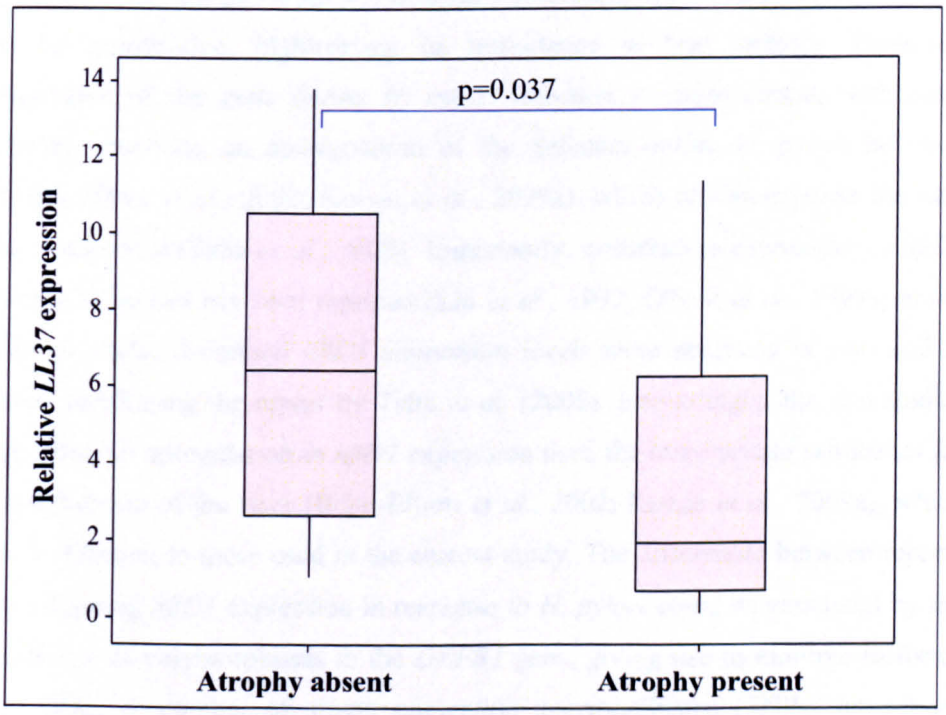


Figure 8.9: The association between *LL37* expression and atrophy.

LL37 expression was measured in 25 antral gastric biopsies for samples and stratified according to the presence (n=9) or absence (n=16) of glandular atrophy.

8.3 DISCUSSION

Antimicrobial peptides play a vital role during microbial infections, acting as a key line of defence against invading microbes and also as essential components in modulating the immune response to infections. The role of such peptides is made clear by the range of disease associated with dysfunctional AMPs. For instance, inflammatory diseases such as Psoriasis and Rosacea, which develop from increased expression of *hβD2* and *LL37* (Lande *et al.*, 2007; Yamasaki *et al.*, 2007), and the development of Crohn's disease due to increased susceptibility to intestinal infections as a result of decreased expression of intestinal α-defensins (Wehkamp *et al.*, 2007).

8.3.1 The association between *hβD1* expression and *H. pylori* infection

The β-defensin group of AMPs have antimicrobial activity against *H. pylori* (George *et al.*, 2003). Of the 4 β-defensins described, *hβD1* expression is believed to be constitutive, highlighting its importance in host defence. However, regulation of the gene during *H. pylori* infection is controversial, with some studies observing an up-regulation of the defensin during *H. pylori* infection (Bajaj-Elliott *et al.*, 2002; Kocsis *et al.*, 2009a), whilst downregulation has also been observed (Taha *et al.*, 2005). Importantly, constitutive expression of *hβD1* during *H. pylori* has been reported (Liu *et al.*, 1997; O'Neil *et al.*, 1999). In the current study, decreased *hβD1* expression levels were observed *in vivo* and *in vitro*, confirming the report by Taha *et al.* (2005). Interestingly, the two studies reporting an upregulation in *hβD1* expression used the same primer sequences for amplification of the gene (Bajaj-Elliott *et al.*, 2002; Kocsis *et al.*, 2009a), which were different to those used in the current study. The differences between reports investigating *hβD1* expression in response to *H. pylori* could be explained by the existence of polymorphisms in the *DEFB1* gene, giving rise to multiple isoforms of hβD1. A number of single nucleotide polymorphisms (SNPs) have been described for *DEFB1* (Prado-Montes de Oca, 2010), and Valore *et al.* have previously reported the presence of 6 isoforms in the urogenital tract, all with varying antimicrobial activity under different conditions (Valore *et al.*, 1998). The presence of hβD1 isoforms was later confirmed in caco-2 cell lines (O'Neil *et al.*, 1999). Bajaj-Elliott *et al.* (2002) observed upregulated expression of *hβD1* in this

cell line, contrary to the reduced expression levels reported in the current study when using different primer sets, suggesting the presence of isoforms. However, only 1 isoform has previously been reported in AGS cells (O'Neil *et al.*, 2000), Furthermore, increased expression levels using the same primer set as Bajaj-Elliott *et al.* were also reported in gastric tissue taken from *H. pylori* infected patients (Kocsis *et al.*, 2009a), which may suggest the presence of gene polymorphisms and potentially hβD1 isoforms in the human gastric mucosa. It would be interesting to assess *hβD1* expression in the samples used in the current study with the primer set described by Bajaj-Elliott *et al.* (2002), as this may give insight into the presence of polymorphic *hβD1* transcripts.

Differences in culture conditions must also be considered as a possible source for the observed differences in *hβD1* regulation observed between studies. One difference in the culture conditions between studies would be the length of time for which cells were cultured with the bacterium. In the current study, no difference in *hβD1* expression was observed after an 8 hour incubation, which suggests that mechanisms involved in downregulation of gene expression do not occur immediately after infection. Although maximal expression of *hβD1* has been observed at 24hours (Kocsis *et al.*, 2009a), earlier time points have been suggested in other gastric cell lines (Bajaj-Elliott *et al.*, 2002), and a time course analysis of *hβD1* expression of infected cells to demonstrate the time at which expression levels begin to alter has not yet been examined. The current experiment has demonstrated that regulation of *hβD1* expression does occur between 8 and 24 hours post-infection. It is possible that downregulation may be a result of cell death, particularly given reduced levels were observed after 24 hours. However, increased IL8 secretion was detected in these culture experiments as described in Chapter 5 (section 6.2.3), which suggest that *hβD1* downregulation was not a result of global suppression of gene transcription or cell death, and reduced levels during *H. pylori* infection were also found *in vivo*.

The importance of *hβD1* regulation was demonstrated here with the negative association observed between *H. pylori* colonisation density and *hβD1* expression in gastric tissue, consistent with antimicrobial activity of the peptide against the bacterium. Downregulation of gene expression may therefore be a bacterial driven

mechanism of survival. However, *in vivo* observations do not prove a causal relationship between *H. pylori* and hβD1. The bactericidal activity of hβD1 must therefore be tested with recombinant peptide *in vitro* to demonstrate this relationship exists. Although previous studies have observed activity of the peptide against *H. pylori* (George et al., 2003), it is important to consider the possible presence of isoforms as discussed earlier, and the use of a recombinant peptide must reflect the mRNA variant that is detected with the primer set used here. Of interest would be to determine the cellular response with respect to *hβD1* expression to various concentrations of bacteria. The increased expression levels of the peptide observed previously was found to be induced in a bacterial dose dependent manner (Bajaj-Elliott *et al.*, 2002). If bacterial numbers are in fact driving the response observed, then the association between bacterial loads and *hβD1* expression in gastric tissue may not reflect the antimicrobial activity of the peptide.

It is also important to consider the presence of other AMPs in the gastric mucosa that may contribute to killing the bacteria. As previously observed, hβD3 was found to have the greatest activity against *H. pylori* compared to hβD2 and hβD1, and expression of this defensin is associated with *H. pylori* infection (George *et al.*, 2003). Immunohistochemistry may prove useful in determining a relationship between *H. pylori* density and *hβD1* expression.

8.3.1.1 The effect of known H. pylori *virulence determinants on* hβD1 *expression*

Clearly, *hβD1* expression is regulated during *H. pylori* infection. Bacterial factors that may influence gene expression were not associated with *hβD1* levels *in vivo*. However, larger sample numbers are required. Further *in vitro* analysis of bacterial factors demonstrated that the major virulence factors CagA and VacA were not involved. Interestingly, decreased *hβD1* levels was not limited to a specific *H. pylori* strain, as strains Tx30a and 11637 also showed the same response with regards to *hβD1* expression. This implies a common bacterial factor such as LPS or an outer membrane protein may be driving the observed response. The 60190Δ*cagE* mutant strain was associated with less reduction of *hβD1* concentrations compared to the 60190wt strain, albeit a small difference. This

may represent an NF-κB-dependent regulation of *hβD1* given the known association between the CagPAI and this signalling molecule (Brandt *et al.*, 2005). Furthermore, NF-κB response elements have been described in the *hβD1* promoter sequence (Prado-Montes de Oca *et al.*, 2009). If *cag*PAI induced signalling is involved in regulating *hβD1* expression, a similar level of expression to the *cag*PAI mutant strain should have been observed with the Tx30a strain which does not possess the CagPAI. However, other differences between these strains may play a role in regulating *hβD1*. Therefore, the possible effect of such a mutation in a different *H. pylori* strain background would be necessary to rule out strain specific associations.

Interestingly, Bajaj-Elliott *et al.* demonstrated an increase in *hβD1* expression when infecting AGS cells with the 11637 strain. This strain was also examined in the current study, and reduced levels comparable to that observed with the 60190 strain was observed, therefore suggesting that the observed difference in *hβD1* expression between the current study and that of Bajaj-Elliott *et al.* (2002) was not due to differences in virulence of the infecting strain.

It is difficult to ascertain from this data whether decreased *hβD1* expression is a result of bacterial factors modulating signalling pathways, or if *hβD1* transcription is regulated by inflammatory mediators that are induced by the bacterium. Previous reports have demonstrated the importance of bacterial virulence factors in downregulation of *hβD1*. For instance, cholera toxin was found to inhibit gene transcription through ERK/MAPK/p38 related signalling pathways (Chakraborty *et al.*, 2008). Further investigation of possible virulence determinants of *H. pylori* that are involved in regulation of *hβD1* is needed.

8.3.1.2 The effect of inflammatory mediators on hβD1 *expression*

The role of immune effectors must also be considered. *IL8*, *IL10* and *TGFβ* expression levels did not correlate with *hβD1* expression levels *in vivo*. Stimulation of AGS cells with IL-1β, TGF-β, IL-17and IL-10 had no effect on *hβD1* expression. However, stimulation with TNFα induced a reduction in *hβD1* levels. Stimulation of *H. pylori* infected cells with TNFα did not result in a further

reduction in *hβD1* expression. TNFα signals through numerous pathways, including NF-κB (Curfs *et al.*, 1997). This, along with the observed difference in *hβD1* expression between the 60190Δ*cagE* mutant strain and the wild type strain, suggests an NF-κB-dependent suppression of *hβD1* expression. Interestingly, NF-κB activation is known to induce expression of genes such as *IL8* and *hβD2* (Boughan *et al.*, 2006; Brandt *et al.*, 2005). The NF-κB family of transcription factors consists of 5 members. NF-κB exists as a homo- or heterodimer of these subunits. Of these, p50 and p52 lack the transcription activation domain necessary for transcription, and promoter binding of these homodimers can block transcription of the target gene (Hayden & Ghosh, 2008). Furthermore, *hβD1* is known to have a p50 binding domain (Prado-Montes de Oca *et al.*, 2009). Therefore, activation of such suppressive NF-κB transcription factors may result in the reduction of *hβD1* expression.

Interestingly, the TIPα protein of *H. pylori* has been shown to induce TNF-α gene expression and NF-κB activation (Suganuma *et al.*, 2005). Furthermore, of the clinical isolates tested in the study by Suganuma *et al.*, the TIPα protein was found to be produced at a similar level in all strains, indicating that it is constitutively expressed. It would be of interest to further investigate this protein as a potential regulator of *hβD1* expression.

The *in vitro* studies reported here have provided an insight into the association between *hβD1* expression and *H. pylori* infection in epithelial cell lines. However, another consideration is the effect of *H. pylori* infection on *hβD1* expression in inflammatory cells. As hβD1 is known to be secreted from these cells (Prado-Montes de Oca, 2010), it is important to determine if the response observed in gastric tissue was solely an epithelial response.

IL-22 is known to function as an inducer of antimicrobial peptide production. In a recent study, increased levels of hβD2 and hβD3 were reported in keratinocytes stimulated with IL-22. However, this was not found with hβD1 (Wolk *et al.*, 2004). It is not known whether the IL-22 specific receptor is expressed in AGS cells, and was therefore not investigated in the current study for the effects of the cytokine on *hβD1* expression. Importantly, IL-22 and IL-17 were found to work

co-operatively to induce expression of hβD2 (Liang *et al.*, 2006), although their effects on *hβD1* expression are unknown.

As mentioned, the *in vitro* model used in this study was limited to a single time point of 24 hours. Of consideration is the study by Bajaj-Elliott (2002) who demonstrated the impact of IL-1β stimulation on *hβD1* and *hβD2* expression levels. It was found that the time of maximum expression for both of these defensins in IL-1β stimulated MKN7 cells was delayed in comparison to *H. pylori* infected cells that were not stimulated with cytokine. This highlights the importance of investigating numerous time points when studying the expression levels of *hβD1* in response to cytokine and *H. pylori* stimulation *in vitro*.

From these results, we can only speculate possible regulatory pathways that are involved in regulation of *hβD1* expression. Importantly, *H. pylori* infection did not completely abrogate *hβD1* expression, which suggests other response elements are involved in *hβD1* expression. Previously, a *Defb1*$^{-/-}$ mouse model has been used to assess the physiological *in vivo* functions of the defensin in response to *Staphylococcus aureus* infection (Morrison *et al.*, 2002). A similar approach may be useful for determining the role of β-defensin 1 during infection with *H. pylori*.

8.3.1.3 The relationship between hβD1 *expression and pathology*

The tumour suppressive properties of hβD1 have not yet been studied in *H. pylori* related gastric cancer. Decreased *hβD1* levels are observed in numerous cancers (Bose *et al.*, 2009; Wenghoefer *et al.*, 2008). Although the mechanisms involved in *hβD1* regulation in cancerous cells have not been elucidated, a cancer specific response has been implicated as higher expression levels have been observed in normal tissue surrounding cancerous tissue (Donald *et al.*, 2003). Transcriptional regulators have been found to modulate gene expression, including c-Myc, a key factor in cell proliferation and apoptosis (Sherman & Froy, 2008), and PAX2, an oncogene found to down-regulate *hβD1* expression (Bose *et al.*, 2009).

As no patients in the current study were diagnosed with gastric cancer, it was not possible to assess this relationship. However, premalignant pathology was assessed, but an association was not observed.

It would be interesting to observe the relationship between *H. pylori*-associated gastric cancer and *hβD1* expression in gastric tissue, and to determine if downregulation of the gene is another factor that contributes to the progression of disease to a cancerous state. Elucidating the possible *H. pylori* specific component that relates to the decreased expression would be significant in understanding the risk associated with developing cancer. Furthermore, previous studies have observed rapid cell death in prostate cancer cell lines in the presence of hβD1 (Bullard *et al.*, 2008), demonstrating the therapeutic advantage of the AMP.

The reported downregulation of *hβD1* in a number of cancers raises a limitation within the *in vitro* model used in the current study. AGS cells are a cancerous cell line, and it is possible that down-regulation of *hβD1* expression occurs with AGS cells. However, downregulation of *hβD1* was still observed when infected with *H. pylori* demonstrating a specific response when infected with the bacterium. Furthermore, decreased levels of *hβD1* were also observed *in vivo*, therefore suggesting a non-cancer related regulation of the gene here. However, to rule out the possibility of an additional effect of cancerous cells, the *in vitro* effects of *H. pylori* infection on *hβD1* expression should be assessed in primary gastric cells (Wee *et al.*, 2010).

Overall, a mechanism is activated during *H. pylori* infection that downregulates the expression of *hβD1*. This may be a response triggered by the bacterium in order to minimise the deleterious effect this peptide may have on the bacterium. As well as the antimicrobial activity of the peptide, the activity of hβD1 in recruiting DCs to the site of infection may be important (Yang *et al.*, 2002). In this respect, downregulation of gene expression may protect the bacterium from a specific antibody response by interfering with the number of APCs.

8.3.2 The association between *LL37* expression and *H. pylori* infection

LL37 is another important AMP that has activity against *H. pylori*. As previously demonstrated (Hase *et al.*, 2003; Leszczynska *et al.*, 2009), expression levels of

this AMP were increased in gastric tissue taken from patients infected with *H. pylori* compared to uninfected tissue in the current study. This was also confirmed *in vitro*. However, in this study AGS cells were not a suitable model for monitoring expression of *LL37* due to low expression of the transcript. This may suggest that other cell types, possibly underlying immune cells which are known to express the AMP (Durr *et al.*, 2006), may be involved in the LL-37 response to *H. pylori* infection. Further investigation of *LL37* expression during *H. pylori* infection in other epithelial cell lines is necessary in order to determine if the lack of detection observed in the current study is specific to AGS cells, or if LL-37 is not released by epithelial cells. Interestingly, AGS cells have previously been demonstrated to express *LL37* (Hase *et al.*, 2003), and differences in expression levels may reflect varying culture conditions, or the amplification of different *LL37* mRNA products. As with hβD1, isoforms of LL-37 have been found in human skin which exhibit variable antimicrobial activity, but show reduced immunological response (Murakami *et al.*, 2004). Importantly, these isoforms were produced as a result of proteolytic processing of mature LL-37, and this highlights the fact that expression of a gene does not reflect the active protein. Therefore, although these small peptides prove difficult to assess *in vitro*, it is essential to look at the association of functional LL-37 protein with *H. pylori* infection as well as mRNA transcript. An important difference between the processing of *hβD1* and *LL37* is that the latter is stored as a proprotein and is only activated by signal induced protease cleavage (Braff *et al.*, 2005; Dudal *et al.*, 2006). Again, the measure of *LL37* transcript may not reflect the amount of functional protein during infection. However, a clear association between *LL37* and *H. pylori* was observed, suggesting the bacterium may regulate *LL37* gene expression.

The importance of LL-37 with respect to *H. pylori* infection was demonstrated by *in vitro* analysis of antimicrobial activity against the bacterium, with studies showing a strong killing activity. The most convincing data comes from Bubki *et al.* (2010) who demonstrated membrane disruption and blebbing of *H. pylori* using AFM after treatment with LL-37. However, an association between *H. pylori* density and *LL37* expression was not observed in the current study. This may reflect physiological peptide levels that are incapable of killing enough bacterial cells to impact overall bacterial densities. Hase *et al.* (2003) found that

LL-37 concentrations of 4μM were needed to cause a log reduction in CFU counts after 6 hours, and 16μmol/L were required to eradicate an inoculum of *H. pylori*. Furthermore, culture conditions appear to affect the minimum bactericidal concentration (MBC) of LL-37. Leszczynska *et al.* (2009) demonstrated MBCs of over 100μg/ml when cultured in Brucella Broth Bulion, whereas MBCs of 10μg/ml on average were required when *H. pylori* was cultured in HEPES. These studies indicate the need for appropriate culture conditions that represent that of the gastric mucosa in order to assess the concentrations of LL-37 required to impact colonisation density.

8.3.2.1 The association between LL37 *expression and* H. pylori *virulence determinants*

In vivo assessment of the relationship between bacterial virulence factors and *LL37* expression showed no association, although a trend towards decreased expression levels with *dupA+* infections was observed. Interestingly, *in vitro* culture of the virulent 60190 strain did significantly induce expression of *LL37*, but this was not seen with the non-virulent Tx30a strain. This would suggest a role for *cagA* or *cagPAI* in the modulation of *LL37*. However, other differences exist between these strains in addition to the *cagA* status. In support of the strain specific regulation of the gene is a previous study that demonstrated a *cagPAI* dependent regulation of *LL37*, which implicates NF-κB as a modulator of its expression (Hase *et al.*, 2003). In addition, NF-κB promoter sites have been identified in the *LL37* gene (Brown & Hancock, 2006). However, addition of NF-κB activators were not found to induce increased expression of *LL37*, therefore it may not be involved, or may not be the sole transcriptional regulator of the gene (Hase *et al.*, 2003). Of note would be the observed ERK/MAPK/p38 dependent downregulation of *LL37* by cholera toxin. This suggests similar signalling pathways that are involved in *hβD1* modulation (Chakraborty *et al.*, 2008). Identification of an appropriate cell line in which *LL37* expression is detectable would be of benefit in order to determine and assess the impact of *H. pylori* mutant strains on expression levels of this AMP.

In further support of a bacterial-specific regulation of *LL37* was the finding that infection with *H. pylori*, but not stimulation with pro-inflammatory cytokines,

resulted in increased *LL37* expression (Hase *et al.*, 2003). In addition, the lack of *LL37* expression in non-infected patients that exhibited NSAID associated inflammation suggests that regulation of gene expression is driven by a bacterial specific factor (Hase *et al.*, 2003). However, LL-37 activity has been suggested in neutrophils, and expression has been induced in response to cytokine stimulation in keratinocytes (Bucki *et al.*, 2004; Chertov *et al.*, 1996; Frohm *et al.*, 1997). Therefore, further investigation of possible immune regulators of *LL37* expression must be considered.

Overall, increased *LL37* expression is observed in gastric epithelial cells when infected with *H. pylori*, possibly in an NF-κB dependent manner, although other signalling pathways may contribute to regulation of gene expression. Upregulation of *LL37* may also be an indirect response, as hβD2 has been shown to induce expression levels of the cathelicidin in breast and colon tissue (Stroinigg & Srivastava, 2005). This along with the observed regulation by NF-κB, which is a known regulator of *hβD2*, is suggestive of an indirect modulation of *LL37*.

As with hβD1, LL-37 has tumour suppressive activity, and was found to be undetectable in gastric adenocarcinoma (Hase *et al.*, 2003). Along with this finding, decreased expression was detected in *H. pylori* infected atrophic tissue in comparison to non-atrophic infections in the present study. This suggests that cellular changes resulting in the development of premalignant pathology may activate cellular responses that result in the downregulation of the peptide. LL-37 has been hypothesised to target and kill cancer cells in a similar mechanism as that known for killing bacterial cells (Li *et al.*, 2006c). However, decreased *LL37* expression was not observed in the presence of IM, which may imply that the observed reduction in expression may result from the loss of glandular tissue which is characteristic of atrophy.

In summary, the expression of both *hβD1* and *LL37* are regulated during *H. pylori* infection by mechanisms involving specific bacterial factors. However, the exact mechanisms involved in the regulation of these AMPs are not fully understood, and the effect of such negative regulation on colonisation of the bacterium requires further study.

Chapter 9

9. FINAL DISCUSSION

Helicobacter pylori persistently colonises the human stomach even though inflammatory responses are mounted in response to the infection. Furthermore, *H. pylori* is the only known bacterium capable of establishing a lifelong infection in an otherwise sterile environment. This is mediated by numerous factors, and the relationship between the bacterium and host is complex. This is highlighted by the fact that it is not completely understood why a small proportion of infected individuals develop disease. Whether colonisation density greatly influences disease development, and the role of individual bacterial virulence factors and host immune responses in determining colonisation density is yet to be determined in detail. The focus of this study was to investigate bacterial and host factors that influence *H. pylori* burden and localisation in the human gastric mucosa.

9.1 DISCUSSION OF THE RESULTS

An intimate relationship is known to exist between gastric inflammation and disease progression (Reviewed by Kusters *et al.*, 2006). Corpus-predominant or pan-gastritis and reduced acidity is associated with an increased risk of developing gastric ulcer disease (GUD) and gastric cancer. Certain host factors have been attributed to driving such gastric conditions such as polymorphism of cytokine genes. The risk of developing duodenal ulcer disease (DUD) on the other hand is associated with an antral-predominant gastritis and an increased acid load. However, little is known of the factors that drive this pattern of gastritis (Figure 9.1).

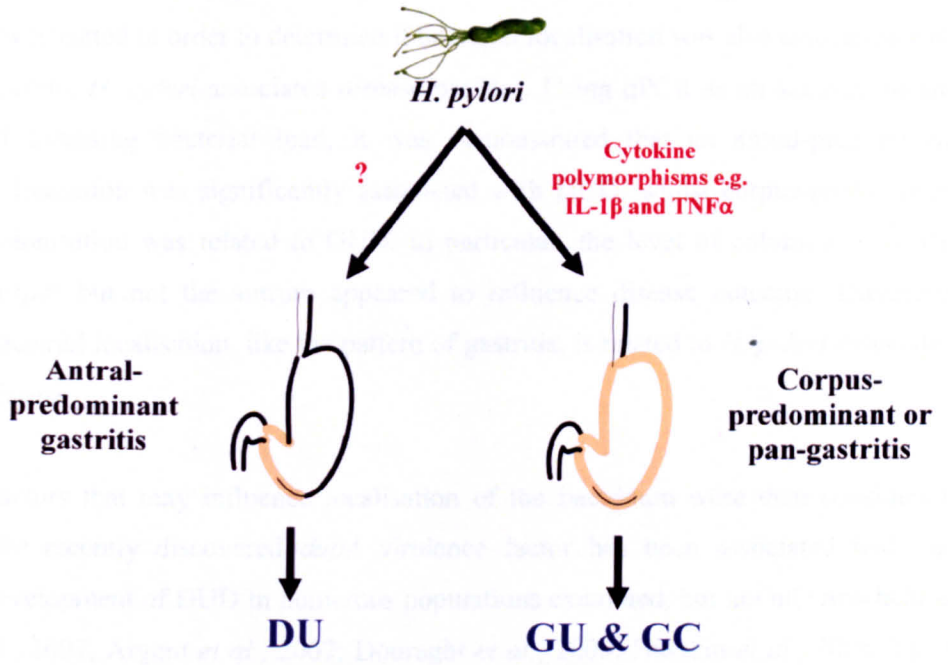


Figure 9.1: Schematic representation of the relationship between gastritis and *H. pylori*-associated disease.

H. pylori infection is associated with the development of duodenal ulcers (DU), gastric ulcers (GU) and gastric cancer (GC). The pattern of gastritis in the stomach is a known determinant of disease outcome, although the factors that influence the development of such patterns are still unclear. Corpus-predominant or pan-gastritis is associated with the development of GU and GC, and polymorphisms in cytokine genes such as IL-1β and TNFα have been identified as factors that influence these disease profiles to develop. Antral-predominant gastritis is associated with the development of DU. However, it is not known what drives the development of this pattern of inflammation.

In the current study, particular patterns of colonisation in the gastric mucosa were investigated in order to determine if bacterial localisation was also associated with specific *H. pylori*-associated disease profiles. Using qPCR as an accurate means of assessing bacterial load, it was demonstrated that an antral-predominant colonisation was significantly associated with DUD, whilst corpus-predominant colonisation was related to GUD. In particular, the level of colonisation in the corpus but not the antrum appeared to influence disease outcome. Therefore, bacterial localisation, like the pattern of gastritis, is related to *H. pylori*-associated disease.

Factors that may influence localisation of the bacterium were then considered. The recently discovered *dupA* virulence factor has been associated with the development of DUD in numerous populations examined, but not all (Arachchi *et al.*, 2007; Argent *et al.*, 2007; Douraghi *et al.*, 2008; Hussein *et al.*, 2008; Lu *et al.*, 2005; Zhang *et al.*, 2008). This suggests that DupA may drive the development of this disease profile, although the mechanisms behind this association are not understood.

In this study, it was demonstrated for the first time that the *dupA* status of the colonising strain is associated with different patterns of *H. pylori* colonisation in the gastric mucosa. *dupA* positive strains were associated with antral-predominant colonisation, and *dupA* negative strains with corpus-predominant colonisation. Therefore, it is possible that the observed association between *dupA*$^+$ strains and DUD may result from a preferential colonisation of the antrum by bacteria expressing this factor. No significant association was observed between DUD and *dupA* status in this study however.

Little is known about the function of DupA. Analysis of the gene sequence has indicated homology to the VirB4 ATPase which is a component of the VirB/D complex of type IV secretion systems known from bacteria such as *Agrobacterium tumefaciens*. Lu *et al.* (2005) showed that strains carrying *dupA* were linked to acid tolerance. As antral-predominant gastritis is associated with increased gastric acid secretion, the observed association between *dupA* and DUD may be reflective of a selective pressure in the gastric environment that favours growth of these strains. Whether DupA has a functional role in inducing the

development of DUD or if the association observed by previous studies is due to preferential colonisation of the antrum due to increased acid levels and antral-predominant gastritis is unknown. VirB4 homologues are components of type IV secretion systems (T4SS), suggesting that DupA may be part of another T4SS in the *H. pylori* genome. Therefore, it is possible that an as yet unidentified virulence determinant is translocated through this T4SS, which may have a role in inducing conditions in the gastric mucosa that increase the risk of developing DUD.

As mentioned, other factors contribute to the progression of disease, including the *vacA* status of the colonising strain (Atherton *et al.*, 1995; Basso *et al.*, 1998; Rhead *et al.*, 2007). In the current study, m1-type strains were associated with increased colonisation densities in the corpus, among *dupA+* strains. A trend towards increased density with m1 and i1-type strains was also observed in both the antrum and corpus among *dupA-* strains too. Previously, the virulent m1 form of the toxin has been associated with increased colonisation densities (Han *et al.*, 1999), suggesting that VacA may influence colonisation in the gastric mucosa. The mechanisms involved are not fully established, although it is possible that increased vacuolating activity induces leakage of key nutrients needed for bacterial growth and survival.

There is a difficulty in establishing whether a particular virulence determinant is independently associated with influencing bacterial load. Strains frequently harbour more virulent forms of more than one virulence determinant, for example, *vacA* s1/m1 strains commonly express *cagA*. Therefore, a very large data set would need to be considered in order to analyse all combinations of *H. pylori* virulence factors. A way around these problems could be to perform *in vitro* studies. In contrast to *in vivo* data however, *vacA* status was not found to affect CFU counts when cultured with gastric epithelial cell lines *in vitro*. This may be due to the selective pressures *in vivo*, e.g. paucity of nutrients, that are not present in the *in vitro* model.

Host factors such as cytokine production are also known to induce a gastric environment that is key for the development of GU and GC. Whether host immune responses are also related to *H. pylori* colonisation was assessed. The relationship between bacterial load and *IL8* expression was not a simple linear

association. The majority of samples tested appeared to fall into a region of relatively low bacterial colonisation density and low *IL8* expression. However, samples colonised with very high levels of *H. pylori* were also found to express low levels of *IL8*. Conversely, extremely high expression of *IL8* was found in tissue samples for which low colonisation levels were measured. This suggests that the reciprocal relationship that was hypothesised may exist in extreme cases, and in most infected individuals a balance between inflammation and colonisation exists.

Interestingly, anti-inflammatory cytokine responses were associated with increased bacterial CFU counts *in vitro* but no association with bacterial load was observed *in vivo*. Our group have previously shown a significant positive association between *IL10* expression and bacterial density (Robinson *et al.*, 2008). However, this relationship did not hold when additional patient data were included. Cytokines such as IL-15 and IL-21 have previously been demonstrated to alter the suppressive functions of Tregs (Ben Ahmed *et al.*, 2009; Peluso *et al.*, 2007), and may therefore alter the capability of suppressive cytokines to mediate bacterial survival, and hence colonisation density, *in vivo*. However, in the reductionist cell culture models used in this study, suppressive cytokines do have the capacity to induce epithelial responses that encourage bacterial growth.

An important finding in this study was that Treg-associated gene expression levels in gastric antral biopsies were linked to the incidence of gastric atrophy and IM. These data suggest that increased Treg responses may be protective against the development of premalignant pathology, as well as PUD which we have previously shown (Robinson *et al.*, 2008). As Treg-associated gene expression levels were negatively associated with inflammation, the mechanism behind such protection may involve dampening of the deleterious inflammatory response. Interestingly, *dupA* positive strains were associated with increased levels of *FOXP3* mRNA expression, yet *dupA* has been associated with increased levels of *IL8* in the gastric mucosa, and stimulation of the proinflammatory cytokine IL-12 *in vitro* (Hussein *et al.*, 2010). Further analysis of *IL8* mRNA expression levels in the gastric mucosa showed no statistical association with *dupA* status. However, the recently reported polymorphic nature of the virulence determinant (Hussein *et al.*, 2010) was not taken into consideration, and may explain the discrepancy

observed. Although our group have previously shown that increasing levels of IL-10 is associated with reduced Th1 responses *in vitro*, it is not known if this is also true for *FOXP3* expressing cells. It is possible that Tregs are recruited to sites of severe inflammation in the gastric mucosa in order to counteract the deleterious effects of inflammation, and therefore increased Th1 responses may be found with increased $FOXP3^+$ Treg responses. If pro- and anti-inflammatory responses are induced in response to *dupA*, then the outcome of infection with respect to the development of disease may be dependent on the overall Treg/Th1 ratio, with disease developing when the immune response is skewed towards inflammation. This may also hold true for other bacterial factors such as *cagA* which is a known risk factor for the development of disease and induces IL-8 secretion (Brandt *et al.*, 2005; Viala *et al.*, 2004). In addition, it has recently been suggested that CagA may be linked to the induction of Treg responses (Chen & Blaser, 2007).

Although bacterial burden is related to bacterial virulence determinants, which induce inflammation, bacterial and host cytokine responses are not directly responsible for killing bacteria. The host immune response utilises numerous mechanisms for the direct killing of bacteria, including the release of antimicrobial peptides (AMPs). *H. pylori* infection is associated with increased expression of numerous AMPs that have demonstrated bactericidal activity against the bacterium (George *et al.*, 2003; Hase *et al.*, 2003), yet infection persists. In the current study, we show *H. pylori* infection to be associated with reduced expression levels of *hβD1 in vivo* and *in vitro*. This modulation was partly dependent on the *cag*PAI, and in line with this our group have now demonstrated an NFκB-dependent downregulation *in vitro* (manuscript in preparation). Furthermore, we have shown that bacterial load is negatively associated with expression of *hβD1* in gastric tissue, suggesting it may have bactericidal activity against *H. pylori*, and current work now shows the bactericidal activity of recombinant *hβD1* against *H. pylori*. The expression of *hβD1* is constitutive, reflecting the importance of the defensin in innate protection against infection. It is therefore possible that *H. pylori* has developed mechanisms to downregulate expression of the AMP in order to allow persistent infection.

Of interest is the synergistic relationship that has been observed between LL-37 and hβD1. Hase *et al.* (2003) demonstrated little bactericidal activity of hβD1 against the *H. pylori* 26695 strain. However, in combination with LL-37, antimicrobial activity was significantly potentiated. Therefore, downregulation of *hβD1* may benefit *H. pylori* persistence not only directly through the function of *hβD1*, but also in reducing the activity of LL-37 which also has antimicrobial activity against the bacterium (Hase *et al.*, 2003).

9.2 EXPERIMENTAL LIMITATIONS

9.2.1 Patients and samples

Samples in this study were collected from patients attending a routine upper GI endoscopy, who were referred for examination based on dyspeptic symptoms. The *H. pylori* negative control therefore may not be a true representation of an uninfected "normal" control group. Also, given the small sample size, it was not possible to match for parameters such as age and gender, which are known to affect immune responses. However, a wide age range and similar male:female ratios were found in the data set examined, and therefore these parameters were not anticipated to have an impact on data analysis. As mentioned, a major influence of the outcome of infection is host genetics, particularly polymorphisms in cytokine genes such as IL-1β (El-Omar *et al.*, 2000; El-Omar *et al.*, 2003). Although this was not covered in the current study, it would be interesting to see how such factors would relate to colonisation density and impact the observed association between disease and distribution of the bacterium in the gastric mucosa. In line with this, it would also be interesting to determine the acidity in the gastric corpus as demonstrated by Derakhshan *et al.* (2006). However, collection of these samples and human genotype analysis was not covered by the current ethics approval for this study.

H. pylori infected patients were stratified based on the presence of disease at the time of endoscopy, and all others were categorised as having gastritis. However, this group may encompass patients that may develop disease later in life, and therefore investigation of parameters that may influence disease progression is

difficult. Importantly, it was possible to assess for atrophy and IM within this group, which are known risk determinants for the development of disease. Also, tissue samples were not collected from the duodenum although patients were stratified based on the presence of duodenal ulceration. This leaves the possibility that the immune responses in the duodenum may not reflect those in the stomach.

It was not possible to perform all analyses on the same gastric tissue. In particular, histological parameters could not be assessed in the same tissue sample as other analyses. Therefore, sampling errors may have confounded the association studied. However, it was possible to assess bacterial load by qPCR and to quantify mRNA transcripts in the same tissue sample. Furthermore, in this study the analysis of Treg responses and pre-malignant pathology was assessed by RT-PCR and confirmed by flow cytometry, suggesting that sampling errors may not greatly impact association studies. Also, our group are now intending to use zoom endoscopy, which will allow for the identification of areas of atrophy during endoscopy and the collection of tissue from these areas, therefore minimising sampling errors.

Importantly, it was not possible to assess the degree of patchy colonisation in the gastric mucosa, which may also introduce sampling errors. Investigation of patchiness in bacterial burden would require assessing a number of gastric tissue samples from the antrum and corpus. However, it was not possible to complete such an analysis based on ethical restrictions on the number of tissue samples collected.

9.2.2 Bacterial virulence typing

The virulence status of the colonising strain was determined based on the presence or absence of the gene or the existence of specific allelic variants, but the presence of functional protein was not assessed. In the case of *cagA*, the functionality of the type IV secretion system should ideally also be considered, as the presence of the gene does not indicate delivery of functional protein into the cell. However, assessing CagA protein levels is labour intensive and involves culture of isolated strains and western blot analysis. An additional problem with this method is the

frequent difficulty in isolating *H. pylori* clinical isolates. Intactness of the CagPAI-encoded type IV secretion system can be measured by the IL-8 response, but again involves *in vitro* analysis of strains isolated from all samples tested in the study. The toxicity of CagA is dependent on the extent of tyrosine-phosphorylation (Argent *et al.*, 2004), which was not determined for strains assessed in this study.

As mentioned, the recent discovery of *dupA* polymorphisms (Hussein *et al.*, 2010) highlights the limitation of typing for just the presence or absence of this virulence factor. Importantly, it is not known which forms of *dupA* are expressed as protein or if expression levels vary, making it difficult to assess the functionality of this virulence determinant.

Therefore, time pressures limited the assessment of strain virulence to the presence or absence of the virulence gene in the current study.

9.2.3 Investigation of the host immune response

The quantification of mRNA transcripts by real time RT-PCR is a key tool for establishing how expression levels differ under certain conditions. However, mRNA levels may not always reflect the amount of protein produced. Furthermore, it was not possible to ascertain the cellular source of mRNA transcript expression using this method. This can be overcome to some extent by the use of flow cytometry, which was used in the current study to estimate the number of Tregs in the gastric mucosa and confirm the association between Treg-associated gene expression and the presence of atrophy and IM.

9.2.4 *In vitro* culture assays

As mentioned earlier, determination of bacterial CFU counts after the treatment of cultures with recombinant cytokine, or when investigating the effects of virulence determinants with mutant strains may be influenced by the culture conditions used. The use of serum-rich medium has been shown to support higher growth rates of *H. pylori* (Douraghi *et al.*, 2010; Sainsus *et al.*, 2008) which may mask small effects on bacterial numbers.

9.3 FUTURE WORK

9.3.1 Further investigation of the relationship between colonisation density and *H. pylori* virulence factors

In vivo investigation of the distribution of the bacterium in the stomach suggested an association with *dupA* status of the infecting strain. To further confirm this relationship, we would need to determine the impact of strains carrying polymorphic variants of this virulence factor as well as *dupA* mutant constructs, which would be best studied in an animal model. The use of two-photon microscopy could be used to visualise the localisation of *H. pylori* in the stomach of mice or Mongolian gerbils to confirm the relationship between bacterial localisation and *dupA* status. A similar approach was used by Kao *et al.* (2010) who investigated the localisation of DCs in the stomachs of *H. pylori* infected mice.

Another consideration is the relationship between acid secretion and bacterial localisation of *dupA* positive strains within the stomach. One way to assess this relationship would be to determine the patterns of colonisation that develop with *dupA* positive or negative strains in gastrin knock-out (GAS-KO) mice in comparison to wild-type mice. This may give insight as to the importance of acidity to DupA function.

9.3.2 Further investigation of the relationship between bacterial density and the immune response

The level of *IL8* expression was shown to be related to bacterial density in the corpus, but IL-8 is not the only inflammatory cytokine that is upregulated during infection with *H. pylori*. Previous murine studies have demonstrated increased colonisation in IFN$\gamma^{-/-}$ mice (Eaton *et al.*, 2001b). As IFNγ is a hallmark cytokine released by Th1 cells (Murphy & Reiner, 2002), the relationship between this cytokine and colonisation in the human gastric mucosa merits investigation. Also, numerous studies have now demonstrated the importance of Th17 cells and IL-17 during *H. pylori* infection (Caruso *et al.*, 2008; Luzza *et al.*, 2000; Mizuno *et al.*, 2005) and therefore is also worth consideration with respect to bacterial load.

To further assess the inflammatory response, it may be important to determine the balance of Th1, Th2 and Th17 responses in the gastric mucosa. Although a Th1 polarised immune response to *H. pylori* is widely accepted (Bamford *et al.*, 1998; Karttunen *et al.*, 1995; Mohammadi *et al.*, 1996; Sommer *et al.*, 1998), Th2 responses are also induced, and the degree of polarisation may be related to colonisation (Goll *et al.*, 2007). Evidence for the role of Th17 in *H. pylori* infection is expanding, and a recent study in mice has demonstrated a negative correlation between Th17 cells and bacterial density (Kao *et al.*, 2010); therefore meriting investigation in humans.

Suppressive cytokines were not found to be associated with bacterial load in the antrum. Given that *IL8* expression was related to bacterial density in the corpus but not the antrum, it would be interesting to investigate whether this was also true for anti-inflammatory cytokine expression.

The immune response that is mounted during *H. pylori* infection is mixed, with upregulation of Th1, Th17 and Treg responses (Goll *et al.*, 2007). Although no association was observed between Treg-associated gene expression levels and bacterial load, it is possible that the impact of these responses on density may be dependent on the predominant response, whether it is overwhelming inflammation or a skew towards a suppressive response. One approach to investigate this would be to determine the ratio of Th1 and Treg cells in the gastric mucosa using flow cytometry.

9.3.3 The relationship between immune responses and *H. pylori* virulence determinants

dupA1 type strains have now been shown to be associated with increased IL-12 production in monocytic cells (Hussein *et al.*, 2010). Whether *dupA1* has an impact on cytokine production in other immune cell types would be of interest. Also, given the association between *FOXP3* expression levels and $dupA^+$ $cagA^+$ strains, it would be interesting to further investigate the impact of these strains on suppressive responses in a larger data set.

Although we have shown *in vitro* culture models to be sufficient to determine the effects of *dupA* status on immune cells (Hussein *et al.*, 2010), it is possible that localisation of the bacterium in the gastric mucosa may play a role in the function of this virulence factor, and may therefore have varying effects on cytokine expression depending on the site of infection. It may be useful to investigate this relationship in an animal model.

The observed association between *TGFB1* expression and the *vacA* i-region status of the infecting strain also merits further investigation. Whether this relationship remains when additional samples are included should be investigated. Also, examining the effect of i1- and i2-type strains on *TGFB1* expression in different cell lines is necessary in order to determine the extent of this relationship.

9.3.4 Antimicrobial peptides and colonisation density

Too few samples were tested in order to determine if bacterial load estimated by qPCR was related to *hβD1* expression levels, however histological analysis of *H. pylori* density did demonstrate a negative association. This relationship should be confirmed with qPCR in a larger data set as this technique was found to be a more accurate means of determining colonisation density (Chapter 3).

Although a small difference in *hβD1* expression was found with a *H. pylori cagE* mutant strain, this mutation did not account for most of the reduction in *hβD1* expression observed. It is possible that another bacterial factor may be involved in regulating *hβD1* expression. Establishing the cellular compartment which induces such modulation would be useful in determining this bacterial factor. A similar approach to that used by Chakraborty *et al.* (2008) to determine the bacterial factor involved in modulating *hβD1* expression in *Vibrio cholerae* infection *in vitro* could be utilised.

We have now shown that the modulation of *hβD1* expression in gastric cell lines when infected with *H. pylori* is not specific to AGS cells, but was also observed in MKN7 cells. However, these are cell lines derived from tumour tissue. Given that the downregulation of *hβD1* has also been reported in a number of cancers (Bose

et al., 2009; Wenghoefer *et al.*, 2008), it may be more appropriate to confirm this relationship in primary gastric epithelial cells. This may also be useful for confirming the upregulation of *LL37* during *H. pylori* infection as observed in gastric tissue. Determining a cell line that expresses detectable levels of *LL37* is necessary to investigate the effects of this AMP on *H. pylori* colonisation. Chakraborty *et al.* (2008) detected *LL37* expression in HT-29 cells. Also, identification of such a cell line would be ideal for assessing the relationship between *hβD1* and *LL37*. Based on a study by Hase *et al.* (2003) hβD1 may function to enhance the activity of LL-37.

9.4 FINAL SUMMARY

The data presented in this study demonstrate the complex relationship between *H. pylori* and its human host. This has implications for the overall infectious load in the gastric mucosa. In this study, the importance of colonisation density is highlighted, with the distribution of the bacterium in the stomach being associated with the disease that develops (summarised in Figure 9.2). This may be influenced by bacterial factors such as *dupA*. Whether density establishes an environment that drives disease progression, or if the gastric environment encourages the distribution of the bacterium is not clear. Further investigation using animal models is necessary to assess the factors that drive patterns of colonisation. While suppressive immune responses were not associated with bacterial burden, their role in *H. pylori* infection is likely to involve protecting the host against the deleterious effects of inflammation, and thus reduce the risk of developing precancerous changes in the stomach as well as peptic ulcer disease. Finally, the direct modulation of bacterial load is regulated through the expression of AMPs, but *H. pylori* can still persistently colonise the human stomach, possibly through modulating the expression of *hβD1*.

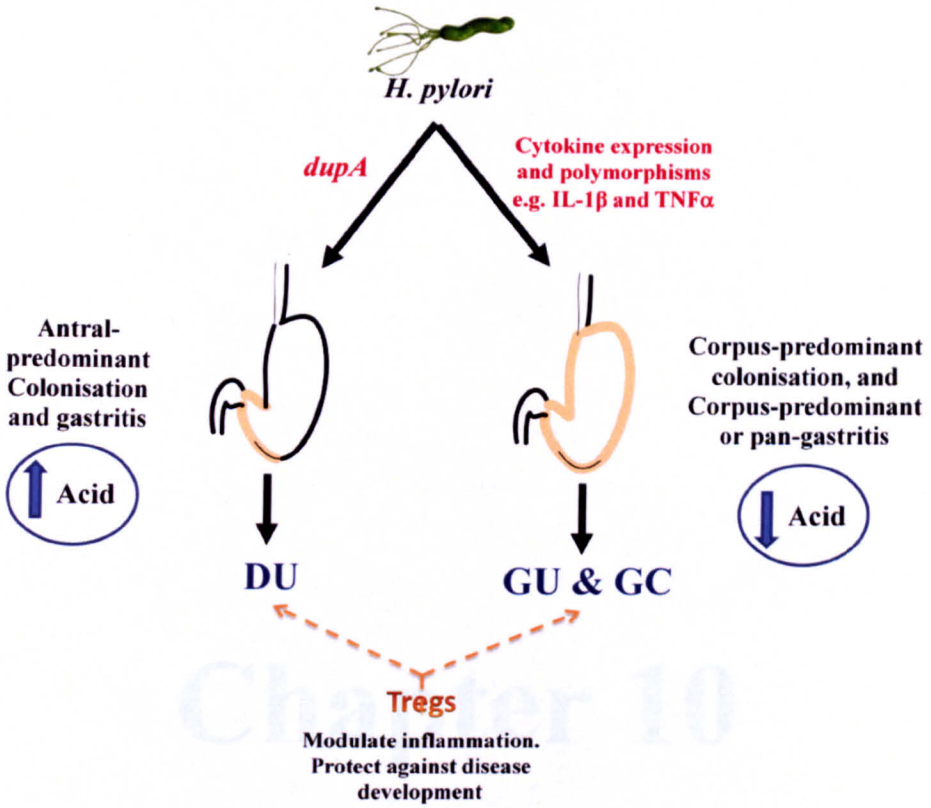


Figure 9.2: Factors that influence the pattern of bacterial colonisation and contribute to *H. pylori*-associated disease progression.

The current study has demonstrated that the pattern of colonisation is associated with disease. Antral-predominant colonisation was found to be associated with DU, whilst corpus-predominant colonisation was associated with GU. It is possible that a tight association exists between the level of acidity, the pattern of colonisation and gastritis. In this study, it is now shown that *dupA* status of the colonising strain may influence the development of DU. Disease development is also related to the number of Tregs in the gastric mucosa, which may have a protective role against gastric disease possibly through the modulation of inflammation. Tregs were not found to be associated with bacterial load.

Chapter 10

10. REFERENCES

Achyut, B. R., Ghoshal, U. C., Moorchung, N. & Mittal, B. (2009). Transforming growth factor-B1 and matrix metalloproteinase-7 promoter variants induce risk for *Helicobacter pylori*-associated gastric precancerous lesions. *DNA Cell Biol* **28**, 295-301.

Adamsky, K., Arnold, K., Sabanay, H. & Peles, E. (2003). Junctional protein MAGI-3 interacts with receptor tyrosine phosphatase beta (RPTP beta) and tyrosine-phosphorylated proteins. *J Cell Sci* **116**, 1279-1289.

Agerberth, B., Gunne, H., Odeberg, J., Kogner, P., Boman, H. G. & Gudmundsson, G. H. (1995). FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. *Proc Natl Acad Sci U S A* **92**, 195-199.

Aggarwal, S. & Gurney, A. L. (2002). IL-17: prototype member of an emerging cytokine family. *J Leukoc Biol* **71**, 1-8.

Agha, A. & Graham, D. Y. (2005). Evidence-based examination of the African enigma in relation to *Helicobacter pylori* infection. *Scand J Gastroenterol* **40**, 523-529.

Akhiani, A. A., Schon, K., Franzen, L. E., Pappo, J. & Lycke, N. (2004). Helicobacter pylori-specific antibodies impair the development of gastritis, facilitate bacterial colonization, and counteract resistance against infection. *J Immunol* **172**, 5024-5033.

Akhiani, A. A., Stensson, A., Schon, K. & Lycke, N. Y. (2005). IgA antibodies impair resistance against Helicobacter pylori infection: studies on immune evasion in IL-10-deficient mice. *J Immunol* **174**, 8144-8153.

Akopyants, N. S., Clifton, S. W., Kersulyte, D. & other authors (1998). Analyses of the cag pathogenicity island of Helicobacter pylori. *Mol Microbiol* **28**, 37-53.

Allaker, R. P., Young, K. A., Hardie, J. M., Domizio, P. & Meadows, N. J. (2002). Prevalence of Helicobacter pylori at oral and gastrointestinal sites in children: evidence for possible oral-to-oral transmission. . *J Med Microbiol* **51**, 312-317.

Allan, S. E., Crome, S. Q., Crellin, N. K., Passerini, L., Steiner, T. S., Bacchetta, R., Roncarolo, M. G. & Levings, M. K. (2007). Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* **19**, 345-354.

Allen, L. A. (2001). The role of the neutrophil and phagocytosis in infection caused by *Helicobacter pylori*. *Curr Opin Infect Dis* **14**, 273-277.

Alm, R. A., Ling, L. S., Moir, D. T. & other authors (1999). Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* **397**, 176-180.

Amedei, A., Cappon, A., Codolo, G. & other authors (2006). The neutrophil-activating protein of *Helicobacter pylori* promotes Th1 immune responses. *J Clin Invest* **116**, 1092-1101.

Amieva, M. R., Vogelmann, R., Covacci, A., Tompkins, L. S., Nelson, W. J. & Falkow, S. (2003). Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science* **300**, 1430-1434.

Annacker, O., Pimenta-Araujo, R., Burlen-Defranoux, O., Barbosa, T. C., Cumano, A. & Bandeira, A. (2001). CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J Immunol* **166**, 3008-3018.

Antony, P. A., Piccirillo, C. A., Akpinarli, A. & other authors (2005). CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J Immunol* **174**, 2591-2601.

Arachchi, H. S., Kalra, V., Lal, B. & other authors (2007). Prevalence of duodenal ulcer-promoting gene (*dupA*) of *Helicobacter pylori* in patients with duodenal ulcer in North Indian population. *Helicobacter* **12**, 591-597.

Argent, R. H., Kidd, M., Owen, R. J., Thomas, R. J., Limb, M. C. & Atherton, J. C. (2004). Determinants and consequences of different levels of CagA phosphorylation for clinical isolates of *Helicobacter pylori*. *Gastroenterology* **127**, 514-523.

Argent, R. H., Burette, A., Miendje Deyi, V. Y. & Atherton, J. C. (2007). The presence of *dupA* in *Helicobacter pylori* is not significantly associated with duodenal ulceration in Belgium, South Africa, China, or North America. *Clin Infect Dis* **45**, 1204-1206.

Argent, R. H., Thomas, R. J., Letley, D. P., Rittig, M. G., Hardie, K. R. & Atherton, J. C. (2008). Functional association between the *Helicobacter pylori* virulence factors VacA and CagA. *J Med Microbiol* **57**, 145-150.

Arnold, I., Lee, J. Y., Amieva, M. R., Roers, A., Flavell, R. A., Sparwasser, T. & Muller, A. (2010). Tolerance rather than immunity protects from *Helicobacter pylori* -induced gastric preneoplasia. *Gastroenterology*.

Asim, M., Chaturvedi, R., Hoge, S. & other authors (2010). *Helicobacter pylori* induces ERK-dependent formation of a phospho-C-FOS/C-JUN AP-1 complex that causes apoptosis in macrophages. *J Biol Chem*.

Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. (1999). An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* **190**, 995-1004.

Atherton, J. C., Cao, P., Peek, R. M., Jr., Tummuru, M. K., Blaser, M. J. & Cover, T. L. (1995). Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. *J Biol Chem* **270**, 17771-17777.

Atherton, J. C., Tham, K. T., Peek, R. M., Jr., Cover, T. L. & Blaser, M. J. (1996). Density of *Helicobacter pylori* infection *in vivo* as assessed by quantitative culture and histology. *J Infect Dis* **174**, 552-556.

Atherton, J. C. (1997). Non-endoscopic tests in the diagnosis of *Helicobacter pylori* infection. *Aliment Pharmacol Ther* **11 Suppl 1**, 11-20.

Atherton, J. C. (2006). The pathogenesis of *Helicobacter pylori*-induced gastro-duodenal diseases. *Annu Rev Pathol* **1**, 63-96.

Aujla, S. J., Chan, Y. R., Zheng, M. & other authors (2008). IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med* **14**, 275-281.

Auroux, J., Lamarque, D., Tankovic, J., Benamouzig, R., Mahe, S., Chaumette, M. T. & Delchier, J. C. (1998). Comparison of quantifying *Helicobacter pylori* gastric infection by culture, histology and C13 urea breath test. *Gastroenterol Clin Biol* **22**, 407-412.

Aydin, O., Egilmez, R., Karabacak, T. & Kanik, A. (2003). Interobserver variation in histopathological assessment of *Helicobacter pylori* gastritis. *World J Gastroenterol* **9**, 2232-2235.

Azevedo, M., Eriksson, S., Mendes, N. & other authors (2008). Infection by *Helicobacter pylori* expressing the BabA adhesin is influenced by the secretor phenotype. *J Pathol* **215**, 308-316.

Azuma, T., Yamazaki, S., Yamakawa, A. & other authors (2004). Association between diversity in the Src homology 2 domain--containing tyrosine phosphatase binding site of Helicobacter pylori CagA protein and gastric atrophy and cancer. *The Journal of infectious diseases* **189**, 820-827.

Baccala, R., Kono, D. H. & Theofilopoulos, A. N. (2005). Interferons as pathogenic effectors in autoimmunity. *Immunol Rev* **204**, 9-26.

Backhed, F., Rokbi, B., Torstensson, E., Zhao, Y., Nilsson, C., Seguin, D., Normark, S., Buchan, A. M. & Richter-Dahlfors, A. (2003). Gastric mucosal recognition of *Helicobacter pylori* is independent of Toll-like receptor 4. *J Infect Dis* **187**, 829-836.

Backstrom, A., Lundberg, C., Kersulyte, D., Berg, D. E., Boren, T. & Arnqvist, A. (2004). Metastability of *Helicobacter pylori bab* adhesin genes and dynamics in Lewis b antigen binding. *Proc Natl Acad Sci U S A* **101**, 16923-16928.

Baecher-Allan, C., Brown, J. A., Freeman, G. J. & Hafler, D. A. (2001). CD4+CD25high regulatory cells in human peripheral blood. *J Immunol* **167**, 1245-1253.

Bajaj-Elliott, M., Fedeli, P., Smith, G. V., Domizio, P., Maher, L., Ali, R. S., Quinn, A. G. & Farthing, M. J. (2002). Modulation of host antimicrobial peptide (beta-defensins 1 and 2) expression during gastritis. *Gut* **51**, 356-361.

Bamford, K. B., Fan, X., Crowe, S. E. & other authors (1998). Lymphocytes in the human gastric mucosa during *Helicobacter pylori* have a T helper cell 1 phenotype. *Gastroenterology* **114**, 482-492.

Basso, D., Navaglia, F., Brigato, L. & other authors (1998). Analysis of *Helicobacter pylori vacA* and *cagA* genotypes and serum antibody profile in benign and malignant gastroduodenal diseases. *Gut* **43**, 182-186.

Basso, D., Zambon, C. F., Letley, D. P. & other authors (2008). Clinical relevance of *Helicobacter pylori cagA* and *vacA* gene polymorphisms. *Gastroenterology* **135**, 91-99.

Bayraktaroglu, T., Aras, A. S., Aydemir, S., Davutoglu, C., Ustundag, Y., Atmaca, H. & Borazan, A. (2004). Serum levels of tumor necrosis factor-alpha, interleukin-6 and interleukin-8 are not increased in dyspeptic patients with *Helicobacter pylori*-associated gastritis. *Mediators Inflamm* **13**, 25-28.

Beales, I., Blaser, M. J., Srinivasan, S., Calam, J., Perez-Perez, G. I., Yamada, T., Schelman, J., Post, L. & Del Valle, J. (1997). Effect of *Helicobacter pylori* products and recombinant cytokines on gastrin release from cultured canine G cells. *Gastroenterology* **113**, 465-471.

Beales, I. L. & Calam, J. (1998). Interleukin 1 beta and tumour necrosis factor alpha inhibit acid secretion in cultured rabbit parietal cells by multiple pathways. *Gut* **42**, 227-234.

Becker-Andre, M. & Hahlbrock, K. (1989). Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). *Nucleic Acids Res* **17**, 9437-9446.

Becker, S., Boger, P., Oehlmann, R. & Ernst, A. (2000). PCR bias in ecological analysis: a case study for quantitative Taq nuclease assays in analyses of microbial communities. *Appl Environ Microbiol* **66**, 4945-4953.

Belas, R., Manos, J. & Suvanasuthi, R. (2004). Proteus mirabilis ZapA metalloprotease degrades a broad spectrum of substrates, including antimicrobial peptides. *Infect Immun* **72**, 5159-5167.

Ben Ahmed, M., Belhadj Hmida, N., Moes, N., Buyse, S., Abdeladhim, M., Louzir, H. & Cerf-Bensussan, N. (2009). IL-15 renders conventional lymphocytes resistant to suppressive functions of regulatory T cells through activation of the phosphatidylinositol 3-kinase pathway. *J Immunol* **182**, 6763-6770.

Ben Mansour, K., Fendri, C., Zribi, M. & other authors (2010). Prevalence of *Helicobacter pylori vacA, cagA, iceA* and *oipA* genotypes in Tunisian patients. *Ann Clin Microbiol Antimicrob* **9**, 10.

Bennett, C. L., Christie, J., Ramsdell, F. & other authors (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of *FOXP3*. *Nat Genet* **27**, 20-21.

Bensch, K. W., Raida, M., Magert, H. J., Schulz-Knappe, P. & Forssmann, W. G. (1995). hBD-1: a novel beta-defensin from human plasma. *FEBS Lett* **368**, 331-335.

Berg, D. J., Lynch, N. A., Lynch, R. G. & Lauricella, D. M. (1998). Rapid development of severe hyperplastic gastritis with gastric epithelial dedifferentiation in *Helicobacter felis*-infected IL-10(-/-) mice. *Am J Pathol* **152**, 1377-1386.

Bergman, P., Johansson, L., Asp, V., Plant, L., Gudmundsson, G. H., Jonsson, A. B. & Agerberth, B. (2005). *Neisseria gonorrhoeae* downregulates expression of the human antimicrobial peptide LL-37. *Cell Microbiol* **7**, 1009-1017.

Bettelli, E., Dastrange, M. & Oukka, M. (2005). Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proc Natl Acad Sci U S A* **102**, 5138-5143.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L. & Kuchroo, V. K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235-238.

Biragyn, A., Ruffini, P. A., Leifer, C. A. & other authors (2002). Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science* **298**, 1025-1029.

Biragyn, A., Coscia, M., Nagashima, K., Sanford, M., Young, H. A. & Olkhanud, P. (2008). Murine beta-defensin 2 promotes TLR-4/MyD88-mediated and NF-kappaB-dependent atypical death of APCs via activation of TNFR2. *J Leukoc Biol* **83**, 998-1008.

Bizzozero, G. (1893). Ueber die schlauchförmigen Drüsen des Magendarmkanals und die Beziehungen ihres Epithels zu dem Oberflächenepithel der Schleimhaut. Dritte mitteilung. *Archiv Mikroskopische Anat* **43**, :82–152.

Blanchard, T. G., Yu, F., Hsieh, C. L. & Redline, R. W. (2003). Severe inflammation and reduced bacteria load in murine *Helicobacter* infection caused by lack of phagocyte oxidase activity. *J Infect Dis* **187**, 1609-1615.

Blaser, M. J. & Parsonnet, J. (1994). Parasitism by the "slow" bacterium *Helicobacter pylori* leads to altered gastric homeostasis and neoplasia. *J Clin Invest* **94**, 4-8.

Blaser, M. J., Perez-Perez, G. I., Kleanthous, H., Cover, T. L., Peek, R. M., Chyou, P. H., Stemmermann, G. N. & Nomura, A. (1995). Infection with *Helicobacter pylori* strains possessing *cagA* is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res* **55**, 2111-2115.

Blaser, M. J. & Crabtree, J. E. (1996). CagA and the outcome of Helicobacter pylori infection. *Am J Clin Pathol* **106**, 565-567.

Blaser, M. J. & Atherton, J. C. (2004). Helicobacter pylori persistence: biology and disease. *J Clin Invest* **113**, 321-333.

Blaser, M. J., Chen, Y. & Reibman, J. (2008). Does *Helicobacter pylori* protect against asthma and allergy? *Gut* **57**, 561-567.

Bluestone, J. A. & Abbas, A. K. (2003). Natural versus adaptive regulatory T cells. *Nat Rev Immunol* **3**, 253-257.

Bodger, K., Wyatt, J. I. & Heatley, R. V. (2001). Variation in serum pepsinogens with severity and topography of *Helicobacter pylori*-associated chronic gastritis in dyspeptic patients referred for endoscopy. *Helicobacter* **6**, 216-224.

Boncristiano, M., Paccani, S. R., Barone, S. & other authors (2003). The *Helicobacter pylori* vacuolating toxin inhibits T cell activation by two independent mechanisms. *J Exp Med* **198**, 1887-1897.

Borden, E. C., Sen, G. C., Uze, G., Silverman, R. H., Ransohoff, R. M., Foster, G. R. & Stark, G. R. (2007). Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov* **6**, 975-990.

Boren, T., Falk, P., Roth, K. A., Larson, G. & Normark, S. (1993). Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. *Science* **262**, 1892-1895.

Bose, S. K., Gibson, W., Bullard, R. S. & Donald, C. D. (2009). PAX2 oncogene negatively regulates the expression of the host defense peptide human beta defensin-1 in prostate cancer. *Mol Immunol* **46**, 1140-1148.

Boughan, P. K., Argent, R. H., Body-Malapel, M. & other authors (2006). Nucleotide-binding oligomerization domain-1 and epidermal growth factor receptor: critical regulators of beta-defensins during *Helicobacter pylori* infection. *J Biol Chem* **281**, 11637-11648.

Bourzac, K. M., Botham, C. M. & Guillemin, K. (2007). *Helicobacter pylori* CagA induces AGS cell elongation through a cell retraction defect that is independent of Cdc42, Rac1, and Arp2/3. *Infect Immun* **75**, 1203-1213.

Boyanova, L. (2009). Prevalence of multidrug-resistant *Helicobacter pylori* in Bulgaria. *J Med Microbiol* **58**, 930-935.

Braff, M. H., Di Nardo, A. & Gallo, R. L. (2005). Keratinocytes store the antimicrobial peptide cathelicidin in lamellar bodies. *J Invest Dermatol* **124**, 394-400.

Brandt, S., Kwok, T., Hartig, R., Konig, W. & Backert, S. (2005). NF-kappaB activation and potentiation of proinflammatory responses by the *Helicobacter pylori* CagA protein. *Proc Natl Acad Sci U S A* **102**, 9300-9305.

Brandt, S., Shafikhani, S., Balachandran, P., Jin, S., Hartig, R., Konig, W., Engel, J. & Backert, S. (2007). Use of a novel coinfection system reveals a role for Rac1, H-Ras, and CrkII phosphorylation in *Helicobacter pylori*-induced host cell actin cytoskeletal rearrangements. *FEMS Immunol Med Microbiol* **50**, 190-205.

Brown, K. L. & Hancock, R. E. (2006). Cationic host defense (antimicrobial) peptides. *Curr Opin Immunol* **18**, 24-30.

Brown, L. M. (2000). *Helicobacter pylori*: epidemiology and routes of transmission. *Epidemiol Rev* **22**, 283-297.

Bucki, R., Pastore, J. J., Randhawa, P., Vegners, R., Weiner, D. J. & Janmey, P. A. (2004). Antibacterial activities of rhodamine B-conjugated gelsolin-derived peptides compared to those of the antimicrobial peptides cathelicidin LL37, magainin II, and melittin. *Antimicrob Agents Chemother* **48**, 1526-1533.

Buckner, J. H. & Ziegler, S. F. (2008). Functional analysis of FOXP3. *Ann N Y Acad Sci* **1143**, 151-169.

Bullard, R. S., Gibson, W., Bose, S. K. & other authors (2008). Functional analysis of the host defense peptide Human Beta Defensin-1: new insight into its potential role in cancer. *Mol Immunol* **45**, 839-848.

Bumann, D., Aksu, S., Wendland, M., Janek, K., Zimny-Arndt, U., Sabarth, N., Meyer, T. F. & Jungblut, P. R. (2002). Proteome analysis of secreted proteins of the gastric pathogen *Helicobacter pylori*. *Infect Immun* **70**, 3396-3403.

Bustin, S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* **25**, 169-193.

Bustin, S. A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* **29**, 23-39.

Bustin, S. A. & Nolan, T. (2004). Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J Biomol Tech* **15**, 155-166.

Calado, D. P., Paixao, T., Holmberg, D. & Haury, M. (2006). Stochastic monoallelic expression of IL-10 in T cells. *J Immunol* **177**, 5358-5364.

Camilleri, M., Andrews, C. N., Bharucha, A. E. & other authors (2007). Alterations in expression of p11 and SERT in mucosal biopsy specimens of patients with irritable bowel syndrome. *Gastroenterology* **132**, 17-25.

Camorlinga-Ponce, M., Aviles-Jimenez, F., Cabrera, L., Hernandez-Pando, R., Munoz, O., Soza, J. & Torres, J. (2003). Intensity of inflammation, density of colonization and interleukin-8 response in the gastric mucosa of children infected with *Helicobacter pylori*. *Helicobacter* **8**, 554-560.

Camorlinga-Ponce, M., Romo, C., Gonzalez-Valencia, G., Munoz, O. & Torres, J. (2004). Topographical localisation of *cagA* positive and *cagA* negative *Helicobacter pylori* strains in the gastric mucosa; an in situ hybridisation study. *J Clin Pathol* **57**, 822-828.

Canedo, P., Castanheira-Vale, A. J., Lunet, N. & other authors (2008). The interleukin-8-251*T/*A polymorphism is not associated with risk for gastric carcinoma development in a Portuguese population. *Eur J Cancer Prev* **17**, 28-32.

Caruso, R., Fina, D., Peluso, I. & other authors (2007). IL-21 is highly produced in *Helicobacter pylori*-infected gastric mucosa and promotes gelatinases synthesis. *J Immunol* **178**, 5957-5965.

Caruso, R., Fina, D., Paoluzi, O. A. & other authors (2008). IL-23-mediated regulation of IL-17 production in *Helicobacter pylori*-infected gastric mucosa. *Eur J Immunol* **38**, 470-478.

Castellino, F. & Germain, R. N. (2006). Cooperation between CD4+ and CD8+ T cells: when, where, and how. *Annu Rev Immunol* **24**, 519-540.

Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R. & Covacci, A. (1996). *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci U S A* **93**, 14648-14653.

Chakraborty, K., Ghosh, S., Koley, H. & other authors (2008). Bacterial exotoxins downregulate cathelicidin (hCAP-18/LL-37) and human beta-defensin 1 (HBD-1) expression in the intestinal epithelial cells. *Cell Microbiol* **10**, 2520-2537.

Chalifour, A., Jeannin, P., Gauchat, J. F., Blaecke, A., Malissard, M., N'Guyen, T., Thieblemont, N. & Delneste, Y. (2004). Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers alpha-defensin production. *Blood* **104**, 1778-1783.

Chatenoud, L. & Bach, J. F. (2006). Adaptive human regulatory T cells: myth or reality? *J Clin Invest* **116**, 2325-2327.

Chatila, T. A., Blaeser, F., Ho, N., Lederman, H. M., Voulgaropoulos, C., Helms, C. & Bowcock, A. M. (2000). JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J Clin Invest* **106**, R75-81.

Chattopadhyay, S., Chakraborty, N. G. & Mukherji, B. (2005). Regulatory T cells and tumor immunity. *Cancer Immunol Immunother* **54**, 1153-1161.

Chen, H., Wilkins, L. M., Aziz, N. & other authors (2006a). Single nucleotide polymorphisms in the human interleukin-1B gene affect transcription according to haplotype context. *Hum Mol Genet* **15**, 519-529.

Chen, H., Xu, Z., Peng, L., Fang, X., Yin, X., Xu, N. & Cen, P. (2006b). Recent advances in the research and development of human defensins. *Peptides* **27**, 931-940.

Chen, W., Shu, D. & Chadwick, V. S. (2001). *Helicobacter pylori* infection: mechanism of colonization and functional dyspepsia Reduced colonization of gastric mucosa by *Helicobacter pylori* in mice deficient in interleukin-10. *J Gastroenterol Hepatol* **16**, 377-383.

Chen, Y. & Blaser, M. J. (2007). Inverse associations of *Helicobacter pylori* with asthma and allergy. *Arch Intern Med* **167**, 821-827.

Chen, Y. & Blaser, M. J. (2008). *Helicobacter pylori* colonization is inversely associated with childhood asthma. *J Infect Dis* **198**, 553-560.

Chertov, O., Michiel, D. F., Xu, L., Wang, J. M., Tani, K., Murphy, W. J., Longo, D. L., Taub, D. D. & Oppenheim, J. J. (1996). Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. *J Biol Chem* **271**, 2935-2940.

Chey, W. D. & Wong, B. C. (2007). American College of Gastroenterology guideline on the management of *Helicobacter pylori* infection. *Am J Gastroenterol* **102**, 1808-1825.

Chong, S. K., Lou, Q., Fitzgerald, J. F. & Lee, C. H. (1996). Evaluation of *16S rRNA* gene PCR with primers Hp1 and Hp2 for detection of *Helicobacter pylori*. *J Clin Microbiol* **34**, 2728-2730.

Chowers, M. Y., Keller, N., Bar-Meir, S. & Chowers, Y. (2002). A defined human gastrin sequence stimulates the growth of Helicobacter pylori. *FEMS Microbiol Lett* **217**, 231-236.

Churin, Y., Al-Ghoul, L., Kepp, O., Meyer, T. F., Birchmeier, W. & Naumann, M. (2003). *Helicobacter pylori* CagA protein targets the c-Met receptor and enhances the motogenic response. *J Cell Biol* **161**, 249-255.

Codolo, G., Mazzi, P., Amedei, A., Del Prete, G., Berton, G., D'Elios, M. M. & de Bernard, M. (2008). The neutrophil-activating protein of Helicobacter pylori down-modulates Th2 inflammation in ovalbumin-induced allergic asthma. *Cell Microbiol* **10**, 2355-2363.

Cohen, H. (2000). Peptic ulcer and Helicobacter pylori. *Gastroenterol Clin North Am* **29**, 775-789.

Cole, A. M., Hong, T., Boo, L. M. & other authors (2002). Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. *Proc Natl Acad Sci U S A* **99**, 1813-1818.

Coombes, J. L., Siddiqui, K. R., Arancibia-Carcamo, C. V., Hall, J., Sun, C. M., Belkaid, Y. & Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* **204**, 1757-1764.

Correa, P. (1995). Helicobacter pylori and gastric carcinogenesis. *Am J Surg Pathol* **19 Suppl 1,** S37-43.

Correa, P. & Houghton, J. (2007). Carcinogenesis of *Helicobacter pylori. Gastroenterology* **133,** 659-672.

Correa, P., Piazuelo, M. B. & Wilson, K. T. (2010). Pathology of gastric intestinal metaplasia: clinical implications. *Am J Gastroenterol* **105,** 493-498.

Couper, K. N., Blount, D. G. & Riley, E. M. (2008). IL-10: the master regulator of immunity to infection. *J Immunol* **180,** 5771-5777.

Cover, T. L. & Blaser, M. J. (1992). Purification and characterization of the vacuolating toxin from *Helicobacter pylori. J Biol Chem* **267,** 10570-10575.

Cover, T. L., Reddy, L. Y. & Blaser, M. J. (1993). Effects of ATPase inhibitors on the response of HeLa cells to *Helicobacter pylori* vacuolating toxin. *Infect Immun* **61,** 1427-1431.

Cover, T. L., Tummuru, M. K., Cao, P., Thompson, S. A. & Blaser, M. J. (1994). Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. *J Biol Chem* **269,** 10566-10573.

Cover, T. L., Hanson, P. I. & Heuser, J. E. (1997). Acid-induced dissociation of VacA, the Helicobacter pylori vacuolating cytotoxin, reveals its pattern of assembly. *J Cell Biol* **138,** 759-769.

Crabtree, G. R. & Olson, E. N. (2002). NFAT signaling: choreographing the social lives of cells. *Cell* **109 Suppl,** S67-79.

Crabtree, J. E., Shallcross, T. M., Heatley, R. V. & Wyatt, J. I. (1991a). Mucosal tumour necrosis factor alpha and interleukin-6 in patients with *Helicobacter pylori* associated gastritis. *Gut* **32,** 1473-1477.

Crabtree, J. E., Taylor, J. D., Wyatt, J. I., Heatley, R. V., Shallcross, T. M., Tompkins, D. S. & Rathbone, B. J. (1991b). Mucosal IgA recognition of *Helicobacter pylori* 120 kDa protein, peptic ulceration, and gastric pathology. *Lancet* **338,** 332-335.

Crabtree, J. E., Wyatt, J. I., Trejdosiewicz, L. K., Peichl, P., Nichols, P. H., Ramsay, N., Primrose, J. N. & Lindley, I. J. (1994). Interleukin-8 expression in *Helicobacter pylori* infected, normal, and neoplastic gastroduodenal mucosa. *J Clin Pathol* **47,** 61-66.

Crabtree, J. E. (1996). Gastric mucosal inflammatory responses to *Helicobacter pylori. Aliment Pharmacol Ther* **10 Suppl 1,** 29-37.

Croxen, M. A., Sisson, G., Melano, R. & Hoffman, P. S. (2006). The *Helicobacter pylori* chemotaxis receptor TlpB (HP0103) is required for pH taxis and for colonization of the gastric mucosa. *J Bacteriol* **188,** 2656-2665.

Cua, D. J. & Tato, C. M. (2010). Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* **10,** 479-489.

Cullen, D. J., Collins, B. J., Christiansen, K. J., Epis, J., Warren, J. R., Surveyor, I. & Cullen, K. J. (1993). When is Helicobacter pylori infection acquired? *Gut* **34,** 1681-1682.

Cunliffe, R. N., Rose, F. R., Keyte, J., Abberley, L., Chan, W. C. & Mahida, Y. R. (2001). Human defensin 5 is stored in precursor form in normal Paneth cells and is expressed by some villous epithelial cells and by metaplastic Paneth cells in the colon in inflammatory bowel disease. *Gut* **48,** 176-185.

Cunliffe, R. N., Kamal, M., Rose, F. R., James, P. D. & Mahida, Y. R. (2002). Expression of antimicrobial neutrophil defensins in epithelial cells of active inflammatory bowel disease mucosa. *J Clin Pathol* **55**, 298-304.

Curfs, J. H., Meis, J. F. & Hoogkamp-Korstanje, J. A. (1997). A primer on cytokines: sources, receptors, effects, and inducers. *Clin Microbiol Rev* **10**, 742-780.

D'Elios, M. M., Manghetti, M., Almerigogna, F. & other authors (1997). Different cytokine profile and antigen-specificity repertoire in *Helicobacter pylori*-specific T cell clones from the antrum of chronic gastritis patients with or without peptic ulcer. *Eur J Immunol* **27**, 1751-1755.

D'Elios, M. M., Amedei, A. & Del Prete, G. (2003). *Helicobacter pylori* antigen-specific T-cell responses at gastric level in chronic gastritis, peptic ulcer, gastric cancer and low-grade mucosa-associated lymphoid tissue (MALT) lymphoma. *Microbes Infect* **5**, 723-730.

D'Elios, M. M., Codolo, G., Amedei, A., Mazzi, P., Berton, G., Zanotti, G., Del Prete, G. & de Bernard, M. (2009). *Helicobacter pylori*, asthma and allergy. *FEMS Immunol Med Microbiol* **56**, 1-8.

Dabiri, H., Maleknejad, P., Yamaoka, Y., Feizabadi, M. M., Jafari, F., Rezadehbashi, M., Nakhjavani, F. A., Mirsalehian, A. & Zali, M. R. (2009). Distribution of *Helicobacter pylori cagA*, *cagE*, *oipA* and *vacA* in different major ethnic groups in Tehran, Iran. *J Gastroenterol Hepatol* **24**, 1380-1386.

Danese, S., Cremonini, F., Armuzzi, A. & other authors (2001). *Helicobacter pylori* CagA-positive strains affect oxygen free radicals generation by gastric mucosa. *Scand J Gastroenterol* **36**, 247-250.

Davidson, T. S., DiPaolo, R. J., Andersson, J. & Shevach, E. M. (2007). Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J Immunol* **178**, 4022-4026.

de Bernard, M., Papini, E., de Filippis, V., Gottardi, E., Telford, J., Manetti, R., Fontana, A., Rappuoli, R. & Montecucco, C. (1995). Low pH activates the vacuolating toxin of *Helicobacter pylori*, which becomes acid and pepsin resistant. *J Biol Chem* **270**, 23937-23940.

de Bernard, M. & D'Elios, M. M. (2009). The immune modulating activity of the *Helicobacter pylori* HP-NAP: Friend or foe? *Toxicon*.

de Jonge, R., Kuipers, E. J., Langeveld, S. C., Loffeld, R. J., Stoof, J., van Vliet, A. H. & Kusters, J. G. (2004a). The *Helicobacter pylori* plasticity region locus *jhp0947-jhp0949* is associated with duodenal ulcer disease and interleukin-12 production in monocyte cells. *FEMS Immunol Med Microbiol* **41**, 161-167.

de Jonge, R., Pot, R. G., Loffeld, R. J., van Vliet, A. H., Kuipers, E. J. & Kusters, J. G. (2004b). The functional status of the *Helicobacter pylori sabB* adhesin gene as a putative marker for disease outcome. *Helicobacter* **9**, 158-164.

De, Y., Chen, Q., Schmidt, A. P., Anderson, G. M., Wang, J. M., Wooters, J., Oppenheim, J. J. & Chertov, O. (2000). LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* **192**, 1069-1074.

Del Prete, G. (1998). The concept of type-1 and type-2 helper T cells and their cytokines in humans. *Int Rev Immunol* **16**, 427-455.

Demangel, C., Bertolino, P. & Britton, W. J. (2002). Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production. *Eur J Immunol* **32**, 994-1002.

Demirturk, L., Ozel, A. M., Yazgan, Y., Solmazgul, E., Yildirim, S., Gultepe, M. & Gurbuz, A. K. (2001). *CagA* status in dyspeptic patients with and without peptic ulcer disease in Turkey: association with histopathologic findings. *Helicobacter* **6**, 163-168.

Denman, S. E., Tomkins, N. W. & McSweeney, C. S. (2007). Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol Ecol* **62**, 313-322.

Derakhshan, M. H., El-Omar, E., Oien, K., Gillen, D., Fyfe, V., Crabtree, J. E. & McColl, K. E. (2006). Gastric histology, serological markers and age as predictors of gastric acid secretion in patients infected with *Helicobacter pylori*. *J Clin Pathol* **59**, 1293-1299.

Derynck, R. & Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**, 577-584.

Di Mario, F., Cavallaro, L. G., Moussa, A. M. & other authors (2006). Usefulness of serum pepsinogens in *Helicobacter pylori* chronic gastritis: relationship with inflammation, activity, and density of the bacterium. *Dig Dis Sci* **51**, 1791-1795.

di Mario, F. & Cavallaro, L. G. (2008). Non-invasive tests in gastric diseases. *Dig Liver Dis* **40**, 523-530.

Dinarello, C. A. (1996). Biologic basis for interleukin-1 in disease. *Blood* **87**, 2095-2147.

Dixon, M. F., Genta, R. M., Yardley, J. H. & Correa, P. (1996). Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* **20**, 1161-1181.

Doherty, N. & Atherton, J. C. (2009). Human gastrin: a true growth factor for *Helicobacter pylori*? *Helicobacter* **14**, 303.

Dominici, P., Bellentani, S., Di Biase, A. R. & other authors (1999). Familial clustering of *Helicobacter pylori* infection: population based study. *BMJ* **319**, 537-540.

Donald, C. D., Sun, C. Q., Lim, S. D. & other authors (2003). Cancer-specific loss of beta-defensin 1 in renal and prostatic carcinomas. *Lab Invest* **83**, 501-505.

Dossumbekova, A., Prinz, C., Mages, J. & other authors (2006). *Helicobacter pylori* HopH (OipA) and bacterial pathogenicity: genetic and functional genomic analysis of *hopH* gene polymorphisms. *J Infect Dis* **194**, 1346-1355.

Douraghi, M., Mohammadi, M., Oghalaie, A. & other authors (2008). *dupA* as a risk determinant in *Helicobacter pylori* infection. *J Med Microbiol* **57**, 554-562.

Douraghi, M., Kashani, S. S., Zeraati, H., Esmaili, M., Oghalaie, A. & Mohammadi, M. (2010). Comparative evaluation of three supplements for *Helicobacter pylori* growth in liquid culture. *Curr Microbiol* **60**, 254-262.

Dubois, A., Fiala, N., Heman-Ackah, L. M., Drazek, E. S., Tarnawski, A., Fishbein, W. N., Perez-Perez, G. I. & Blaser, M. J. (1994). Natural gastric infection with *Helicobacter pylori* in monkeys: a model for spiral bacteria infection in humans. *Gastroenterology* **106**, 1405-1417.

Dudal, S., Turriere, C., Bessoles, S., Fontes, P., Sanchez, F., Liautard, J., Liautard, J. P. & Lafont, V. (2006). Release of LL-37 by activated human Vgamma9Vdelta2 T cells: a microbicidal weapon against *Brucella suis*. *J Immunol* **177**, 5533-5539.

Duhen, T., Geiger, R., Jarrossay, D., Lanzavecchia, A. & Sallusto, F. (2009). Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol* **10**, 857-863.

Dumonceaux, T. J., Hill, J. E., Briggs, S. A., Amoako, K. K., Hemmingsen, S. M. & Van Kessel, A. G. (2006). Enumeration of specific bacterial populations in complex intestinal communities using quantitative PCR based on the chaperonin-60 target. *J Microbiol Methods* **64**, 46-62.

Durr, U. H., Sudheendra, U. S. & Ramamoorthy, A. (2006). LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochim Biophys Acta* **1758**, 1408-1425.

Dzierzanowska-Fangrat, K., Crabtree, J. E., Rozynek, E., Dura, W., Celinska-Cedro, D., Wojda, U. & Dzierzanowska, D. (2002). *Helicobacter pylori cagA* genotype and density of colonization in relation to gastric inflammation in children. *Eur J Gastroenterol Hepatol* **14**, 1303-1307.

Eaton, K. A. & Krakowka, S. (1994). Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori*. *Infect Immun* **62**, 3604-3607.

Eaton, K. A., Kersulyte, D., Mefford, M., Danon, S. J., Krakowka, S. & Berg, D. E. (2001a). Role of *Helicobacter pylori cag* region genes in colonization and gastritis in two animal models. *Infect Immun* **69**, 2902-2908.

Eaton, K. A., Mefford, M. & Thevenot, T. (2001b). The role of T cell subsets and cytokines in the pathogenesis of *Helicobacter pylori* gastritis in mice. *J Immunol* **166**, 7456-7461.

Ekstrom, A. M., Held, M., Hansson, L. E., Engstrand, L. & Nyren, O. (2001). *Helicobacter pylori* in gastric cancer established by CagA immunoblot as a marker of past infection. *Gastroenterology* **121**, 784-791.

El-Omar, E. M., Penman, I. D., Ardill, J. E., Chittajallu, R. S., Howie, C. & McColl, K. E. (1995). *Helicobacter pylori* infection and abnormalities of acid secretion in patients with duodenal ulcer disease. *Gastroenterology* **109**, 681-691.

El-Omar, E. M., Carrington, M., Chow, W. H. & other authors (2000). Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* **404**, 398-402.

El-Omar, E. M. (2001). The importance of interleukin 1beta in *Helicobacter pylori* associated disease. *Gut* **48**, 743-747.

El-Omar, E. M., Rabkin, C. S., Gammon, M. D. & other authors (2003). Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. *Gastroenterology* **124**, 1193-1201.

El-Zimaity, H. M., Graham, D. Y., al-Assi, M. T., Malaty, H., Karttunen, T. J., Graham, D. P., Huberman, R. M. & Genta, R. M. (1996). Interobserver variation in the histopathological assessment of *Helicobacter pylori* gastritis. *Hum Pathol* **27**, 35-41.

Elitsur, Y., Neace, C., Werthammer, M. C. & Triest, W. E. (1999). Prevalence of CagA, VacA antibodies in symptomatic and asymptomatic children with *Helicobacter pylori* infection. *Helicobacter* **4**, 100-105.

Engelhardt, J. J., Sullivan, T. J. & Allison, J. P. (2006). CTLA-4 overexpression inhibits T cell responses through a CD28-B7-dependent mechanism. *J Immunol* **177**, 1052-1061.

Erdag, G. & Morgan, J. R. (2002). Interleukin-1alpha and interleukin-6 enhance the antibacterial properties of cultured composite keratinocyte grafts. *Ann Surg* **235**, 113-124.

Erdman, S. E., Poutahidis, T., Tomczak, M., Rogers, A. B., Cormier, K., Plank, B., Horwitz, B. H. & Fox, J. G. (2003a). CD4+ CD25+ regulatory T lymphocytes inhibit microbially induced colon cancer in Rag2-deficient mice. *Am J Pathol* **162**, 691-702.

Erdman, S. E., Rao, V. P., Poutahidis, T. & other authors (2003b). CD4(+)CD25(+) regulatory lymphocytes require interleukin 10 to interrupt colon carcinogenesis in mice. *Cancer Res* **63**, 6042-6050.

Ernst, P. B. & Gold, B. D. (2000). The disease spectrum of Helicobacter pylori: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. *Annu Rev Microbiol* **54**, 615-640.

Ertem, D., Harmanci, H. & Pehlivanoglu, E. (2003). *Helicobacter pylori* infection in Turkish preschool and school children: role of socioeconomic factors and breast feeding. *Turk J Pediatr* **45**, 114-122.

Evans, D. G., Queiroz, D. M., Mendes, E. N. & Evans, D. J., Jr. (1998). *Helicobacter pylori cagA* status and s and m alleles of *vacA* in isolates from individuals with a variety of *H. pylori*-associated gastric diseases. *J Clin Microbiol* **36**, 3435-3437.

Everhart, J. E. (2000). Recent developments in the epidemiology of Helicobacter pylori. *Gastroenterol Clin North Am* **29**, 559-578.

Falk, P., Roth, K. A., Boren, T., Westblom, T. U., Gordon, J. I. & Normark, S. (1993). An *in vitro* adherence assay reveals that *Helicobacter pylori* exhibits cell lineage-specific tropism in the human gastric epithelium. *Proc Natl Acad Sci U S A* **90**, 2035-2039.

Fallone, C. A., Barkun, A. N., Gottke, M. U. & other authors (2000). Association of Helicobacter pylori genotype with gastroesophageal reflux disease and other upper gastrointestinal diseases. *The American journal of gastroenterology* **95**, 659-669.

Farrell, S., Doherty, G. M., Milliken, I., Shield, M. D. & McCallion, W. A. (2005). Risk factors for *Helicobacter pylori* infection in children: an examination of the role played by intrafamilial bed sharing. *Pediatr Infect Dis J* **24**, 149-152.

Fassi Fehri, L., Koch, M., Belogolova, E. & other authors (2010). *Helicobacter pylori* induces miR-155 in T cells in a cAMP-Foxp3-dependent manner. *PLoS One* **5**, e9500.

Fernandez-Botran, R., Sanders, V. M., Mosmann, T. R. & Vitetta, E. S. (1988). Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells. *J Exp Med* **168**, 543-558.

Ferrandez, A., Benito, R., Arenas, J., Garcia-Gonzalez, M. A., Sopena, F., Alcedo, J., Ortego, J., Sainz, R. & Lanas, A. (2006). CagA-positive Helicobacter pylori infection is not associated with decreased risk of Barrett's esophagus in a population with high H. pylori infection rate. *BMC gastroenterology* **6**, 7.

Figueiredo, C., Quint, W., Nouhan, N. & other authors (2001). Assessment of *Helicobacter pylori vacA* and *cagA* genotypes and host serological response. *J Clin Microbiol* **39**, 1339-1344.

Figueiredo, C., Machado, J. C., Pharoah, P. & other authors (2002). *Helicobacter pylori* and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. *J Natl Cancer Inst* **94**, 1680-1687.

Figura, N., Crabtree, J. E. & Dattilo, M. (1997). *In vitro* activity of lansoprazole against *Helicobacter pylori*. *J Antimicrob Chemother* **39**, 585-590.

Fischer, W., Puls, J., Buhrdorf, R., Gebert, B., Odenbreit, S. & Haas, R. (2001). Systematic mutagenesis of the *Helicobacter pylori cag* pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. *Mol Microbiol* **42**, 1337-1348.

Ford, A. C., Malfertheiner, P., Giguere, M., Santana, J., Khan, M. & Moayyedi, P. (2008). Adverse events with bismuth salts for *Helicobacter pylori* eradication: systematic review and meta-analysis. *World J Gastroenterol* **14**, 7361-7370.

Forman, D., Sitas, F., Newell, D. G., Stacey, A. R., Boreham, J., Peto, R., Campbell, T. C., Li, J. & Chen, J. (1990). Geographic association of *Helicobacter pylori* antibody prevalence and gastric cancer mortality in rural China. *Int J Cancer* **46**, 608-611.

Foss, D. L., Baarsch, M. J. & Murtaugh, M. P. (1998). Regulation of hypoxanthine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase and beta-actin mRNA expression in porcine immune cells and tissues. *Anim Biotechnol* **9**, 67-78.

Fox, J. G., Yan, L. L., Dewhirst, F. E., Paster, B. J., Shames, B., Murphy, J. C., Hayward, A., Belcher, J. C. & Mendes, E. N. (1995). *Helicobacter bilis* sp. nov., a novel *Helicobacter* species isolated from bile, livers, and intestines of aged, inbred mice. *J Clin Microbiol* **33**, 445-454.

Fox, J. G., Beck, P., Dangler, C. A., Whary, M. T., Wang, T. C., Shi, H. N. & Nagler-Anderson, C. (2000). Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces *helicobacter*-induced gastric atrophy. *Nat Med* **6**, 536-542.

Fox, J. G. & Wang, T. C. (2001). *Helicobacter pylori*--not a good bug after all! *N Engl J Med* **345**, 829-832.

Fox, J. G. (2002). The non-H pylori helicobacters: their expanding role in gastrointestinal and systemic diseases. *Gut* **50**, 273-283.

Fox, J. G. & Wang, T. C. (2007). Inflammation, atrophy, and gastric cancer. *J Clin Invest* **117**, 60-69.

Francois, F., Roper, J., Goodman, A. J. & other authors (2008). The association of gastric leptin with oesophageal inflammation and metaplasia. *Gut* **57**, 16-24.

Freeman, W. M., Walker, S. J. & Vrana, K. E. (1999). Quantitative RT-PCR: pitfalls and potential. *Biotechniques* **26**, 112-122, 124-115.

Frenck, R. W., Jr. & Clemens, J. (2003). *Helicobacter* in the developing world. *Microbes Infect* **5**, 705-713.

Frenck, R. W., Jr., Fathy, H. M., Sherif, M. & other authors (2006). Sensitivity and specificity of various tests for the diagnosis of *Helicobacter pylori* in Egyptian children. *Pediatrics* **118**, e1195-1202.

Frohm, M., Agerberth, B., Ahangari, G., Stahle-Backdahl, M., Liden, S., Wigzell, H. & Gudmundsson, G. H. (1997). The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J Biol Chem* **272**, 15258-15263.

Fu, S., Ramanujam, K. S., Wong, A., Fantry, G. T., Drachenberg, C. B., James, S. P., Meltzer, S. J. & Wilson, K. T. (1999). Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis. *Gastroenterology* **116**, 1319-1329.

Fujimoto, S., Olaniyi Ojo, O., Arnqvist, A., Wu, J. Y., Odenbreit, S., Haas, R., Graham, D. Y. & Yamaoka, Y. (2007). *Helicobacter pylori* BabA expression, gastric mucosal injury, and clinical outcome. *Clin Gastroenterol Hepatol* **5**, 49-58.

Fujimura, T., Ohta, T., Oyama, K., Miyashita, T. & Miwa, K. (2006). Role of cyclooxygenase-2 in the carcinogenesis of gastrointestinal tract cancers: a review and report of personal experience. *World J Gastroenterol* **12**, 1336-1345.

Furuta, T., Kaneko, E., Suzuki, M., Arai, H. & Futami, H. (1996). Quantitative study of *Helicobacter pylori* in gastric mucus by competitive PCR using synthetic DNA fragments. *J Clin Microbiol* **34**, 2421-2425.

Furuta, T., El-Omar, E. M., Xiao, F., Shirai, N., Takashima, M. & Sugimura, H. (2002). Interleukin 1beta polymorphisms increase risk of hypochlorhydria and atrophic gastritis and reduce risk of duodenal ulcer recurrence in Japan. *Gastroenterology* **123**, 92-105.

Gajewski, T. F. & Fitch, F. W. (1988). Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J Immunol* **140**, 4245-4252.

Galmiche, A., Rassow, J., Doye, A. & other authors (2000). The N-terminal 34 kDa fragment of Helicobacter pylori vacuolating cytotoxin targets mitochondria and induces cytochrome c release. *The EMBO journal* **19**, 6361-6370.

Ganz, T. & Lehrer, R. I. (1994). Defensins. *Curr Opin Immunol* **6**, 584-589.

Ganz, T. (2003). Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* **3**, 710-720.

Garcia-Gonzalez, M. A., Lanas, A., Quintero, E. & other authors (2007). Gastric cancer susceptibility is not linked to pro-and anti-inflammatory cytokine gene polymorphisms in whites: a Nationwide Multicenter Study in Spain. *Am J Gastroenterol* **102**, 1878-1892.

Garcia, J. R., Krause, A., Schulz, S. & other authors (2001). Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. *FASEB J* **15**, 1819-1821.

Garhart, C. A., Heinzel, F. P., Czinn, S. J. & Nedrud, J. G. (2003). Vaccine-induced reduction of *Helicobacter pylori* colonization in mice is interleukin-12 dependent but gamma interferon and inducible nitric oxide synthase independent. *Infect Immun* **71**, 910-921.

Genta, R. M. & Dixon, M. F. (1995). The Sydney System revisited: the Houston International Gastritis Workshop. *Am J Gastroenterol* **90**, 1039-1041.

Genta, R. M. (1996). Recognizing atrophy: another step toward a classification of gastritis. *Am J Surg Pathol* **20 Suppl 1**, S23-30.

Genta, R. M., Gurer, I. E., Graham, D. Y., Krishnan, B., Segura, A. M., Gutierrez, O., Kim, J. G. & Burchette, J. L., Jr. (1996). Adherence of *Helicobacter pylori* to areas of incomplete intestinal metaplasia in the gastric mucosa. *Gastroenterology* **111**, 1206-1211.

Genta, R. M. (1997a). The immunobiology of *Helicobacter pylori* gastritis. *Semin Gastrointest Dis* **8**, 2-11.

Genta, R. M. (1997b). *Helicobacter pylori*, inflammation, mucosal damage, and apoptosis: pathogenesis and definition of gastric atrophy. *Gastroenterology* **113**, S51-55.

George, J. T., Boughan, P. K., Karageorgiou, H. & Bajaj-Elliott, M. (2003). Host anti-microbial response to *Helicobacter pylori* infection. *Mol Immunol* **40**, 451-456.

Gerhard, M., Lehn, N., Neumayer, N., Boren, T., Rad, R., Schepp, W., Miehlke, S., Classen, M. & Prinz, C. (1999). Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin. *Proc Natl Acad Sci U S A* **96**, 12778-12783.

Gerrits, M. M., van Vliet, A. H., Kuipers, E. J. & Kusters, J. G. (2006). *Helicobacter pylori* and antimicrobial resistance: molecular mechanisms and clinical implications. *Lancet Infect Dis* **6**, 699-709.

Gewirtz, A. T., Yu, Y., Krishna, U. S., Israel, D. A., Lyons, S. L. & Peek, R. M., Jr. (2004). *Helicobacter pylori* flagellin evades toll-like receptor 5-mediated innate immunity. *J Infect Dis* **189**, 1914-1920.

Ghiringhelli, F., Puig, P. E., Roux, S. & other authors (2005). Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. *J Exp Med* **202**, 919-929.

Giannakis, M., Chen, S. L., Karam, S. M., Engstrand, L. & Gordon, J. I. (2008). *Helicobacter pylori* evolution during progression from chronic atrophic gastritis to gastric cancer and its impact on gastric stem cells. *Proc Natl Acad Sci U S A* **105**, 4358-4363.

Gibson, U. E., Heid, C. A. & Williams, P. M. (1996). A novel method for real time quantitative RT-PCR. *Genome Res* **6**, 995-1001.

Giglio, S., Monis, P. T. & Saint, C. P. (2003). Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR. *Nucleic Acids Res* **31**, e136.

Gionchetti, P., Vaira, D., Campieri, M. & other authors (1994). Enhanced mucosal interleukin-6 and -8 in *Helicobacter pylori*-positive dyspeptic patients. *Am J Gastroenterol* **89**, 883-887.

Gisbert, J. P. & Calvet, X. (2009). Review article: *Helicobacter pylori*-negative duodenal ulcer disease. *Aliment Pharmacol Ther* **30**, 791-815.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R. & Mathieu, C. (2001). An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* **25**, 386-401.

Glynn, M. K., Friedman, C. R., Gold, B. D., Khanna, B., Hutwagner, L., Iihoshi, N., Revollo, C. & Quick, R. (2002). Seroincidence of *Helicobacter pylori* infection in a cohort of rural Bolivian children: acquisition and analysis of possible risk factors. *Clin Infect Dis* **35**, 1059-1065.

Gobert, A. P., McGee, D. J., Akhtar, M., Mendz, G. L., Newton, J. C., Cheng, Y., Mobley, H. L. & Wilson, K. T. (2001). Helicobacter pylori arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. *Proc Natl Acad Sci U S A* **98**, 13844-13849.

Goll, R., Gruber, F., Olsen, T., Cui, G., Raschpichler, G., Buset, M., Asfeldt, A. M., Husebekk, A. & Florholmen, J. (2007). *Helicobacter pylori* stimulates a mixed adaptive immune response with a strong T-regulatory component in human gastric mucosa. *Helicobacter* **12**, 185-192.

Gomes, L. I., Rocha, G. A., Rocha, A. M., Soares, T. F., Oliveira, C. A., Bittencourt, P. F. & Queiroz, D. M. (2008). Lack of association between *Helicobacter pylori* infection with *dupA*-positive strains and gastroduodenal diseases in Brazilian patients. *Int J Med Microbiol* **298**, 223-230.

Goodwin, C. S., Blincow, E. D., Warren, J. R., Waters, T. E., Sanderson, C. R. & Easton, L. (1985). Evaluation of cultural techniques for isolating *Campylobacter pyloridis* from endoscopic biopsies of gastric mucosa. *J Clin Pathol* **38**, 1127-1131.

Goodwin, C. S., Armstrong, J. A., Chilvers, T., Peters, M., Collins, M. D., Sly, L., McConnell, W. & W.E.S., H. (1989). Transfer of Campylobacter pylori and Campylobacter mustelae to Helicobacter gen. nov. as Helicobacter pylori comb. nov. and Helicobacter mustelae comb. nov., respectively. . *Int J Syst Bacteriol* **39**, 397–405.

Graham, D. Y., Go, M. F., Lew, G. M., Genta, R. M. & Rehfeld, J. F. (1993). *Helicobacter pylori* infection and exaggerated gastrin release. Effects of inflammation and progastrin processing. *Scand J Gastroenterol* **28**, 690-694.

Graham, D. Y. & Klein, P. D. (2000). Accurate diagnosis of *Helicobacter pylori*. 13C-urea breath test. *Gastroenterol Clin North Am* **29**, 885-893, x.

Group, E. S. (1993). Epidemiology of, and risk factors for, *Helicobacter pylori* infection among 3194 asymptomatic subjects in 17 populations. The EUROGAST Study Group. *Gut* **34**, 1672-1676.

Gudmundsson, G. H., Agerberth, B., Odeberg, J., Bergman, T., Olsson, B. & Salcedo, R. (1996). The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. *Eur J Biochem* **238**, 325-332.

Gunn, M. C., Stephens, J. C., Stewart, J. A., Rathbone, B. J. & West, K. P. (1998). The significance of *cagA* and *vacA* subtypes of *Helicobacter pylori* in the pathogenesis of inflammation and peptic ulceration. *J Clin Pathol* **51**, 761-764.

Hafsi, N., Voland, P., Schwendy, S., Rad, R., Reindl, W., Gerhard, M. & Prinz, C. (2004). Human dendritic cells respond to Helicobacter pylori, promoting NK cell and Th1-effector responses in vitro. *J Immunol* **173**, 1249-1257.

Han, S. R., Schneider, T., Loos, M., Bhakdi, S. & Maeurer, M. J. (1999). One-step polymerase chain reaction-based typing of *Helicobacter pylori vacA* gene: association with gastric histopathology. *Med Microbiol Immunol* **188**, 131-138.

Hanninen, M. L., Happonen, I., Saari, S. & Jalava, K. (1996). Culture and characteristics of *Helicobacter bizzozeronii*, a new canine gastric *Helicobacter sp. Int J Syst Bacteriol* **46**, 160-166.

Hara, M., Kingsley, C. I., Niimi, M., Read, S., Turvey, S. E., Bushell, A. R., Morris, P. J., Powrie, F. & Wood, K. J. (2001). IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol* **166**, 3789-3796.

Harder, J., Bartels, J., Christophers, E. & Schroder, J. M. (2001). Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem* **276**, 5707-5713.

Harris, P. R., Cover, T. L., Crowe, D. R., Orenstein, J. M., Graham, M. F., Blaser, M. J. & Smith, P. D. (1996). Helicobacter pylori cytotoxin induces vacuolation of primary human mucosal epithelial cells. *Infect Immun* **64**, 4867-4871.

Harris, P. R., Wright, S. W., Serrano, C. & other authors (2008). *Helicobacter pylori* gastritis in children is associated with a regulatory T-cell response. *Gastroenterology* **134**, 491-499.

Hase, K., Eckmann, L., Leopard, J. D., Varki, N. & Kagnoff, M. F. (2002). Cell differentiation is a key determinant of cathelicidin LL-37/human cationic antimicrobial protein 18 expression by human colon epithelium. *Infect Immun* **70**, 953-963.

Hase, K., Murakami, M., Iimura, M. & other authors (2003). Expression of LL-37 by human gastric epithelial cells as a potential host defense mechanism against *Helicobacter pylori*. *Gastroenterology* **125**, 1613-1625.

Hatakeyama, M. (2008). SagA of CagA in *Helicobacter pylori* pathogenesis. *Curr Opin Microbiol* **11**, 30-37.

Hayashi, S., Sugiyama, T., Yokota, K., Isogai, H., Isogai, E., Oguma, K., Asaka, M., Fujii, N. & Hirai, Y. (1998). Analysis of immunoglobulin A antibodies to *Helicobacter pylori* in serum and gastric juice in relation to mucosal inflammation. *Clin Diagn Lab Immunol* **5**, 617-621.

Hayden, M. S. & Ghosh, S. (2008). Shared principles in NF-kappaB signaling. *Cell* **132**, 344-362.

He, Q., Wang, J. P., Osato, M. & Lachman, L. B. (2002). Real-time quantitative PCR for detection of *Helicobacter pylori*. *J Clin Microbiol* **40**, 3720-3728.

Heid, C. A., Stevens, J., Livak, K. J. & Williams, P. M. (1996). Real time quantitative PCR. *Genome Res* 6, 986-994.

Hellmig, S., Hampe, J., Folsch, U. R. & Schreiber, S. (2005). Role of IL-10 promoter haplotypes in *Helicobacter pylori* associated gastric inflammation. *Gut* **54**, 888.

Henry, S., Bru, D., Stres, B., Hallet, S. & Philippot, L. (2006). Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of *16S rRNA, narG, nirK, and nosZ* genes in soils. *Appl Environ Microbiol* **72**, 5181-5189.

Hida, N., Shimoyama, T., Jr., Neville, P., Dixon, M. F., Axon, A. T., Shimoyama, T., Sr. & Crabtree, J. E. (1999). Increased expression of IL-10 and IL-12 (p40) mRNA in Helicobacter pylori infected gastric mucosa: relation to bacterial cag status and peptic ulceration. *J Clin Pathol* **52**, 658-664.

Higashi, H., Tsutsumi, R., Fujita, A., Yamazaki, S., Asaka, M., Azuma, T. & Hatakeyama, M. (2002a). Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. *Proc Natl Acad Sci U S A* **99**, 14428-14433.

Higashi, H., Tsutsumi, R., Muto, S., Sugiyama, T., Azuma, T., Asaka, M. & Hatakeyama, M. (2002b). SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. *Science* **295**, 683-686.

Higashi, H., Yokoyama, K., Fujii, Y., Ren, S., Yuasa, H., Saadat, I., Murata-Kamiya, N., Azuma, T. & Hatakeyama, M. (2005). EPIYA motif is a membrane-targeting signal of *Helicobacter pylori* virulence factor CagA in mammalian cells. *J Biol Chem* **280**, 23130-23137.

Higuchi, R., Fockler, C., Dollinger, G. & Watson, R. (1993). Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (N Y)* **11**, 1026-1030.

Ho, S. B., Shekels, L. L., Toribara, N. W., Kim, Y. S., Lyftogt, C., Cherwitz, D. L. & Niehans, G. A. (1995). Mucin gene expression in normal, preneoplastic, and neoplastic human gastric epithelium. *Cancer Res* **55**, 2681-2690.

Hocker, M., Rosenberg, I., Xavier, R., Henihan, R. J., Wiedenmann, B., Rosewicz, S., Podolsky, D. K. & Wang, T. C. (1998). Oxidative stress activates the human histidine decarboxylase promoter in AGS gastric cancer cells. *J Biol Chem* **273**, 23046-23054.

Hojo, M., Miwa, H., Nagahara, A. & Sato, N. (2001). Pooled analysis on the efficacy of the second-line treatment regimens for *Helicobacter pylori* infection. *Scand J Gastroenterol* **36**, 690-700.

Holland, P. M., Abramson, R. D., Watson, R. & Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci U S A* **88**, 7276-7280.

Homan, M., Luzar, B., Kocjan, B. J., Orel, R., Mocilnik, T., Shrestha, M., Kveder, M. & Poljak, M. (2009). Prevalence and clinical relevance of *cagA, vacA, and iceA* genotypes of *Helicobacter pylori* isolated from Slovenian children. *J Pediatr Gastroenterol Nutr* **49**, 289-296.

Honda, S., Fujioka, T., Tokieda, M., Gotoh, T., Nishizono, A. & Nasu, M. (1998). Gastric ulcer, atrophic gastritis, and intestinal metaplasia caused by *Helicobacter pylori* infection in Mongolian gerbils. *Scand J Gastroenterol* **33**, 454-460.

Hoover, D. M., Wu, Z., Tucker, K., Lu, W. & Lubkowski, J. (2003). Antimicrobial characterization of human beta-defensin 3 derivatives. *Antimicrob Agents Chemother* **47**, 2804-2809.

Horwitz, D. A., Zheng, S. G. & Gray, J. D. (2008a). Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other. *Trends Immunol* **29**, 429-435.

Horwitz, D. A., Zheng, S. G., Wang, J. & Gray, J. D. (2008b). Critical role of IL-2 and TGF-beta in generation, function and stabilization of Foxp3+CD4+ Treg. *Eur J Immunol* **38**, 912-915.

Hosseini, M. E., Oghalaie, A., Habibi, G., Nahvijoo, A., Hosseini, Z. M., Tashakoripoor, M. & Mohammadi, M. (2010). Molecular detection of host cytokine expression in *Helicobacter pylori* infected patients via semi-quantitative RT-PCR. *Indian J Med Microbiol* **28**, 40-44.

Hotchin, N. A., Cover, T. L. & Akhtar, N. (2000). Cell vacuolation induced by the VacA cytotoxin of Helicobacter pylori is regulated by the Rac1 GTPase. *The Journal of biological chemistry* **275**, 14009-14012.

Hou, L., El-Omar, E. M., Chen, J. & other authors (2007). Polymorphisms in Th1-type cell-mediated response genes and risk of gastric cancer. *Carcinogenesis* **28**, 118-123.

Houghton, J., Fox, J. G. & Wang, T. C. (2002). Gastric cancer: laboratory bench to clinic. *J Gastroenterol Hepatol* **17**, 495-502.

Houghton, J., Stoicov, C., Nomura, S. & other authors (2004). Gastric cancer originating from bone marrow-derived cells. *Science* **306**, 1568-1571.

Hsieh, C. S., Zheng, Y., Liang, Y., Fontenot, J. D. & Rudensky, A. Y. (2006). An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol* **7**, 401-410.

Hsu, P. I., Li, C. N., Tseng, H. H. & other authors (2004). The interleukin-1 RN polymorphism and *Helicobacter pylori* infection in the development of duodenal ulcer. *Helicobacter* **9**, 605-613.

Huang, J. Q., Sridhar, S., Chen, Y. & Hunt, R. H. (1998). Meta-analysis of the relationship between Helicobacter pylori seropositivity and gastric cancer. *Gastroenterology* **114**, 1169-1179.

Hurov, J. B., Watkins, J. L. & Piwnica-Worms, H. (2004). Atypical PKC phosphorylates PAR-1 kinases to regulate localization and activity. *Curr Biol* **14**, 736-741.

Hussein, N. R., Mohammadi, M., Talebkhan, Y., Doraghi, M., Letley, D. P., Muhammad, M. K., Argent, R. H. & Atherton, J. C. (2008). Differences in virulence markers between *Helicobacter pylori* strains from Iraq and those from Iran: potential importance of regional differences in *H. pylori*-associated disease. *J Clin Microbiol* **46**, 1774-1779.

Hussein, N. R., Argent, R. H., Marx, C. K., Patel, S. R., Robinson, K. & Atherton, J. C. (2010). *Helicobacter pylori dupA* is polymorphic, and its active form induces proinflammatory cytokine secretion by mononuclear cells. *J Infect Dis* **202**, 261-269.

Ilver, D., Arnqvist, A., Ogren, J. & other authors (1998). *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science* **279**, 373-377.

Ishihara, S., Rumi, M. A., Kadowaki, Y. & other authors (2004). Essential role of MD-2 in TLR4-dependent signaling during *Helicobacter pylori*-associated gastritis. *J Immunol* **173**, 1406-1416.

Islam, D., Bandholtz, L., Nilsson, J., Wigzell, H., Christensson, B., Agerberth, B. & Gudmundsson, G. (2001). Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. *Nat Med* **7**, 180-185.

Islami, F. & Kamangar, F. (2008). *Helicobacter pylori* and esophageal cancer risk: a meta-analysis. *Cancer Prev Res (Phila Pa)* **1**, 329-338.

Ismail, H. F., Fick, P., Zhang, J., Lynch, R. G. & Berg, D. J. (2003). Depletion of neutrophils in IL-10(-/-) mice delays clearance of gastric *Helicobacter* infection and decreases the Th1 immune response to *Helicobacter*. *J Immunol* **170**, 3782-3789.

Isomoto, H., Mukae, H., Ishimoto, H. & other authors (2004). Elevated concentrations of alpha-defensins in gastric juice of patients with *Helicobacter pylori* infection. *Am J Gastroenterol* **99**, 1916-1923.

Ito, Y., Azuma, T., Ito, S. & other authors (1997). Analysis and typing of the vacA gene from cagA-positive strains of Helicobacter pylori isolated in Japan. *Journal of clinical microbiology* **35**, 1710-1714.

Jamora, C. & Fuchs, E. (2002). Intercellular adhesion, signalling and the cytoskeleton. *Nat Cell Biol* **4**, E101-108.

Jang, T. J. (2010). The number of FOXP3-positive regulatory T cells is increased in *Helicobacter pylori* gastritis and gastric cancer. *Pathol Res Pract* **206**, 34-38.

Janzon, A., Sjoling, A., Lothigius, A., Ahmed, D., Qadri, F. & Svennerholm, A. M. (2009). Failure to detect *Helicobacter pylori* DNA in drinking and environmental water in Dhaka, Bangladesh, using highly sensitive real-time PCR assays. *Appl Environ Microbiol* **75**, 3039-3044.

Ji, X., Fernandez, T., Burroni, D., Pagliaccia, C., Atherton, J. C., Reyrat, J. M., Rappuoli, R. & Telford, J. L. (2000). Cell specificity of *Helicobacter pylori* cytotoxin is determined by a short region in the polymorphic midregion. *Infect Immun* **68**, 3754-3757.

Jo, Y., Han, S. U., Kim, Y. J., Kim, J. H., Kim, S. T., Kim, S. J. & Hahm, K. B. (2010). Suppressed Gastric Mucosal TGF-beta1 Increases Susceptibility to *H. pylori*-Induced Gastric Inflammation and Ulceration: A Stupid Host Defense Response. *Gut Liver* **4**, 43-53.

Jones, D. E. & Bevins, C. L. (1992). Paneth cells of the human small intestine express an antimicrobial peptide gene. *J Biol Chem* **267**, 23216-23225.

Jones, D. E. & Bevins, C. L. (1993). Defensin-6 mRNA in human Paneth cells: implications for antimicrobial peptides in host defense of the human bowel. *FEBS Lett* **315**, 187-192.

Jonkers, D., Stobberingh, E., de Bruine, A., Arends, J. W. & Stockbrugger, R. (1997). Evaluation of immunohistochemistry for the detection of *Helicobacter pylori* in gastric mucosal biopsies. *J Infect* **35**, 149-154.

Jonkers, D., Houben, G., de Bruine, A., Arends, J. W., Stobberingh, E. & Stockbrugger, R. (1998). Prevalence of gastric metaplasia in the duodenal bulb and distribution of *Helicobacter pylori* in the gastric mucosa. A clinical and histopathological study in 96 consecutive patients. *Ital J Gastroenterol Hepatol* **30**, 481-483.

Jose, J., Wolk, U., Lorenzen, D., Wenschuh, H. & Meyer, T. F. (2000). Human T-cell response to meningococcal immunoglobulin A1 protease associated alpha-proteins. *Scand J Immunol* **51**, 176-185.

Joss, A., Akdis, M., Faith, A., Blaser, K. & Akdis, C. A. (2000). IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway. *Eur J Immunol* **30**, 1683-1690.

Jovanovic, D. V., Di Battista, J. A., Martel-Pelletier, J., Jolicoeur, F. C., He, Y., Zhang, M., Mineau, F. & Pelletier, J. P. (1998). IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* **160**, 3513-3521.

Jung, H. C., Kim, J. M., Song, I. S. & Kim, C. Y. (1997). *Helicobacter pylori* induces an array of pro-inflammatory cytokines in human gastric epithelial cells: quantification of mRNA for

interleukin-8, -1 alpha/beta, granulocyte-macrophage colony-stimulating factor, monocyte chemoattractant protein-1 and tumour necrosis factor-alpha. *J Gastroenterol Hepatol* **12**, 473-480.

Kabir, S. (2004). Detection of Helicobacter pylori DNA in feces and saliva by polymerase chain reaction: a review. *Helicobacter* **9**, 115-123.

Kadayifci, A., Buyukhatipoglu, H., Cemil Savas, M. & Simsek, I. (2006). Eradication of *Helicobacter pylori* with triple therapy: an epidemiologic analysis of trends in Turkey over 10 years. *Clin Ther* **28**, 1960-1966.

Kaklikkaya, N., Cubukcu, K., Aydin, F. & other authors (2006). Significance of *cagA* status and *vacA* subtypes of *Helicobacter pylori* in determining gastric histopathology: virulence markers of *H. pylori* and histopathology. *J Gastroenterol Hepatol* **21**, 1042-1047.

Kandulski, A., Wex, T., Kuester, D., Peitz, U., Gebert, I., Roessner, A. & Malfertheiner, P. (2008). Naturally occurring regulatory T cells (CD4+, CD25high, FOXP3+) in the antrum and cardia are associated with higher *H. pylori* colonization and increased gene expression of TGF-beta1. *Helicobacter* **13**, 295-303.

Kang, J. M., Kim, N., Lee, D. H., Park, J. H., Lee, M. K., Kim, J. S., Jung, H. C. & Song, I. S. (2009). The effects of genetic polymorphisms of IL-6, IL-8, and IL-10 on *Helicobacter pylori*-induced gastroduodenal diseases in Korea. *J Clin Gastroenterol* **43**, 420-428.

Kao, C. Y., Chen, Y., Thai, P., Wachi, S., Huang, F., Kim, C., Harper, R. W. & Wu, R. (2004). IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways. *J Immunol* **173**, 3482-3491.

Kao, J. Y., Zhang, M., Miller, M. J. & other authors (2010). *Helicobacter pylori* immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. *Gastroenterology* **138**, 1046-1054.

Kaparakis, M., Walduck, A. K., Price, J. D., Pedersen, J. S., van Rooijen, N., Pearse, M. J., Wijburg, O. L. & Strugnell, R. A. (2008). Macrophages are mediators of gastritis in acute *Helicobacter pylori* infection in C57BL/6 mice. *Infect Immun* **76**, 2235-2239.

Karita, M., Tummuru, M. K., Wirth, H. P. & Blaser, M. J. (1996). Effect of growth phase and acid shock on *Helicobacter pylori cagA* expression. *Infect Immun* **64**, 4501-4507.

Karita, M. & Blaser, M. J. (1998). Acid-tolerance response in *Helicobacter pylori* and differences between *cagA*+ and *cagA*- strains. *J Infect Dis* **178**, 213-219.

Karttunen, R., Karttunen, T., Ekre, H. P. & MacDonald, T. T. (1995). Interferon gamma and interleukin 4 secreting cells in the gastric antrum in *Helicobacter pylori* positive and negative gastritis. *Gut* **36**, 341-345.

Karttunen, R. A., Karttunen, T. J., Yousfi, M. M., el-Zimaity, H. M., Graham, D. Y. & el-Zaatari, F. A. (1997). Expression of mRNA for interferon-gamma, interleukin-10, and interleukin-12 (p40) in normal gastric mucosa and in mucosa infected with *Helicobacter pylori*. *Scand J Gastroenterol* **32**, 22-27.

Karttunen, T., Niemela, S. & Lehtola, J. (1991). *Helicobacter pylori* in dyspeptic patients: quantitative association with severity of gastritis, intragastric pH, and serum gastrin concentration. *Scand J Gastroenterol Suppl* **186**, 124-134.

Kato, I., Nomura, A. M., Stemmermann, G. N. & Chyou, P. H. (1992). A prospective study of gastric and duodenal ulcer and its relation to smoking, alcohol, and diet. *Am J Epidemiol* **135**, 521-530.

Kato, S., Nakajima, S., Nishino, Y. & other authors (2006). Association between gastric atrophy and *Helicobacter pylori* infection in Japanese children: a retrospective multicenter study. *Dig Dis Sci* **51**, 99-104.

Kauser, F., Khan, A. A., Hussain, M. A. & other authors (2004). The *cag* pathogenicity island of *Helicobacter pylori* is disrupted in the majority of patient isolates from different human populations. *J Clin Microbiol* **42**, 5302-5308.

Kawahara, T., Kuwano, Y., Teshima-Kondo, S., Kawai, T., Nikawa, T., Kishi, K. & Rokutan, K. (2001). Toll-like receptor 4 regulates gastric pit cell responses to *Helicobacter pylori* infection. *J Med Invest* **48**, 190-197.

Kawaida, H., Kono, K., Takahashi, A., Sugai, H., Mimura, K., Miyagawa, N., Omata, H., Ooi, A. & Fujii, H. (2005). Distribution of CD4+CD25high regulatory T-cells in tumor-draining lymph nodes in patients with gastric cancer. *J Surg Res* **124**, 151-157.

Kawauchi, K., Yagihashi, A., Tsuji, N., Uehara, N., Furuya, D., Kobayashi, D. & Watanabe, N. (2006). Human beta-defensin-3 induction in *H. pylori*-infected gastric mucosal tissues. *World J Gastroenterol* **12**, 5793-5797.

Kearney, D. J. & Brousal, A. (2000). Treatment of *Helicobacter pylori* infection in clinical practice in the United States: results from 224 patients. *Dig Dis Sci* **45**, 265-271.

Kekki, M., Maaroos, H. I., Sipponen, P., Uibo, R., Tammur, R., Tamm, A. & Villako, K. (1991). Grade of *Helicobacter pylori* colonisation in relation to gastritis: a six-year population-based follow-up study. *Scand J Gastroenterol Suppl* **186**, 142-150.

Kersulyte, D., Chalkauskas, H. & Berg, D. E. (1999). Emergence of recombinant strains of Helicobacter pylori during human infection. *Mol Microbiol* **31**, 31-43.

Khamri, W., Walker, M. M., Clark, P., Atherton, J. C., Thursz, M. R., Bamford, K. B., Lechler, R. I. & Lombardi, G. (2010). *Helicobacter pylori* stimulates dendritic cells to induce interleukin-17 expression from CD4+ T lymphocytes. *Infect Immun* **78**, 845-853.

Khattri, R., Cox, T., Yasayko, S. A. & Ramsdell, F. (2003). An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* **4**, 337-342.

Khulusi, S., Badve, S., Patel, P., Lloyd, R., Marrero, J. M., Finlayson, C., Mendall, M. A. & Northfield, T. C. (1996). Pathogenesis of gastric metaplasia of the human duodenum: role of Helicobacter pylori, gastric acid, and ulceration. *Gastroenterology* **110**, 452-458.

Kida, Y., Shimizu, T. & Kuwano, K. (2006). Sodium butyrate up-regulates cathelicidin gene expression via activator protein-1 and histone acetylation at the promoter region in a human lung epithelial cell line, EBC-1. *Mol Immunol* **43**, 1972-1981.

Kikuchi, S., Crabtree, J. E., Forman, D. & Kurosawa, M. (1999). Association between infections with CagA-positive or -negative strains of *Helicobacter pylori* and risk for gastric cancer in young adults. Research Group on Prevention of Gastric Carcinoma Among Young Adults. *Am J Gastroenterol* **94**, 3455-3459.

Kingsley, C. I., Karim, M., Bushell, A. R. & Wood, K. J. (2002). CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J Immunol* **168**, 1080-1086.

Kiriya, K., Watanabe, N., Nishio, A. & other authors (2007). Essential role of Peyer's patches in the development of *Helicobacter*-induced gastritis. *Int Immunol* **19**, 435-446.

Kivi, M., Johansson, A. L., Reilly, M. & Tindberg, Y. (2005). *Helicobacter pylori* status in family members as risk factors for infection in children. *Epidemiol Infect* **133**, 645-652.

Klein, P. D., Graham, D. Y., Gaillour, A., Opekun, A. R. & Smith, E. O. (1991). Water source as risk factor for *Helicobacter pylori* infection in Peruvian children. Gastrointestinal Physiology Working Group. *Lancet* **337**, 1503-1506.

Kluver, E., Schulz-Maronde, S., Scheid, S., Meyer, B., Forssmann, W. G. & Adermann, K. (2005). Structure-activity relation of human beta-defensin 3: influence of disulfide bonds and cysteine substitution on antimicrobial activity and cytotoxicity. *Biochemistry* **44**, 9804-9816.

Ko, J. S., Kim, K. M., Oh, Y. L. & Seo, J. K. (2008). *cagA*, *vacA*, and *iceA* genotypes of *Helicobacter pylori* in Korean children. *Pediatr Int* **50**, 628-631.

Kobayashi, D., Eishi, Y., Ohkusa, T., Ishige, Suzuki, T., Minami, J., Yamada, T., Takizawa, T. & Koike, M. (2002). Gastric mucosal density of *Helicobacter pylori* estimated by real-time PCR compared with results of urea breath test and histological grading. *J Med Microbiol* **51**, 305-311.

Kocsis, A. K., Kiss, Z. F., Tiszlavicz, L., Tiszlavicz, Z. & Mandi, Y. (2009a). Potential role of human beta-defensin 1 in *Helicobacter pylori*-induced gastritis. *Scand J Gastroenterol* **44**, 289-295.

Kocsis, A. K., Ocsovszky, I., Tiszlavicz, L., Tiszlavicz, Z. & Mandi, Y. (2009b). *Helicobacter pylori* induces the release of alpha-defensin by human granulocytes. *Inflamm Res* **58**, 241-247.

Koike, T., Ohara, S., Sekine, H., Iijima, K., Abe, Y., Kato, K., Toyota, T. & Shimosegawa, T. (2001). Helicobacter pylori infection prevents erosive reflux oesophagitis by decreasing gastric acid secretion. *Gut* **49**, 330-334.

Kolls, J. K. & Linden, A. (2004). Interleukin-17 family members and inflammation. *Immunity* **21**, 467-476.

Kono, K., Kawaida, H., Takahashi, A., Sugai, H., Mimura, K., Miyagawa, N., Omata, H. & Fujii, H. (2006). CD4(+)CD25high regulatory T cells increase with tumor stage in patients with gastric and esophageal cancers. *Cancer Immunol Immunother* **55**, 1064-1071.

Konturek, P. C., Konturek, S. J. & Brzozowski, T. (2009). *Helicobacter pylori* infection in gastric cancerogenesis. *J Physiol Pharmacol* **60**, 3-21.

Kosmaczewska, A., Ciszak, L., Potoczek, S. & Frydecka, I. (2008). The significance of Treg cells in defective tumor immunity. *Arch Immunol Ther Exp (Warsz)* **56**, 181-191.

Kranzer, K., Eckhardt, A., Aigner, M., Knoll, G., Deml, L., Speth, C., Lehn, N., Rehli, M. & Schneider-Brachert, W. (2004). Induction of maturation and cytokine release of human dendritic cells by Helicobacter pylori. *Infect Immun* **72**, 4416-4423.

Kranzer, K., Sollner, L., Aigner, M., Lehn, N., Deml, L., Rehli, M. & Schneider-Brachert, W. (2005). Impact of Helicobacter pylori virulence factors and compounds on activation and maturation of human dendritic cells. *Infect Immun* **73**, 4180-4189.

Krienitz, W. (1906). Ueber das auftreten von mageninhalt bei carcinoma ventriculi. . *Disch Med Wochenschr* **22**, 872.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. & Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**, 263-274.

Kuipers, E. J., Thijs, J. C. & Festen, H. P. (1995a). The prevalence of *Helicobacter pylori* in peptic ulcer disease. *Aliment Pharmacol Ther* **9 Suppl 2**, 59-69.

Kuipers, E. J., Uyterlinde, A. M., Pena, A. S., Hazenberg, H. J., Bloemena, E., Lindeman, J., Klinkenberg-Knol, E. C. & Meuwissen, S. G. (1995b). Increase of *Helicobacter pylori*-

associated corpus gastritis during acid suppressive therapy: implications for long-term safety. *Am J Gastroenterol* **90**, 1401-1406.

Kuipers, E. J., Uyterlinde, A. M., Pena, A. S., Roosendaal, R., Pals, G., Nelis, G. F., Festen, H. P. & Meuwissen, S. G. (1995c). Long-term sequelae of Helicobacter pylori gastritis. *Lancet* **345**, 1525-1528.

Kuipers, E. J. (1999). Review article: exploring the link between Helicobacter pylori and gastric cancer. *Aliment Pharmacol Ther* **13 Suppl 1**, 3-11.

Kuipers, E. J. & Meijer, G. A. (2000). *Helicobacter pylori* gastritis in Africa. *Eur J Gastroenterol Hepatol* **12**, 601-603.

Kusters, J. G., van Vliet, A. H. & Kuipers, E. J. (2006). Pathogenesis of Helicobacter pylori infection. *Clin Microbiol Rev* **19**, 449-490.

Laan, M., Cui, Z. H., Hoshino, H., Lotvall, J., Sjostrand, M., Gruenert, D. C., Skoogh, B. E. & Linden, A. (1999). Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol* **162**, 2347-2352.

Lai, C. H., Poon, S. K., Chen, Y. C., Chang, C. S. & Wang, W. C. (2005). Lower prevalence of Helicobacter pylori infection with vacAs1a, cagA-positive, and babA2-positive genotype in erosive reflux esophagitis disease. *Helicobacter* **10**, 577-585.

Lande, R., Gregorio, J., Facchinetti, V. & other authors (2007). Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* **449**, 564-569.

Laouar, Y., Sutterwala, F. S., Gorelik, L. & Flavell, R. A. (2005). Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. *Nat Immunol* **6**, 600-607.

Laouar, Y., Town, T., Jeng, D., Tran, E., Wan, Y., Kuchroo, V. K. & Flavell, R. A. (2008). TGF-beta signaling in dendritic cells is a prerequisite for the control of autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* **105**, 10865-10870.

Lascols, C., Lamarque, D., Costa, J. M. & other authors (2003). Fast and accurate quantitative detection of *Helicobacter pylori* and identification of clarithromycin resistance mutations in *H. pylori* isolates from gastric biopsy specimens by real-time PCR. *J Clin Microbiol* **41**, 4573-4577.

Le Gros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F. D. & Paul, W. E. (1990). Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J Exp Med* **172**, 921-929.

Le, J. & Vilcek, J. (1987). Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. *Lab Invest* **56**, 234-248.

Lee, A., O'Rourke, J., De Ungria, M. C., Robertson, B., Daskalopoulos, G. & Dixon, M. F. (1997). A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology* **112**, 1386-1397.

Lee, C. W., Rao, V. P., Rogers, A. B., Ge, Z., Erdman, S. E., Whary, M. T. & Fox, J. G. (2007a). Wild-type and interleukin-10-deficient regulatory T cells reduce effector T-cell-mediated gastroduodenitis in Rag2-/- mice, but only wild-type regulatory T cells suppress *Helicobacter pylori* gastritis. *Infect Immun* **75**, 2699-2707.

Lee, H. S., Choe, G., Kim, W. H., Kim, H. H., Song, J. & Park, K. U. (2006). Expression of Lewis antigens and their precursors in gastric mucosa: relationship with *Helicobacter pylori* infection and gastric carcinogenesis. *J Pathol* **209**, 88-94.

Lee, K. A., Park, J. H., Sohn, T. S., Kim, S., Rhee, J. C. & Kim, J. W. (2007b). Interaction of polymorphisms in the interleukin 1B-31 and general transcription factor 2A1 genes on the susceptibility to gastric cancer. *Cytokine* **38**, 96-100.

Lee, S. K., Stack, A., Katzowitsch, E., Aizawa, S. I., Suerbaum, S. & Josenhans, C. (2003). *Helicobacter pylori* flagellins have very low intrinsic activity to stimulate human gastric epithelial cells via TLR5. *Microbes Infect* **5**, 1345-1356.

Lehmann, F. S., Terracciano, L., Carena, I., Baeriswyl, C., Drewe, J., Tornillo, L., De Libero, G. & Beglinger, C. (2002). In situ correlation of cytokine secretion and apoptosis in *Helicobacter pylori*-associated gastritis. *Am J Physiol Gastrointest Liver Physiol* **283**, G481-488.

Lehours, P., Menard, A., Dupouy, S., Bergey, B., Richy, F., Zerbib, F., Ruskone-Fourmestraux, A., Delchier, J. C. & Megraud, F. (2004). Evaluation of the association of nine *Helicobacter pylori* virulence factors with strains involved in low-grade gastric mucosa-associated lymphoid tissue lymphoma. *Infect Immun* **72**, 880-888.

Leszczynska, K., Namiot, A., Fein, D. E., Wen, Q., Namiot, Z., Savage, P. B., Diamond, S., Janmey, P. A. & Bucki, R. (2009). Bactericidal activities of the cationic steroid CSA-13 and the cathelicidin peptide LL-37 against *Helicobacter pylori* in simulated gastric juice. *BMC Microbiol* **9**, 187.

Letley, D. P., Lastovica, A., Louw, J. A., Hawkey, C. J. & Atherton, J. C. (1999). Allelic diversity of the *Helicobacter pylori* vacuolating cytotoxin gene in South Africa: rarity of the *vacA* s1a genotype and natural occurrence of an s2/m1 allele. *J Clin Microbiol* **37**, 1203-1205.

Letley, D. P. & Atherton, J. C. (2000). Natural diversity in the N terminus of the mature vacuolating cytotoxin of *Helicobacter pylori* determines cytotoxin activity. *J Bacteriol* **182**, 3278-3280.

Letley, D. P., Rhead, J. L., Twells, R. J., Dove, B. & Atherton, J. C. (2003). Determinants of non-toxicity in the gastric pathogen *Helicobacter pylori*. *J Biol Chem* **278**, 26734-26741.

Leunk, R. D., Johnson, P. T., David, B. C., Kraft, W. G. & Morgan, D. R. (1988). Cytotoxic activity in broth-culture filtrates of Campylobacter pylori. *Journal of medical microbiology* **26**, 93-99.

Li, M. O., Sanjabi, S. & Flavell, R. A. (2006a). Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* **25**, 455-471.

Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A. K. & Flavell, R. A. (2006b). Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* **24**, 99-146.

Li, M. O. & Flavell, R. A. (2008). Contextual regulation of inflammation: a duet by transforming growth factor-beta and interleukin-10. *Immunity* **28**, 468-476.

Li, X., Li, Y., Han, H., Miller, D. W. & Wang, G. (2006c). Solution structures of human LL-37 fragments and NMR-based identification of a minimal membrane-targeting antimicrobial and anticancer region. *J Am Chem Soc* **128**, 5776-5785.

Li, Z. & Li, J. (2006). Local expressions of TGF-beta1, TGF-beta1RI, CTGF, and Smad-7 in *Helicobacter pylori*-associated gastritis. *Scand J Gastroenterol* **41**, 1007-1012.

Liang, S. C., Tan, X. Y., Luxenberg, D. P., Karim, R., Dunussi-Joannopoulos, K., Collins, M. & Fouser, L. A. (2006). Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* **203**, 2271-2279.

Lindholm, C., Quiding-Jarbrink, M., Lonroth, H., Hamlet, A. & Svennerholm, A. M. (1998). Local cytokine response in Helicobacter pylori-infected subjects. *Infect Immun* **66**, 5964-5971.

Lindkvist, P., Enquselassie, F., Asrat, D., Muhe, L., Nilsson, I. & Giesecke, J. (1998). Risk factors for infection with *Helicobacter pylori*--a study of children in rural Ethiopia. *Scand J Infect Dis* **30**, 371-376.

Liu, H., Rahman, A., Semino-Mora, C., Doi, S. Q. & Dubois, A. (2008). Specific and sensitive detection of *H. pylori* in biological specimens by real-time RT-PCR and in situ hybridization. *PLoS One* **3**, e2689.

Liu, L., Zhao, C., Heng, H. H. & Ganz, T. (1997). The human beta-defensin-1 and alpha-defensins are encoded by adjacent genes: two peptide families with differing disulfide topology share a common ancestry. *Genomics* **43**, 316-320.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408.

Logan, R. P., Polson, R. J., Misiewicz, J. J. & other authors (1991). Simplified single sample 13Carbon urea breath test for *Helicobacter pylori*: comparison with histology, culture, and ELISA serology. *Gut* **32**, 1461-1464.

Logan, R. P., Walker, M. M., Misiewicz, J. J., Gummett, P. A., Karim, Q. N. & Baron, J. H. (1995). Changes in the intragastric distribution of *Helicobacter pylori* during treatment with omeprazole. *Gut* **36**, 12-16.

Longstreth, G. F. (1995). Epidemiology of hospitalization for acute upper gastrointestinal hemorrhage: a population-based study. *Am J Gastroenterol* **90**, 206-210.

Louw, J. A., Falck, V., van Rensburg, C., Zak, J., Adams, G. & Marks, I. N. (1993). Distribution of *Helicobacter pylori* colonisation and associated gastric inflammatory changes: difference between patients with duodenal and gastric ulcers. *J Clin Pathol* **46**, 754-756.

Lu, H., Hsu, P. I., Graham, D. Y. & Yamaoka, Y. (2005). Duodenal ulcer promoting gene of *Helicobacter pylori*. *Gastroenterology* **128**, 833-848.

Luger, A. (1917). Ueber spirochaeten und fusiforme bazillen im darm, mit einer beitrag zur frage der lamblien-enteritis. . *Wiein Klin Wochenschr* **52**.

Lundgren, A., Stromberg, E., Sjoling, A. & other authors (2005a). Mucosal FOXP3-expressing CD4+ CD25high regulatory T cells in Helicobacter pylori-infected patients. *Infect Immun* **73**, 523-531.

Lundgren, A., Trollmo, C., Edebo, A., Svennerholm, A. M. & Lundin, B. S. (2005b). *Helicobacter pylori*-specific CD4+ T cells home to and accumulate in the human *Helicobacter pylori*-infected gastric mucosa. *Infect Immun* **73**, 5612-5619.

Lundin, B. S., Enarsson, K., Kindlund, B., Lundgren, A., Johnsson, E., Quiding-Jarbrink, M. & Svennerholm, A. M. (2007). The local and systemic T-cell response to *Helicobacter pylori* in gastric cancer patients is characterised by production of interleukin-10. *Clin Immunol* **125**, 205-213.

Lupetti, P., Heuser, J. E., Manetti, R., Massari, P., Lanzavecchia, S., Bellon, P. L., Dallai, R., Rappuoli, R. & Telford, J. L. (1996). Oligomeric and subunit structure of the Helicobacter pylori vacuolating cytotoxin. *The Journal of cell biology* **133**, 801-807.

Luzza, F., Parrello, T., Monteleone, G., Sebkova, L., Imeneo, M., La Vecchia, A., Maletta, M., Docimo, C. & Pallone, F. (1999). Changes in the mucosal expression of interleukin 15 in *Helicobacter pylori*-associated gastritis. *FEMS Immunol Med Microbiol* **24**, 233-238.

Luzza, F., Parrello, T., Monteleone, G., Sebkova, L., Romano, M., Zarrilli, R., Imeneo, M. & Pallone, F. (2000). Up-regulation of IL-17 is associated with bioactive IL-8 expression in *Helicobacter pylori*-infected human gastric mucosa. *J Immunol* **165**, 5332-5337.

Machado, J. C., Pharoah, P., Sousa, S. & other authors (2001). Interleukin 1B and interleukin 1RN polymorphisms are associated with increased risk of gastric carcinoma. *Gastroenterology* **121**, 823-829.

Mahdavi, J., Sonden, B., Hurtig, M. & other authors (2002). *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. *Science* **297**, 573-578.

Mahr, S., Neumayer, N., Gerhard, M., Classen, M. & Prinz, C. (2000). IL-1beta-induced apoptosis in rat gastric enterochromaffin-like cells is mediated by iNOS, NF-kappaB, and Bax protein. *Gastroenterology* **118**, 515-524.

Malaty, H. M. (2007). Epidemiology of *Helicobacter pylori* infection. *Best Pract Res Clin Gastroenterol* **21**, 205-214.

Malfertheiner, P., Megraud, F., O'Morain, C. & other authors (2007). Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. *Gut* **56**, 772-781.

Maloy, K. J., Salaun, L., Cahill, R., Dougan, G., Saunders, N. J. & Powrie, F. (2003). CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. *J Exp Med* **197**, 111-119.

Mandell, L., Moran, A. P., Cocchiarella, A., Houghton, J., Taylor, N., Fox, J. G., Wang, T. C. & Kurt-Jones, E. A. (2004). Intact gram-negative *Helicobacter pylori*, *Helicobacter felis*, and *Helicobacter hepaticus* bacteria activate innate immunity via toll-like receptor 2 but not toll-like receptor 4. *Infect Immun* **72**, 6446-6454.

Mannath, J. & Ragunath, K. (2010). Narrow band imaging and high resolution endoscopy with magnification could be useful in identifying gastric atrophy. *Dig Dis Sci* **55**, 1799-1800.

Marchetti, M. & Rappuoli, R. (2002). Isogenic mutants of the *cag* pathogenicity island of *Helicobacter pylori* in the mouse model of infection: effects on colonization efficiency. *Microbiology* **148**, 1447-1456.

Marshall, B. J. & Warren, J. R. (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **1**, 1311-1315.

Mathews, M., Jia, H. P., Guthmiller, J. M., Losh, G., Graham, S., Johnson, G. K., Tack, B. F. & McCray, P. B., Jr. (1999). Production of beta-defensin antimicrobial peptides by the oral mucosa and salivary glands. *Infect Immun* **67**, 2740-2745.

Matteo, M. J., Granados, G., Perez, C. V., Olmos, M., Sanchez, C. & Catalano, M. (2007). *Helicobacter pylori cag* pathogenicity island genotype diversity within the gastric niche of a single host. *J Med Microbiol* **56**, 664-669.

Matteo, M. J., Armitano, R. I., Granados, G., Wonaga, A. D., Sanches, C., Olmos, M. & Catalano, M. (2010). *Helicobacter pylori oipA, vacA* and *dupA* genetic diversity in individual hosts. *J Med Microbiol* **59**, 89-95.

Mattsson, A., Quiding-Jarbrink, M., Lonroth, H., Hamlet, A., Ahlstedt, I. & Svennerholm, A. (1998). Antibody-secreting cells in the stomachs of symptomatic and asymptomatic *Helicobacter pylori*-infected subjects. *Infect Immun* **66**, 2705-2712.

Maynard, C. L. & Weaver, C. T. (2008). Diversity in the contribution of interleukin-10 to T-cell-mediated immune regulation. *Immunol Rev* **226**, 219-233.

Mbulaiteye, S. M., Hisada, M. & El-Omar, E. M. (2009). *Helicobacter pylori* associated global gastric cancer burden. *Front Biosci* **14**, 1490-1504.

McClain, M. S., Cao, P., Iwamoto, H., Vinion-Dubiel, A. D., Szabo, G., Shao, Z. & Cover, T. L. (2001). A 12-amino-acid segment, present in type s2 but not type s1 *Helicobacter pylori* VacA proteins, abolishes cytotoxin activity and alters membrane channel formation. *J Bacteriol* **183**, 6499-6508.

McDaniels, A. E., Wymer, L., Rankin, C. & Haugland, R. (2005). Evaluation of quantitative real time PCR for the measurement of *Helicobacter pylori* at low concentrations in drinking water. *Water Res* **39**, 4808-4816.

Medalie, J. H., Stange, K. C., Zyzanski, S. J. & Goldbourt, U. (1992). The importance of biopsychosocial factors in the development of duodenal ulcer in a cohort of middle-aged men. *Am J Epidemiol* **136**, 1280-1287.

Megraud, F., Boyanova, L. & Lamouliatte, H. (1991). Activity of lansoprazole against *Helicobacter pylori*. *Lancet* **337**, 1486.

Megraud, F. (2004). *H pylori* antibiotic resistance: prevalence, importance, and advances in testing. *Gut* **53**, 1374-1384.

Megraud, F. & Lehours, P. (2007). *Helicobacter pylori* detection and antimicrobial susceptibility testing. *Clin Microbiol Rev* **20**, 280-322.

Meng, K., Rodriguez-Pena, A., Dimitrov, T., Chen, W., Yamin, M., Noda, M. & Deuel, T. F. (2000). Pleiotrophin signals increased tyrosine phosphorylation of beta beta-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase beta/zeta. *Proc Natl Acad Sci U S A* **97**, 2603-2608.

Miehlke, S., Yu, J., Schuppler, M. & other authors (2001). *Helicobacter pylori vacA, iceA,* and *cagA* status and pattern of gastritis in patients with malignant and benign gastroduodenal disease. *Am J Gastroenterol* **96**, 1008-1013.

Mikula, M., Dzwonek, A., Jagusztyn-Krynicka, K. & Ostrowski, J. (2003). Quantitative detection for low levels of *Helicobacter pylori* infection in experimentally infected mice by real-time PCR. *J Microbiol Methods* **55**, 351-359.

Mills, K. H. & McGuirk, P. (2004). Antigen-specific regulatory T cells--their induction and role in infection. *Semin Immunol* **16**, 107-117.

Mimuro, H., Suzuki, T., Tanaka, J., Asahi, M., Haas, R. & Sasakawa, C. (2002). Grb2 is a key mediator of *helicobacter pylori* CagA protein activities. *Mol Cell* **10**, 745-755.

Miwa, H., Go, M. F. & Sato, N. (2002). *H. pylori* and gastric cancer: the Asian enigma. *Am J Gastroenterol* **97**, 1106-1112.

Mizuno, T., Ando, T., Nobata, K. & other authors (2005). Interleukin-17 levels in *Helicobacter pylori*-infected gastric mucosa and pathologic sequelae of colonization. *World J Gastroenterol* **11**, 6305-6311.

Mohammadi, M., Czinn, S., Redline, R. & Nedrud, J. (1996). *Helicobacter*-specific cell-mediated immune responses display a predominant Th1 phenotype and promote a delayed-type hypersensitivity response in the stomachs of mice. *J Immunol* **156**, 4729-4738.

Molinari, M., Galli, C., Norais, N., Telford, J. L., Rappuoli, R., Luzio, J. P. & Montecucco, C. (1997). Vacuoles induced by *Helicobacter pylori* toxin contain both late endosomal and lysosomal markers. *J Biol Chem* **272**, 25339-25344.

Molinari, M., Salio, M., Galli, C., Norais, N., Rappuoli, R., Lanzavecchia, A. & Montecucco, C. (1998). Selective inhibition of Ii-dependent antigen presentation by Helicobacter pylori toxin VacA. *J Exp Med* **187**, 135-140.

Monteiro, L., Hua, J., Birac, C., Lamouliatte, H. & Megraud, F. (1997). Quantitative polymerase chain reaction for the detection of *Helicobacter pylori* in gastric biopsy specimens. *Eur J Clin Microbiol Infect Dis* **16**, 143-149.

Montemurro, P., Nishioka, H., Dundon, W. G., de Bernard, M., Del Giudice, G., Rappuoli, R. & Montecucco, C. (2002). The neutrophil-activating protein (HP-NAP) of *Helicobacter pylori* is a potent stimulant of mast cells. *Eur J Immunol* **32**, 671-676.

Mookherjee, N., Brown, K. L., Bowdish, D. M. & other authors (2006). Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. *J Immunol* **176**, 2455-2464.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* **19**, 683-765.

Mora, J. R., Bono, M. R., Manjunath, N., Weninger, W., Cavanagh, L. L., Rosemblatt, M. & Von Andrian, U. H. (2003). Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* **424**, 88-93.

Moran, A. P. (1999). *Helicobacter pylori* lipopolysaccharide-mediated gastric and extragastric pathology. *J Physiol Pharmacol* **50**, 787-805.

Morgan, M. E., van Bilsen, J. H., Bakker, A. M. & other authors (2005). Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Hum Immunol* **66**, 13-20.

Morgner, A., Bayerdorffer, E., Neubauer, A. & Stolte, M. (2000). Malignant tumors of the stomach. Gastric mucosa-associated lymphoid tissue lymphoma and Helicobacter pylori. *Gastroenterol Clin North Am* **29**, 593-607.

Mori, N., Ueda, A., Geleziunas, R., Wada, A., Hirayama, T., Yoshimura, T. & Yamamoto, N. (2001). Induction of monocyte chemoattractant protein 1 by *Helicobacter pylori* involves NF-kappaB. *Infect Immun* **69**, 1280-1286.

Morihara, F., Hifumi, E., Yamada, M., Nishizono, A. & Uda, T. (2008). Therapeutic effects of molecularly designed antigen UREB138 for mice infected with *Helicobacter pylori*. *Biotechnol Bioeng* **100**, 634-643.

Morrison, G., Kilanowski, F., Davidson, D. & Dorin, J. (2002). Characterization of the mouse beta defensin 1, Defb1, mutant mouse model. *Infect Immun* **70**, 3053-3060.

Moss, S. F., Legon, S., Bishop, A. E., Polak, J. M. & Calam, J. (1992). Effect of Helicobacter pylori on gastric somatostatin in duodenal ulcer disease. *Lancet* **340**, 930-932.

Mullins, P. D. & Steer, H. W. (1998). *Helicobacter pylori* colonization density and gastric acid output in non-ulcer dyspepsia and duodenal ulcer disease. *Helicobacter* **3**, 86-92.

Muotiala, A., Helander, I. M., Pyhala, L., Kosunen, T. U. & Moran, A. P. (1992). Low biological activity of *Helicobacter pylori* lipopolysaccharide. *Infect Immun* **60**, 1714-1716.

Murakami, M., Lopez-Garcia, B., Braff, M., Dorschner, R. A. & Gallo, R. L. (2004). Postsecretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense. *J Immunol* **172**, 3070-3077.

Murata-Kamiya, N., Kurashima, Y., Teishikata, Y. & other authors (2007). *Helicobacter pylori* CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. *Oncogene* **26**, 4617-4626.

Murphy, K. M. & Reiner, S. L. (2002). The lineage decisions of helper T cells. *Nat Rev Immunol* **2**, 933-944.

Nagai, S., Mimuro, H., Yamada, T. & other authors (2007). Role of Peyer's patches in the induction of *Helicobacter pylori*-induced gastritis. *Proc Natl Acad Sci U S A* **104**, 8971-8976.

Nakachi, N., Klein, T. W., Friedman, H. & Yamamoto, Y. (2000). *Helicobacter pylori* infection of human gastric epithelial cells induces IL-8 and TNFalpha, but not TGFbeta1 mRNA. *FEMS Immunol Med Microbiol* **29**, 23-26.

Namavar, F., Sparrius, M., Veerman, E. C., Appelmelk, B. J. & Vandenbroucke-Grauls, C. M. (1998). Neutrophil-activating protein mediates adhesion of Helicobacter pylori to sulfated carbohydrates on high-molecular-weight salivary mucin. *Infect Immun* **66**, 444-447.

Narayanan, G. L. (2005). Determinants of vacuolating cytotoxin production in the gastric pathogen *Helicobacter pylori*. *Institute of Infection, Immunity and Inflammation* **University of Nottingham**.

Nayak, A. K. & Rose, J. B. (2007). Detection of *Helicobacter pylori* in sewage and water using a new quantitative PCR method with SYBR green. *J Appl Microbiol* **103**, 1931-1941.

Nazarenko, I., Lowe, B., Darfler, M., Ikonomi, P., Schuster, D. & Rashtchian, A. (2002). Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. *Nucleic Acids Res* **30**, e37.

Necchi, V., Candusso, M. E., Tava, F., Luinetti, O., Ventura, U., Fiocca, R., Ricci, V. & Solcia, E. (2007). Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by *Helicobacter pylori*. *Gastroenterology* **132**, 1009-1023.

Necchi, V., Manca, R., Ricci, V. & Solcia, E. (2009). Evidence for transepithelial dendritic cells in human *H. pylori* active gastritis. *Helicobacter* **14**, 208-222.

Neel, B. G., Gu, H. & Pao, L. (2003). The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. *Trends Biochem Sci* **28**, 284-293.

Netea, M. G., van der Graaf, C., Van der Meer, J. W. & Kullberg, B. J. (2004). Toll-like receptors and the host defense against microbial pathogens: bringing specificity to the innate-immune system. *J Leukoc Biol* **75**, 749-755.

Nguyen, B. V., Nguyen, K. G., Phung, C. D., Kremp, O., Kalach, N., Dupont, C., Raymond, J. & Vidal-Trecan, G. (2006). Prevalence of and factors associated with *Helicobacter pylori* infection in children in the north of Vietnam. *Am J Trop Med Hyg* **74**, 536-539.

Nguyen, T. X., Cole, A. M. & Lehrer, R. I. (2003). Evolution of primate theta-defensins: a serpentine path to a sweet tooth. *Peptides* **24**, 1647-1654.

Nishikawa, H., Kato, T., Tawara, I. & other authors (2005). Accelerated chemically induced tumor development mediated by CD4+CD25+ regulatory T cells in wild-type hosts. *Proc Natl Acad Sci U S A* **102**, 9253-9257.

Noach, L. A., Bosma, N. B., Jansen, J., Hoek, F. J., van Deventer, S. J. & Tytgat, G. N. (1994a). Mucosal tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 production in patients with *Helicobacter pylori* infection. *Scand J Gastroenterol* **29**, 425-429.

Noach, L. A., Rolf, T. M. & Tytgat, G. N. (1994b). Electron microscopic study of association between *Helicobacter pylori* and gastric and duodenal mucosa. *J Clin Pathol* **47**, 699-704.

Nogueira, C., Figueiredo, C., Carneiro, F. & other authors (2001). *Helicobacter pylori* genotypes may determine gastric histopathology. *Am J Pathol* **158**, 647-654.

Nogva, H. K., Dromtorp, S. M., Nissen, H. & Rudi, K. (2003). Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. *Biotechniques* **34**, 804-808, 810, 812-803.

Nomura, A. M., Perez-Perez, G. I., Lee, J., Stemmermann, G. & Blaser, M. J. (2002). Relation between *Helicobacter pylori cagA* status and risk of peptic ulcer disease. *Am J Epidemiol* **155**, 1054-1059.

O'Garra, A., Vieira, P. L., Vieira, P. & Goldfeld, A. E. (2004). IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage. *J Clin Invest* **114**, 1372-1378.

O'Garra, A. & Vieira, P. (2007). T(H)1 cells control themselves by producing interleukin-10. *Nat Rev Immunol* **7**, 425-428.

O'Neil, D. A., Porter, E. M., Elewaut, D., Anderson, G. M., Eckmann, L., Ganz, T. & Kagnoff, M. F. (1999). Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. *J Immunol* **163**, 6718-6724.

O'Neil, D. A., Cole, S. P., Martin-Porter, E., Housley, M. P., Liu, L., Ganz, T. & Kagnoff, M. F. (2000). Regulation of human beta-defensins by gastric epithelial cells in response to infection with *Helicobacter pylori* or stimulation with interleukin-1. *Infect Immun* **68**, 5412-5415.

O'Shea, J. J. & Murray, P. J. (2008). Cytokine signaling modules in inflammatory responses. *Immunity* **28**, 477-487.

Obonyo, M., Cole, S. P., Datta, S. K. & Guiney, D. G. (2006). Evidence for interleukin-1-independent stimulation of interleukin-12 and down-regulation by interleukin-10 in Helicobacter pylori-infected murine dendritic cells deficient in the interleukin-1 receptor. *FEMS Immunol Med Microbiol* **47**, 414-419.

Occhialini, A., Marais, A., Alm, R., Garcia, F., Sierra, R. & Megraud, F. (2000). Distribution of open reading frames of plasticity region of strain *J99* in *Helicobacter pylori* strains isolated from gastric carcinoma and gastritis patients in Costa Rica. *Infect Immun* **68**, 6240-6249.

Ohkusa, T., Okayasu, I., Miwa, H., Ohtaka, K., Endo, S. & Sato, N. (2003). Helicobacter pylori infection induces duodenitis and superficial duodenal ulcer in Mongolian gerbils. *Gut* **52**, 797-803.

Okano, Y., Hristova, K. R., Leutenegger, C. M., Jackson, L. E., Denison, R. F., Gebreyesus, B., Lebauer, D. & Scow, K. M. (2004). Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. *Appl Environ Microbiol* **70**, 1008-1016.

Okuda, M., Miyashiro, E., Booka, M., Tsuji, T. & Nakazawa, T. (2007). *Helicobacter pylori* colonization in the first 3 years of life in Japanese children. *Helicobacter* **12**, 324-327.

Olbe, L., Hamlet, A., Dalenback, J. & Fandriks, L. (1996). A mechanism by which *Helicobacter pylori* infection of the antrum contributes to the development of duodenal ulcer. *Gastroenterology* **110**, 1386-1394.

Onizuka, S., Tawara, I., Shimizu, J., Sakaguchi, S., Fujita, T. & Nakayama, E. (1999). Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res* **59**, 3128-3133.

Oppmann, B., Lesley, R., Blom, B. & other authors (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* **13**, 715-725.

Orhan, D., Kalel, G., Saltik-Temizel, I. N., Demir, H., Bulun, A., Karaagaoglu, E. & Caglar, M. (2008). Immunohistochemical detection of *Helicobacter pylori* infection in gastric biopsies of urea breath test-positive and -negative pediatric patients. *Turk J Pediatr* **50**, 34-39.

Otte, J. M., Neumann, H. M., Brand, S., Schrader, H., Schmidt, W. E. & Schmitz, F. (2009). Expression of beta-defensin 4 is increased in human gastritis. *Eur J Clin Invest* **39**, 126-138.

Pacholczyk, R., Ignatowicz, H., Kraj, P. & Ignatowicz, L. (2006). Origin and T cell receptor diversity of Foxp3+CD4+CD25+ T cells. *Immunity* **25**, 249-259.

Pagliaccia, C., de Bernard, M., Lupetti, P. & other authors (1998). The m2 form of the *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. *Proc Natl Acad Sci U S A* **95**, 10212-10217.

Pandolfi, F., Pierdominici, M., Marziali, M., Livia Bernardi, M., Antonelli, G., Galati, V., D'Offizi, G. & Aiuti, F. (2000). Low-dose IL-2 reduces lymphocyte apoptosis and increases naive CD4 cells in HIV-1 patients treated with HAART. *Clin Immunol* **94**, 153-159.

Papini, E., Satin, B., Bucci, C., de Bernard, M., Telford, J. L., Manetti, R., Rappuoli, R., Zerial, M. & Montecucco, C. (1997). The small GTP binding protein rab7 is essential for cellular vacuolation induced by Helicobacter pylori cytotoxin. *The EMBO journal* **16**, 15-24.

Papini, E., Satin, B., Norais, N., de Bernard, M., Telford, J. L., Rappuoli, R. & Montecucco, C. (1998). Selective increase of the permeability of polarized epithelial cell monolayers by *Helicobacter pylori* vacuolating toxin. *J Clin Invest* **102**, 813-820.

Pardoll, D. (2003). Does the immune system see tumors as foreign or self? *Annu Rev Immunol* **21**, 807-839.

Parkin, D. M. (2004). International variation. *Oncogene* **23**, 6329-6340.

Parsonnet, J., Blaser, M. J., Perez-Perez, G. I., Hargrett-Bean, N. & Tauxe, R. V. (1992). Symptoms and risk factors of Helicobacter pylori infection in a cohort of epidemiologists. *Gastroenterology* **102**, 41-46.

Parsonnet, J., Friedman, G. D., Orentreich, N. & Vogelman, H. (1997). Risk for gastric cancer in people with CagA positive or CagA negative Helicobacter pylori infection. *Gut* **40**, 297-301.

Paster, B. J., Lee, A., Fox, J. G., Dewhirst, F. E., Tordoff, L. A., Fraser, G. J., O'Rourke, J. L., Taylor, N. S. & Ferrero, R. (1991). Phylogeny of *Helicobacter felis* sp. nov., *Helicobacter mustelae*, and related bacteria. *Int J Syst Bacteriol* **41**, 31-38.

Pazgier, M., Hoover, D. M., Yang, D., Lu, W. & Lubkowski, J. (2006). Human beta-defensins. *Cell Mol Life Sci* **63**, 1294-1313.

Peek, R. M., Jr., Wirth, H. P., Moss, S. F. & other authors (2000). Helicobacter pylori alters gastric epithelial cell cycle events and gastrin secretion in Mongolian gerbils. *Gastroenterology* **118**, 48-59.

Peek, R. M., Jr. & Blaser, M. J. (2002). Helicobacter pylori and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer* **2**, 28-37.

Pellicano, A., Sebkova, L., Monteleone, G., Guarnieri, G., Imeneo, M., Pallone, F. & Luzza, F. (2007). Interleukin-12 drives the Th1 signaling pathway in *Helicobacter pylori*-infected human gastric mucosa. *Infect Immun* **75**, 1738-1744.

Peluso, I., Fantini, M. C., Fina, D., Caruso, R., Boirivant, M., MacDonald, T. T., Pallone, F. & Monteleone, G. (2007). IL-21 counteracts the regulatory T cell-mediated suppression of human CD4+ T lymphocytes. *J Immunol* **178**, 732-739.

Perez-Paramo, M., Albillos, A., Calleja, J. L., Salas, C., Marin, M. C., Marcos, M. L., Cacho, G., Escartin, P. & Ortiz-Berrocal, J. (1997). Changes in gastrin and serum pepsinogens in monitoring of *Helicobacter pylori* response to therapy. *Dig Dis Sci* **42**, 1734-1740.

Perez-Perez, G. I. & Blaser, M. J. (1987). Conservation and diversity of *Campylobacter pyloridis* major antigens. *Infect Immun* **55**, 1256-1263.

Perez-Perez, G. I., Rothenbacher, D. & Brenner, H. (2004). Epidemiology of Helicobacter pylori infection. *Helicobacter* **9 Suppl 1**, 1-6.

Perri, F., Clemente, R., Pastore, M. & other authors (1998). The 13C-urea breath test as a predictor of intragastric bacterial load and severity of *Helicobacter pylori* gastritis. *Scand J Clin Lab Invest* **58**, 19-27.

Peters, M. N. & Richardson, C. T. (1983). Stressful life events, acid hypersecretion, and ulcer disease. *Gastroenterology* **84**, 114-119.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45.

Plebani, M., Basso, D., Cassaro, M., Brigato, L., Scrigner, M., Toma, A., Di Mario, F. & Rugge, M. (1996). *Helicobacter pylori* serology in patients with chronic gastritis. *Am J Gastroenterol* **91**, 954-958.

Pohlner, J., Halter, R., Beyreuther, K. & Meyer, T. F. (1987). Gene structure and extracellular secretion of Neisseria gonorrhoeae IgA protease. *Nature* **325**, 458-462.

Polenghi, A., Bossi, F., Fischetti, F. & other authors (2007). The neutrophil-activating protein of *Helicobacter pylori* crosses endothelia to promote neutrophil adhesion *in vivo*. *J Immunol* **178**, 1312-1320.

Polonikov, A. V., Ivanov, V. P., Belugin, D. A., Khoroshaya, I. V., Kolchanova, I. O., Solodilova, M. A., Tutochkina, M. P. & Stepchenko, A. A. (2007). Analysis of common transforming growth factor beta-1 gene polymorphisms in gastric and duodenal ulcer disease: pilot study. *J Gastroenterol Hepatol* **22**, 555-564.

Prado-Montes de Oca, E., Velarde-Felix, J. S., Rios-Tostado, J. J., Picos-Cardenas, V. J. & Figuera, L. E. (2009). SNP 668C (-44) alters a NF-kappaB1 putative binding site in non-coding strand of human beta-defensin 1 (DEFB1) and is associated with lepromatous leprosy. *Infect Genet Evol* **9**, 617-625.

Prado-Montes de Oca, E. (2010). Human beta-defensin 1: A restless warrior against allergies, infections and cancer. *Int J Biochem Cell Biol*.

Price, A. B. (1991). The Sydney System: histological division. *J Gastroenterol Hepatol* **6**, 209-222.

Pride, D. T. & Blaser, M. J. (2002). Concerted evolution between duplicated genetic elements in *Helicobacter pylori*. *J Mol Biol* **316**, 629-642.

Prinz, C., Neumayer, N., Mahr, S., Classen, M. & Schepp, W. (1997). Functional impairment of rat enterochromaffin-like cells by interleukin 1 beta. *Gastroenterology* **112**, 364-375.

Prinz, C., Schoniger, M., Rad, R. & other authors (2001). Key importance of the Helicobacter pylori adherence factor blood group antigen binding adhesin during chronic gastric inflammation. *Cancer Res* **61**, 1903-1909.

Rad, R., Gerhard, M., Lang, R., Schoniger, M., Rosch, T., Schepp, W., Becker, I., Wagner, H. & Prinz, C. (2002). The *Helicobacter pylori* blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. *J Immunol* **168**, 3033-3041.

Rad, R., Dossumbekova, A., Neu, B., Lang, R., Bauer, S., Saur, D., Gerhard, M. & Prinz, C. (2004). Cytokine gene polymorphisms influence mucosal cytokine expression, gastric inflammation, and host specific colonisation during *Helicobacter pylori* infection. *Gut* **53**, 1082-1089.

Rad, R., Brenner, L., Bauer, S. & other authors (2006). CD25+/Foxp3+ T cells regulate gastric inflammation and *Helicobacter pylori* colonization *in vivo*. *Gastroenterology* **131**, 525-537.

Rad, R., Brenner, L., Krug, A. & other authors (2007). Toll-like receptor-dependent activation of antigen-presenting cells affects adaptive immunity to *Helicobacter pylori*. *Gastroenterology* **133**, 150-163 e153.

Raghavan, S., Fredriksson, M., Svennerholm, A. M., Holmgren, J. & Suri-Payer, E. (2003). Absence of CD4+CD25+ regulatory T cells is associated with a loss of regulation leading to increased pathology in *Helicobacter pylori*-infected mice. *Clin Exp Immunol* **132**, 393-400.

Raghavan, S. & Holmgren, J. (2005). CD4+CD25+ suppressor T cells regulate pathogen induced inflammation and disease. *FEMS Immunol Med Microbiol* **44**, 121-127.

Rajasekaran, A. K., Hojo, M., Huima, T. & Rodriguez-Boulan, E. (1996). Catenins and zonula occludens-1 form a complex during early stages in the assembly of tight junctions. *J Cell Biol* **132**, 451-463.

Rao, A., Luo, C. & Hogan, P. G. (1997). Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* **15**, 707-747.

Raqib, R., Sarker, P., Bergman, P. & other authors (2006). Improved outcome in shigellosis associated with butyrate induction of an endogenous peptide antibiotic. *Proc Natl Acad Sci U S A* **103**, 9178-9183.

Rautelin, H. I., Oksanen, A. M., Veijola, L. I., Sipponen, P. I., Tervahartiala, T. I., Sorsa, T. A. & Lauhio, A. (2009). Enhanced systemic matrix metalloproteinase response in *Helicobacter pylori* gastritis. *Ann Med* **41**, 208-215.

Rauws, E. A. & Tytgat, G. N. (1990). Cure of duodenal ulcer associated with eradication of *Helicobacter pylori*. *Lancet* **335**, 1233-1235.

Rehnberg-Laiho, L., Rautelin, H., Valle, M. & Kosunen, T. U. (1998). Persisting *Helicobacter* antibodies in Finnish children and adolescents between two and twenty years of age. *Pediatr Infect Dis J* **17**, 796-799.

Rhead, J. L., Letley, D. P., Mohammadi, M., Hussein, N., Mohagheghi, M. A., Eshagh Hosseini, M. & Atherton, J. C. (2007). A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. *Gastroenterology* **133**, 926-936.

Ricci, V., Galmiche, A., Doye, A., Necchi, V., Solcia, E. & Boquet, P. (2000). High cell sensitivity to *Helicobacter pylori* VacA toxin depends on a GPI-anchored protein and is not blocked by inhibition of the clathrin-mediated pathway of endocytosis. *Mol Biol Cell* **11**, 3897-3909.

Rieder, G., Merchant, J. L. & Haas, R. (2005). *Helicobacter pylori cag*-type IV secretion system facilitates corpus colonization to induce precancerous conditions in Mongolian gerbils. *Gastroenterology* **128**, 1229-1242.

Ririe, K. M., Rasmussen, R. P. & Wittwer, C. T. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem* **245**, 154-160.

Rizwan, M., Alvi, A. & Ahmed, N. (2008). Novel protein antigen (JHP940) from the genomic plasticity region of *Helicobacter pylori* induces tumor necrosis factor alpha and interleukin-8 secretion by human macrophages. *J Bacteriol* **190**, 1146-1151.

Robinson, K., Loughlin, M. F., Potter, R. & Jenks, P. J. (2005). Host adaptation and immune modulation are mediated by homologous recombination in *Helicobacter pylori*. *J Infect Dis* **191**, 579-587.

Robinson, K., Argent, R. H. & Atherton, J. C. (2007). The inflammatory and immune response to *Helicobacter pylori* infection. *Best Pract Res Clin Gastroenterol* **21**, 237-259.

Robinson, K., Kenefeck, R., Pidgeon, E. L., Shakib, S., Patel, S., Polson, R. J., Zaitoun, A. M. & Atherton, J. C. (2008). *Helicobacter pylori*-induced peptic ulcer disease is associated with inadequate regulatory T cell responses. *Gut* **57**, 1375-1385.

Robinson, K. & Atherton, J. C. (2010). *Helicobacter pylori*-induced acquired immunity and immunoregulation, . In *Helicobacter pylori in the 21st Century*, pp. 94-115. Edited by P. S. H. Mitchell. Oxfordshire, UK: CABI Press.

Rockall, T. A., Logan, R. F., Devlin, H. B. & Northfield, T. C. (1995). Incidence of and mortality from acute upper gastrointestinal haemorrhage in the United Kingdom. Steering Committee and members of the National Audit of Acute Upper Gastrointestinal Haemorrhage. *BMJ* **311**, 222-226.

Romano, M., Iovene, M. R., Russo, M. I. & other authors (2008). Failure of first-line eradication treatment significantly increases prevalence of antimicrobial-resistant *Helicobacter pylori* clinical isolates. *J Clin Pathol* **61**, 1112-1115.

Roper, J., Francois, F., Shue, P. L., Mourad, M. S., Pei, Z., Olivares de Perez, A. Z., Perez-Perez, G. I., Tseng, C. H. & Blaser, M. J. (2008). Leptin and ghrelin in relation to *Helicobacter pylori* status in adult males. *J Clin Endocrinol Metab* **93**, 2350-2357.

Rosenfeld, Y., Papo, N. & Shai, Y. (2006). Endotoxin (lipopolysaccharide) neutralization by innate immunity host-defense peptides. Peptide properties and plausible modes of action. *J Biol Chem* **281**, 1636-1643.

Rosenstock, S., Jorgensen, T., Bonnevie, O. & Andersen, L. (2003). Risk factors for peptic ulcer disease: a population based prospective cohort study comprising 2416 Danish adults. *Gut* **52**, 186-193.

Rothenbacher, D. & Brenner, H. (2003). Burden of *Helicobacter pylori* and *H. pylori*-related diseases in developed countries: recent developments and future implications. *Microbes Infect* **5**, 693-703.

Roussel, Y., Harris, A., Lee, M. H. & Wilks, M. (2007). Novel methods of quantitative real-time PCR data analysis in a murine *Helicobacter pylori* vaccine model. *Vaccine* **25**, 2919-2929.

Rowland, M., Kumar, D., Daly, L., O'Connor, P., Vaughan, D. & Drumm, B. (1999). Low rates of *Helicobacter pylori* reinfection in children. *Gastroenterology* **117**, 336-341.

Rowland, M., Daly, L., Vaughan, M., Higgins, A., Bourke, B. & Drumm, B. (2006). Age-specific incidence of *Helicobacter pylori*. *Gastroenterology* **130**, 65-72; quiz 211.

Rudensky, A. Y. & Campbell, D. J. (2006). *In vivo* sites and cellular mechanisms of T reg cell-mediated suppression. *J Exp Med* **203**, 489-492.

Rudi, J., Kolb, C., Maiwald, M., Kuck, D., Sieg, A., Galle, P. R. & Stremmel, W. (1998). Diversity of *Helicobacter pylori vacA* and *cagA* genes and relationship to VacA and CagA protein expression, cytotoxin production, and associated diseases. *J Clin Microbiol* **36**, 944-948.

Saadat, I., Higashi, H., Obuse, C. & other authors (2007). *Helicobacter pylori* CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. *Nature* **447**, 330-333.

Sachs, G., Scott, D., Weeks, D. & Melchers, K. (2002). The compartment buffered by the urease of *Helicobacter pylori*: cytoplasm or periplasm? *Trends Microbiol* **10**, 217-218; author reply 218-219.

Sainsus, N., Cattori, V., Lepadatu, C. & Hofmann-Lehmann, R. (2008). Liquid culture medium for the rapid cultivation of *Helicobacter pylori* from biopsy specimens. *Eur J Clin Microbiol Infect Dis* **27**, 1209-1217.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* **155**, 1151-1164.

Sakaguchi, S., Setoguchi, R., Yagi, H. & Nomura, T. (2006). Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in self-tolerance and autoimmune disease. *Curr Top Microbiol Immunol* **305**, 51-66.

Sakaguchi, S. & Powrie, F. (2007). Emerging challenges in regulatory T cell function and biology. *Science* **317**, 627-629.

Sakaguchi, S., Miyara, M., Costantino, C. M. & Hafler, D. A. (2010). FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* **10**, 490-500.

Samloff, I. M. (1971). Cellular localization of group I pepsinogens in human gastric mucosa by immunofluorescence. *Gastroenterology* **61**, 185-188.

Samloff, I. M. & Liebman, W. M. (1973). Cellular localization of the group II pepsinogens in human stomach and duodenum by immunofluorescence. *Gastroenterology* **65**, 36-42.

Santos, A., Queiroz, D. M., Menard, A., Marais, A., Rocha, G. A., Oliveira, C. A., Nogueira, A. M., Uzeda, M. & Megraud, F. (2003). New pathogenicity marker found in the plasticity region of the *Helicobacter pylori* genome. *J Clin Microbiol* **41**, 1651-1655.

Sasada, T., Kimura, M., Yoshida, Y., Kanai, M. & Takabayashi, A. (2003). CD4+CD25+ regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression. *Cancer* **98**, 1089-1099.

Satin, B., Del Giudice, G., Della Bianca, V. & other authors (2000). The neutrophil-activating protein (HP-NAP) of *Helicobacter pylori* is a protective antigen and a major virulence factor. *J Exp Med* **191**, 1467-1476.

Sawai, N., Kita, M., Kodama, T., Tanahashi, T., Yamaoka, Y., Tagawa, Y., Iwakura, Y. & Imanishi, J. (1999). Role of gamma interferon in *Helicobacter pylori*-induced gastric inflammatory responses in a mouse model. *Infect Immun* **67**, 279-285.

Sawyer, D. W., Donowitz, G. R. & Mandell, G. L. (1989). Polymorphonuclear neutrophils: an effective antimicrobial force. *Rev Infect Dis* **11 Suppl 7**, S1532-1544.

Sayi, A., Kohler, E., Hitzler, I., Arnold, I., Schwendener, R., Rehrauer, H. & Muller, A. (2009). The CD4+ T cell-mediated IFN-gamma response to *Helicobacter* infection is essential for clearance and determines gastric cancer risk. *J Immunol* **182**, 7085-7101.

Schauber, J., Svanholm, C., Termen, S. & other authors (2003). Expression of the cathelicidin LL-37 is modulated by short chain fatty acids in colonocytes: relevance of signalling pathways. *Gut* **52**, 735-741.

Schauber, J., Iffland, K., Frisch, S. & other authors (2004). Histone-deacetylase inhibitors induce the cathelicidin LL-37 in gastrointestinal cells. *Mol Immunol* **41**, 847-854.

Scheiman, J. M. & Cutler, A. F. (1999). Helicobacter pylori and gastric cancer. *Am J Med* **106**, 222-226.

Schmidt, H. M., Andres, S., Kaakoush, N. O. & other authors (2009). The prevalence of the duodenal ulcer promoting gene (*dupA*) in *Helicobacter pylori* isolates varies by ethnic group and is not universally associated with disease development: a case-control study. *Gut Pathog* **1**, 5.

Schneeberger, C., Speiser, P., Kury, F. & Zeillinger, R. (1995). Quantitative detection of reverse transcriptase-PCR products by means of a novel and sensitive DNA stain. *PCR Methods Appl* **4**, 234-238.

Schreiber, S., Konradt, M., Groll, C., Scheid, P., Hanauer, G., Werling, H. O., Josenhans, C. & Suerbaum, S. (2004). The spatial orientation of *Helicobacter pylori* in the gastric mucus. *Proc Natl Acad Sci U S A* **101**, 5024-5029.

Schultze, V., Hackelsberger, A., Gunther, T., Miehlke, S., Roessner, A. & Malfertheiner, P. (1998). Differing patterns of *Helicobacter pylori* gastritis in patients with duodenal, prepyloric, and gastric ulcer disease. *Scand J Gastroenterol* **33**, 137-142.

Schutte, B. C., Mitros, J. P., Bartlett, J. A., Walters, J. D., Jia, H. P., Welsh, M. J., Casavant, T. L. & McCray, P. B., Jr. (2002). Discovery of five conserved beta -defensin gene clusters using a computational search strategy. *Proc Natl Acad Sci U S A* **99**, 2129-2133.

Schwarz, S., Morelli, G., Kusecek, B. & other authors (2008). Horizontal versus familial transmission of *Helicobacter pylori*. *PLoS Pathog* **4**, e1000180.

Segal, E. D., Cha, J., Lo, J., Falkow, S. & Tompkins, L. S. (1999). Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *Proc Natl Acad Sci U S A* **96**, 14559-14564.

Segditsas, S. & Tomlinson, I. (2006). Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene* **25**, 7531-7537.

Selbach, M., Moese, S., Hauck, C. R., Meyer, T. F. & Backert, S. (2002). Src is the kinase of the *Helicobacter pylori* CagA protein *in vitro* and *in vivo*. *J Biol Chem* **277**, 6775-6778.

Selbach, M., Moese, S., Hurwitz, R., Hauck, C. R., Meyer, T. F. & Backert, S. (2003). The *Helicobacter pylori* CagA protein induces cortactin dephosphorylation and actin rearrangement by c-Src inactivation. *EMBO J* **22**, 515-528.

Selsted, M. E. & Ouellette, A. J. (2005). Mammalian defensins in the antimicrobial immune response. *Nat Immunol* **6**, 551-557.

Seno, H., Satoh, K., Tsuji, S., Shiratsuchi, T., Harada, Y., Hamajima, N., Sugano, K., Kawano, S. & Chiba, T. (2007). Novel interleukin-4 and interleukin-1 receptor antagonist gene variations associated with non-cardia gastric cancer in Japan: comprehensive analysis of 207 polymorphisms of 11 cytokine genes. *J Gastroenterol Hepatol* **22**, 729-737.

Setoguchi, R., Hori, S., Takahashi, T. & Sakaguchi, S. (2005). Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med* **201**, 723-735.

Sewald, X., Fischer, W. & Haas, R. (2008a). Sticky socks: *Helicobacter pylori* VacA takes shape. *Trends Microbiol* **16**, 89-92.

Sewald, X., Gebert-Vogl, B., Prassl, S. & other authors (2008b). Integrin subunit CD18 Is the T-lymphocyte receptor for the *Helicobacter pylori* vacuolating cytotoxin. *Cell Host Microbe* **3**, 20-29.

Sharma, S. A., Tummuru, M. K., Blaser, M. J. & Kerr, L. D. (1998). Activation of IL-8 gene expression by *Helicobacter pylori* is regulated by transcription factor nuclear factor-kappa B in gastric epithelial cells. *J Immunol* **160**, 2401-2407.

Shen, B., Porter, E. M., Reynoso, E. & other authors (2005). Human defensin 5 expression in intestinal metaplasia of the upper gastrointestinal tract. *J Clin Pathol* **58**, 687-694.

Shen, J., Arnett, D. K., Peacock, J. M. & other authors (2007). Interleukin1beta genetic polymorphisms interact with polyunsaturated fatty acids to modulate risk of the metabolic syndrome. *J Nutr* **137**, 1846-1851.

Sherman, H. & Froy, O. (2008). Expression of human beta-defensin 1 is regulated via c-Myc and the biological clock. *Mol Immunol* **45**, 3163-3167.

Sheu, B. S., Sheu, S. M., Yang, H. B., Huang, A. H. & Wu, J. J. (2003). Host gastric Lewis expression determines the bacterial density of *Helicobacter pylori* in *babA2* genopositive infection. *Gut* **52**, 927-932.

Sheu, B. S., Odenbreit, S., Hung, K. H., Liu, C. P., Sheu, S. M., Yang, H. B. & Wu, J. J. (2006). Interaction between host gastric Sialyl-Lewis X and *H. pylori* SabA enhances *H. pylori* density in patients lacking gastric Lewis B antigen. *Am J Gastroenterol* **101**, 36-44.

Sheu, B. S. & Wu, J. J. (2008). Type 1 and 2 Lewis antigens of *Helicobacter pylori* - a potential marker of the human geographical distribution. *J Med Microbiol* **57**, 543-544.

Shi, Y. & Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.

Shimizu, J., Yamazaki, S. & Sakaguchi, S. (1999). Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* **163**, 5211-5218.

Shimoyama, T. & Crabtree, J. E. (1998). Bacterial factors and immune pathogenesis in Helicobacter pylori infection. *Gut* **43 Suppl 1**, S2-5.

Shimoyama, T., Fukuda, S., Liu, Q., Nakaji, S., Fukuda, Y. & Sugawara, K. (2002). Production of chemokines and reactive oxygen species by human neutrophils stimulated by *Helicobacter pylori*. *Helicobacter* **7**, 170-174.

Siegel, P. M. & Massague, J. (2003). Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* **3**, 807-821.

Sipponen, P., Kosunen, T. U., Samloff, I. M., Heinonen, O. P. & Siurala, M. (1996). Rate of Helicobacter pylori acquisition among Finnish adults: a fifteen year follow-up. *Scand J Gastroenterol* **31**, 229-232.

Smith, M. F., Jr., Mitchell, A., Li, G., Ding, S., Fitzmaurice, A. M., Ryan, K., Crowe, S. & Goldberg, J. B. (2003). Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for *Helicobacter pylori*-induced NF-kappa B activation and chemokine expression by epithelial cells. *J Biol Chem* **278**, 32552-32560.

Smythies, L. E., Waites, K. B., Lindsey, J. R., Harris, P. R., Ghiara, P. & Smith, P. D. (2000). *Helicobacter pylori*-induced mucosal inflammation is Th1 mediated and exacerbated in IL-4, but not IFN-gamma, gene-deficient mice. *J Immunol* **165**, 1022-1029.

Soltermann, A., Koetzer, S., Eigenmann, F. & Komminoth, P. (2007). Correlation of *Helicobacter pylori* virulence genotypes *vacA* and *cagA* with histological parameters of gastritis and patient's age. *Mod Pathol* **20**, 878-883.

Sommer, F., Faller, G., Konturek, P., Kirchner, T., Hahn, E. G., Zeus, J., Rollinghoff, M. & Lohoff, M. (1998). Antrum- and corpus mucosa-infiltrating CD4(+) lymphocytes in *Helicobacter pylori* gastritis display a Th1 phenotype. *Infect Immun* **66**, 5543-5546.

Sonnenberg, A. (2006). Causes underlying the birth-cohort phenomenon of peptic ulcer: analysis of mortality data 1911-2000, England and Wales. *Int J Epidemiol* **35**, 1090-1097.

Souaze, F., Ntodou-Thome, A., Tran, C. Y., Rostene, W. & Forgez, P. (1996). Quantitative RT-PCR: limits and accuracy. *Biotechniques* **21**, 280-285.

Spellberg, B. & Edwards, J. E., Jr. (2001). Type 1/Type 2 immunity in infectious diseases. *Clin Infect Dis* **32**, 76-102.

Stein, M., Bagnoli, F., Halenbeck, R., Rappuoli, R., Fantl, W. J. & Covacci, A. (2002). c-Src/Lyn kinases activate *Helicobacter pylori* CagA through tyrosine phosphorylation of the EPIYA motifs. *Mol Microbiol* **43**, 971-980.

Stroinigg, N. & Srivastava, M. D. (2005). Modulation of toll-like receptor 7 and LL-37 expression in colon and breast epithelial cells by human beta-defensin-2. *Allergy Asthma Proc* **26**, 299-309.

Su, B., Ceponis, P. J., Lebel, S., Huynh, H. & Sherman, P. M. (2003). *Helicobacter pylori* activates Toll-like receptor 4 expression in gastrointestinal epithelial cells. *Infect Immun* **71**, 3496-3502.

Suganuma, M., Kurusu, M., Suzuki, K., Nishizono, A., Murakami, K., Fujioka, T. & Fujiki, H. (2005). New tumor necrosis factor-alpha-inducing protein released from *Helicobacter pylori* for gastric cancer progression. *J Cancer Res Clin Oncol* **131**, 305-313.

Sugimoto, M., Furuta, T., Shirai, N., Nakamura, A., Xiao, F., Kajimura, M., Sugimura, H. & Hishida, A. (2007). Different effects of polymorphisms of tumor necrosis factor-alpha and interleukin-1 beta on development of peptic ulcer and gastric cancer. *J Gastroenterol Hepatol* **22**, 51-59.

Sun, C. Q., Arnold, R., Fernandez-Golarz, C. & other authors (2006). Human beta-defensin-1, a potential chromosome 8p tumor suppressor: control of transcription and induction of apoptosis in renal cell carcinoma. *Cancer Res* **66**, 8542-8549.

Suri-Payer, E. & Cantor, H. (2001). Differential cytokine requirements for regulation of autoimmune gastritis and colitis by CD4(+)CD25(+) T cells. *J Autoimmun* **16**, 115-123.

Sutton, P., Doidge, C., Pinczower, G., Wilson, J., Harbour, S., Swierczak, A. & Lee, A. (2007). Effectiveness of vaccination with recombinant HpaA from *Helicobacter pylori* is influenced by host genetic background. *FEMS Immunol Med Microbiol* **50**, 213-219.

Suzuki, A., Hirata, M., Kamimura, K. & other authors (2004a). aPKC acts upstream of PAR-1b in both the establishment and maintenance of mammalian epithelial polarity. *Curr Biol* **14**, 1425-1435.

Suzuki, H., Miura, S., Imaeda, H., Suzuki, M., Han, J. Y., Mori, M., Fukumura, D., Tsuchiya, M. & Ishii, H. (1996). Enhanced levels of chemiluminescence and platelet activating factor in urease-positive gastric ulcers. *Free Radic Biol Med* **20**, 449-454.

Suzuki, M. T. & Giovannoni, S. J. (1996). Bias caused by template annealing in the amplification of mixtures of *16S rRNA* genes by PCR. *Appl Environ Microbiol* **62**, 625-630.

Suzuki, M. T., Taylor, L. T. & DeLong, E. F. (2000). Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. *Appl Environ Microbiol* **66**, 4605-4614.

Suzuki, T., Ina, K., Nishiwaki, T. & other authors (2004b). Differential roles of interleukin-1beta and interleukin-8 in neutrophil transendothelial migration in patients with *Helicobacter pylori* infection. *Scand J Gastroenterol* **39**, 313-321.

Swain, S. L., Weinberg, A. D., English, M. & Huston, G. (1990). IL-4 directs the development of Th2-like helper effectors. *J Immunol* **145**, 3796-3806.

Taguchi, A., Ohmiya, N., Shirai, K., Mabuchi, N., Itoh, A., Hirooka, Y., Niwa, Y. & Goto, H. (2005). Interleukin-8 promoter polymorphism increases the risk of atrophic gastritis and gastric cancer in Japan. *Cancer Epidemiol Biomarkers Prev* **14**, 2487-2493.

Taha, A. S., Faccenda, E., Angerson, W. J., Balsitis, M. & Kelly, R. W. (2005). Gastric epithelial anti-microbial peptides--histological correlation and influence of anatomical site and peptic ulcer disease. *Dig Liver Dis* **37**, 51-56.

Tahara, T., Shibata, T., Nakamura, M., Yoshioka, D., Okubo, M., Arisawa, T. & Hirata, I. (2009). Gastric mucosal pattern by using magnifying narrow-band imaging endoscopy clearly distinguishes histological and serological severity of chronic gastritis. *Gastrointest Endosc* **70**, 246-253.

Tai, P., Wang, J., Jin, H. & other authors (2008). Induction of regulatory T cells by physiological level estrogen. *J Cell Physiol* **214**, 456-464.

Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W. & Sakaguchi, S. (2000). Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* **192**, 303-310.

Takashima, M., Furuta, T., Hanai, H., Sugimura, H. & Kaneko, E. (2001). Effects of *Helicobacter pylori* infection on gastric acid secretion and serum gastrin levels in Mongolian gerbils. *Gut* **48**, 765-773.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251**, 1451-1455.

Takeshima, E., Tomimori, K., Takamatsu, R., Ishikawa, C., Kinjo, F., Hirayama, T., Fujita, J. & Mori, N. (2009). *Helicobacter pylori* VacA activates NF-kappaB in T cells via the classical but not alternative pathway. *Helicobacter* **14**, 271-279.

Tammer, I., Brandt, S., Hartig, R., Konig, W. & Backert, S. (2007). Activation of Abl by *Helicobacter pylori*: a novel kinase for CagA and crucial mediator of host cell scattering. *Gastroenterology* **132**, 1309-1319.

Tanabe, H., Sato, T., Watari, J., Maemoto, A., Fijiya, M., Kono, T., Ashida, T., Ayabe, T. & Kohgo, Y. (2008). Functional role of metaplastic paneth cell defensins in *Helicobacter pylori*-infected stomach. *Helicobacter* **13**, 370-379.

Tanaka, H., Yoshida, M., Nishiumi, S., Ohnishi, N., Kobayashi, K., Yamamoto, K., Fujita, T., Hatakeyama, M. & Azuma, T. (2010). The CagA protein of *Helicobacter pylori* suppresses the functions of dendritic cell in mice. *Arch Biochem Biophys* **498**, 35-42.

Tang, J. H., Liu, N. J., Cheng, H. T. & other authors (2009). Endoscopic diagnosis of *Helicobacter pylori* infection by rapid urease test in bleeding peptic ulcers: a prospective case-control study. *J Clin Gastroenterol* **43**, 133-139.

Taylor, A., Akdis, M., Joss, A. & other authors (2007). IL-10 inhibits CD28 and ICOS costimulations of T cells via src homology 2 domain-containing protein tyrosine phosphatase 1. *J Allergy Clin Immunol* **120**, 76-83.

Tegtmeyer, N., Zabler, D., Schmidt, D., Hartig, R., Brandt, S. & Backert, S. (2009). Importance of EGF receptor, HER2/Neu and Erk1/2 kinase signalling for host cell elongation and scattering induced by the *Helicobacter pylori* CagA protein: antagonistic effects of the vacuolating cytotoxin VacA. *Cell Microbiol* **11**, 488-505.

Testerman, T. L., Conn, P. B., Mobley, H. L. & McGee, D. J. (2006). Nutritional requirements and antibiotic resistance patterns of *Helicobacter* species in chemically defined media. *J Clin Microbiol* **44**, 1650-1658.

Tham, K. T., Peek, R. M., Jr., Atherton, J. C., Cover, T. L., Perez-Perez, G. I., Shyr, Y. & Blaser, M. J. (2001). *Helicobacter pylori* genotypes, host factors, and gastric mucosal histopathology in peptic ulcer disease. *Hum Pathol* **32**, 264-273.

Thomas-Kaskel, A. K., Waller, C. F., Schultze-Seemann, W. & Veelken, H. (2007). Immunotherapy with dendritic cells for prostate cancer. *Int J Cancer* **121**, 467-473.

Thompson, C. & Powrie, F. (2004). Regulatory T cells. *Curr Opin Pharmacol* **4**, 408-414.

Thoreson, A. C., Hamlet, A., Celik, J., Bystrom, M., Nystrom, S., Olbe, L. & Svennerholm, A. M. (2000). Differences in surface-exposed antigen expression between Helicobacter pylori strains isolated from duodenal ulcer patients and from asymptomatic subjects. *Journal of clinical microbiology* **38**, 3436-3441.

Tindberg, Y., Bengtsson, C., Granath, F., Blennow, M., Nyren, O. & Granstrom, M. (2001). *Helicobacter pylori* infection in Swedish school children: lack of evidence of child-to-child transmission outside the family. *Gastroenterology* **121**, 310-316.

Tombola, F., Carlesso, C., Szabo, I. & other authors (1999). *Helicobacter pylori* vacuolating toxin forms anion-selective channels in planar lipid bilayers: possible implications for the mechanism of cellular vacuolation. *Biophys J* **76**, 1401-1409.

Tombola, F., Morbiato, L., Del Giudice, G., Rappuoli, R., Zoratti, M. & Papini, E. (2001). The *Helicobacter pylori* VacA toxin is a urea permease that promotes urea diffusion across epithelia. *J Clin Invest* **108**, 929-937.

Tomita, T., Jackson, A. M., Hida, N., Hayat, M., Dixon, M. F., Shimoyama, T., Axon, A. T., Robinson, P. A. & Crabtree, J. E. (2001). Expression of Interleukin-18, a Th1 cytokine, in human gastric mucosa is increased in *Helicobacter pylori* infection. *J Infect Dis* **183**, 620-627.

Torres, J., Perez-Perez, G., Goodman, K. J., Atherton, J. C., Gold, B. D., Harris, P. R., la Garza, A. M., Guarner, J. & Munoz, O. (2000). A comprehensive review of the natural history of *Helicobacter pylori* infection in children. *Arch Med Res* **31**, 431-469.

Torres, V. J., McClain, M. S. & Cover, T. L. (2004). Interactions between p-33 and p-55 domains of the Helicobacter pylori vacuolating cytotoxin (VacA). *J Biol Chem* **279**, 2324-2331.

Town, T., Laouar, Y., Pittenger, C., Mori, T., Szekely, C. A., Tan, J., Duman, R. S. & Flavell, R. A. (2008). Blocking TGF-beta-Smad2/3 innate immune signaling mitigates Alzheimer-like pathology. *Nat Med* **14**, 681-687.

Trifari, S., Kaplan, C. D., Tran, E. H., Crellin, N. K. & Spits, H. (2009). Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nat Immunol* **10**, 864-871.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* **3**, 133-146.

Trinchieri, G. (2007). Interleukin-10 production by effector T cells: Th1 cells show self control. *J Exp Med* **204**, 239-243.

Tsai, S. P., Wong, A., Mai, E. & other authors (2003). Nucleic acid capture assay, a new method for direct quantitation of nucleic acids. *Nucleic Acids Res* **31**, e25.

Tsuchiya, M., Imamura, L., Park, J. B. & Kobashi, K. (1995). *Helicobacter pylori* urease inhibition by rabeprazole, a proton pump inhibitor. *Biol Pharm Bull* **18**, 1053-1056.

Tsukada, K., Miyazaki, T., Katoh, H. & other authors (2005). The incidence of reflux oesophagitis after eradication therapy for Helicobacter pylori. *European journal of gastroenterology & hepatology* **17**, 1025-1028.

Tsutsumi, R., Higashi, H., Higuchi, M., Okada, M. & Hatakeyama, M. (2003). Attenuation of *Helicobacter pylori* CagA x SHP-2 signaling by interaction between CagA and C-terminal Src kinase. *J Biol Chem* **278**, 3664-3670.

Tsutsumi, R., Takahashi, A., Azuma, T., Higashi, H. & Hatakeyama, M. (2006). Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with *Helicobacter pylori* CagA. *Mol Cell Biol* **26**, 261-276.

Tu, S., Bhagat, G., Cui, G. & other authors (2008). Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell* **14**, 408-419.

Tummala, S., Sheth, S. G., Goldsmith, J. D. & other authors (2007). Quantifying gastric *Helicobacter pylori* infection: a comparison of quantitative culture, urease breath testing, and histology. *Dig Dis Sci* **52**, 396-401.

Tummuru, M. K., Cover, T. L. & Blaser, M. J. (1993). Cloning and expression of a high-molecular-mass major antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. *Infect Immun* **61**, 1799-1809.

Twisk, M., Kusters, J. G., Balk, A. G., Kuipers, E. J. & Loffeld, R. J. (2001). Colonisation density and topographic localisation of *Helicobacter pylori* do not depend on the *cagA* status. *J Clin Pathol* **54**, 771-773.

Uemura, N., Okamoto, S., Yamamoto, S., Matsumura, N., Yamaguchi, S., Yamakido, M., Taniyama, K., Sasaki, N. & Schlemper, R. J. (2001). *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med* **345**, 784-789.

Umit, H., Tezel, A., Bukavaz, S., Unsal, G., Otkun, M., Soylu, A. R., Tucer, D. & Bilgi, S. (2009). The relationship between virulence factors of *Helicobacter pylori* and severity of gastritis in infected patients. *Dig Dis Sci* **54**, 103-110.

Vaananen, H., Vauhkonen, M., Helske, T. & other authors (2003). Non-endoscopic diagnosis of atrophic gastritis with a blood test. Correlation between gastric histology and serum levels of gastrin-17 and pepsinogen I: a multicentre study. *Eur J Gastroenterol Hepatol* **15**, 885-891.

Vaezi, M. F., Falk, G. W., Peek, R. M., Vicari, J. J., Goldblum, J. R., Perez-Perez, G. I., Rice, T. W., Blaser, M. J. & Richter, J. E. (2000). *CagA*-positive strains of *Helicobacter pylori* may protect against Barrett's esophagus. *Am J Gastroenterol* **95**, 2206-2211.

Valle, J., Kekki, M., Sipponen, P., Ihamaki, T. & Siurala, M. (1996). Long-term course and consequences of *Helicobacter pylori* gastritis. Results of a 32-year follow-up study. *Scand J Gastroenterol* **31**, 546-550.

Valore, E. V., Park, C. H., Quayle, A. J., Wiles, K. R., McCray, P. B., Jr. & Ganz, T. (1998). Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. *J Clin Invest* **101**, 1633-1642.

Van de Bovenkamp, J. H., Mahdavi, J., Korteland-Van Male, A. M., Buller, H. A., Einerhand, A. W., Boren, T. & Dekker, J. (2003). The MUC5AC glycoprotein is the primary receptor for *Helicobacter pylori* in the human stomach. *Helicobacter* **8**, 521-532.

van Doorn, L. J., Figueiredo, C., Sanna, R., Pena, S., Midolo, P., Ng, E. K., Atherton, J. C., Blaser, M. J. & Quint, W. G. (1998). Expanding allelic diversity of *Helicobacter pylori vacA*. *J Clin Microbiol* **36**, 2597-2603.

van Doorn, L. J., Henskens, Y., Nouhan, N. & other authors (2000). The efficacy of laboratory diagnosis of *Helicobacter pylori* infections in gastric biopsy specimens is related to bacterial density and *vacA*, *cagA*, and *iceA* genotypes. *J Clin Microbiol* **38**, 13-17.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, RESEARCH0034.

Viala, J., Chaput, C., Boneca, I. G. & other authors (2004). Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori cag* pathogenicity island. *Nat Immunol* **5**, 1166-1174.

Vicari, J. J., Peek, R. M., Falk, G. W. & other authors (1998). The seroprevalence of *cagA*-positive *Helicobacter pylori* strains in the spectrum of gastroesophageal reflux disease. *Gastroenterology* **115**, 50-57.

von Bothmer, C., Edebo, A., Lonroth, H., Olbe, L., Pettersson, A. & Fandriks, L. (2002). Helicobacter pylori infection inhibits antral mucosal nitric oxide production in humans. *Scand J Gastroenterol* **37**, 404-408.

von Wintzingerode, F., Gobel, U. B. & Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* **21**, 213-229.

Wada, A., Mori, N., Oishi, K. & other authors (1999). Induction of human beta-defensin-2 mRNA expression by *Helicobacter pylori* in human gastric cell line MKN45 cells on cag pathogenicity island. *Biochem Biophys Res Commun* **263**, 770-774.

Wada, A., Ogushi, K., Kimura, T. & other authors (2001). *Helicobacter pylori*-mediated transcriptional regulation of the human beta-defensin 2 gene requires NF-kappaB. *Cell Microbiol* **3**, 115-123.

Wada, A., Yamasaki, E. & Hirayama, T. (2004). Helicobacter pylori vacuolating cytotoxin, VacA, is responsible for gastric ulceration. *Journal of biochemistry* **136**, 741-746.

Wagner, S., Haruma, K., Gladziwa, U., Soudah, B., Gebel, M., Bleck, J., Schmidt, H. & Manns, M. (1994). *Helicobacter pylori* infection and serum pepsinogen A, pepsinogen C, and gastrin in gastritis and peptic ulcer: significance of inflammation and effect of bacterial eradication. *Am J Gastroenterol* **89**, 1211-1218.

Walker, L. S., Chodos, A., Eggena, M., Dooms, H. & Abbas, A. K. (2003). Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells *in vivo*. *J Exp Med* **198**, 249-258.

Walz, A., Odenbreit, S., Mahdavi, J., Boren, T. & Ruhl, S. (2005). Identification and characterization of binding properties of *Helicobacter pylori* by glycoconjugate arrays. *Glycobiology* **15**, 700-708.

Wang, J., Ioan-Facsinay, A., van der Voort, E. I., Huizinga, T. W. & Toes, R. E. (2007). Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* **37**, 129-138.

Wang, Z., Hong, J., Sun, W. & other authors (2006). Role of IFN-gamma in induction of Foxp3 and conversion of CD4+ CD25- T cells to CD4+ Tregs. *J Clin Invest* **116**, 2434-2441.

Watanabe, T., Tada, M., Nagai, H., Sasaki, S. & Nakao, M. (1998). *Helicobacter pylori* infection induces gastric cancer in mongolian gerbils. *Gastroenterology* **115**, 642-648.

Watanabe, T., Higuchi, K., Tominaga, K., Fujiwara, Y. & Arakawa, T. (2001). Acid regulates inflammatory response in a rat model of induction of gastric ulcer recurrence by interleukin 1beta. *Gut* **48**, 774-781.

Waugh, D. J. & Wilson, C. (2008). The interleukin-8 pathway in cancer. *Clin Cancer Res* **14**, 6735-6741.

Webb, P. M., Knight, T., Greaves, S., Wilson, A., Newell, D. G., Elder, J. & Forman, D. (1994). Relation between infection with *Helicobacter pylori* and living conditions in childhood: evidence for person to person transmission in early life. *BMJ* **308**, 750-753.

Wee, J. L., Chionh, Y. T., Ng, G. Z. & other authors (2010). Protease-activated receptor-1 down-regulates the murine inflammatory and humoral response to *Helicobacter pylori*. *Gastroenterology* **138**, 573-582.

Wehkamp, J., Harder, J., Wehkamp, K. & other authors (2004). NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. *Infect Immun* **72**, 5750-5758.

Wehkamp, J., Wang, G., Kubler, I. & other authors (2007). The Paneth cell alpha-defensin deficiency of ileal Crohn's disease is linked to Wnt/Tcf-4. *J Immunol* **179**, 3109-3118.

Weiner, H. L. (2001). Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* **182**, 207-214.

Wenghoefer, M., Pantelis, A., Dommisch, H. & other authors (2008). Decreased gene expression of human beta-defensin-1 in the development of squamous cell carcinoma of the oral cavity. *Int J Oral Maxillofac Surg* **37**, 660-663.

Wilschanski, M., Schlesinger, Y., Faber, J., Rudensky, B., Ohnona, F. S., Freier, S., Rahman, E., Refael, S. & Halle, D. (2007). Combination of *Helicobacter pylori* strain and tumor necrosis factor-alpha polymorphism of the host increases the risk of peptic ulcer disease in children. *J Pediatr Gastroenterol Nutr* **45**, 199-203.

Wolk, K., Kunz, S., Witte, E., Friedrich, M., Asadullah, K. & Sabat, R. (2004). IL-22 increases the innate immunity of tissues. *Immunity* **21**, 241-254.

Wolk, K., Witte, E., Witte, K., Warszawska, K. & Sabat, R. (2010). Biology of interleukin-22. *Semin Immunopathol* **32**, 17-31.

Wong, B. L., Zhu, S. L., Huang, X. R., Ma, J., Xia, H. H., Bucala, R., Wong, B. C. & Lan, H. Y. (2009). Essential role for macrophage migration inhibitory factor in gastritis induced by *Helicobacter pylori*. *Am J Pathol* **174**, 1319-1328.

Wu, M. S., Lin, J. T., Hsu, P. N., Lin, C. Y., Hsieh, Y. T., Chiu, Y. H., Hsueh, P. R. & Liao, K. W. (2007). Preferential induction of transforming growth factor-beta production in gastric

epithelial cells and monocytes by *Helicobacter pylori* soluble proteins. *J Infect Dis* **196**, 1386-1393.

Wu, W. K., Sung, J. J., To, K. F., Yu, L., Li, H. T., Li, Z. J., Chu, K. M., Yu, J. & Cho, C. H. (2010). The host defense peptide LL-37 activates the tumor-suppressing bone morphogenetic protein signaling via inhibition of proteasome in gastric cancer cells. *J Cell Physiol* **223**, 178-186.

Xiao, B., Liu, Z., Li, B. S. & other authors (2009). Induction of microRNA-155 during *Helicobacter pylori* infection and its negative regulatory role in the inflammatory response. *J Infect Dis* **200**, 916-925.

Yahiro, K., Niidome, T., Hatakeyama, T., Aoyagi, H., Kurazono, H., Padilla, P. I., Wada, A. & Hirayama, T. (1997). *Helicobacter pylori* vacuolating cytotoxin binds to the 140-kDa protein in human gastric cancer cell lines, AZ-521 and AGS. *Biochem Biophys Res Commun* **238**, 629-632.

Yahiro, K., Wada, A., Nakayama, M. & other authors (2003). Protein-tyrosine phosphatase alpha, RPTP alpha, is a *Helicobacter pylori* VacA receptor. *J Biol Chem* **278**, 19183-19189.

Yahiro, K., Wada, A., Yamasaki, E. & other authors (2004). Essential domain of receptor tyrosine phosphatase beta (RPTPbeta) for interaction with *Helicobacter pylori* vacuolating cytotoxin. *J Biol Chem* **279**, 51013-51021.

Yakoob, J., Jafri, W., Abid, S. & other authors (2005). Role of rapid urease test and histopathology in the diagnosis of *Helicobacter pylori* infection in a developing country. *BMC Gastroenterol* **5**, 38.

Yamaguchi, T. & Sakaguchi, S. (2006). Regulatory T cells in immune surveillance and treatment of cancer. *Semin Cancer Biol* **16**, 115-123.

Yamaguchi, Y., Nagase, T., Makita, R., Fukuhara, S., Tomita, T., Tominaga, T., Kurihara, H. & Ouchi, Y. (2002). Identification of multiple novel epididymis-specific beta-defensin isoforms in humans and mice. *J Immunol* **169**, 2516-2523.

Yamaji, Y., Mitsushima, T., Ikuma, H. & other authors (2001). Inverse background of Helicobacter pylori antibody and pepsinogen in reflux oesophagitis compared with gastric cancer: analysis of 5732 Japanese subjects. *Gut* **49**, 335-340.

Yamaoka, Y., Kodama, T., Kashima, K., Graham, D. Y. & Sepulveda, A. R. (1998). Variants of the 3' region of the *cagA* gene in *Helicobacter pylori* isolates from patients with different *H. pylori*-associated diseases. *J Clin Microbiol* **36**, 2258-2263.

Yamaoka, Y., Kodama, T., Kita, M., Imanishi, J., Kashima, K. & Graham, D. Y. (1999). Relation between clinical presentation, *Helicobacter pylori* density, interleukin 1beta and 8 production, and *cagA* status. *Gut* **45**, 804-811.

Yamaoka, Y., Kwon, D. H. & Graham, D. Y. (2000). A M(r) 34,000 proinflammatory outer membrane protein (oipA) of *Helicobacter pylori*. *Proc Natl Acad Sci U S A* **97**, 7533-7538.

Yamaoka, Y., Kikuchi, S., el-Zimaity, H. M., Gutierrez, O., Osato, M. S. & Graham, D. Y. (2002a). Importance of *Helicobacter pylori oipA* in clinical presentation, gastric inflammation, and mucosal interleukin 8 production. *Gastroenterology* **123**, 414-424.

Yamaoka, Y., Souchek, J., Odenbreit, S. & other authors (2002b). Discrimination between cases of duodenal ulcer and gastritis on the basis of putative virulence factors of *Helicobacter pylori*. *J Clin Microbiol* **40**, 2244-2246.

Yamaoka, Y., Ojo, O., Fujimoto, S. & other authors (2006). *Helicobacter pylori* outer membrane proteins and gastroduodenal disease. *Gut* **55**, 775-781.

Yamaoka, Y. (2008). Roles of the plasticity regions of *Helicobacter pylori* in gastroduodenal pathogenesis. *J Med Microbiol* **57**, 545-553.

Yamasaki, E., Wada, A., Kumatori, A. & other authors (2006). *Helicobacter pylori* vacuolating cytotoxin induces activation of the proapoptotic proteins Bax and Bak, leading to cytochrome c release and cell death, independent of vacuolation. *J Biol Chem* **281**, 11250-11259.

Yamasaki, K., Di Nardo, A., Bardan, A. & other authors (2007). Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea. *Nat Med* **13**, 975-980.

Yamauchi, K., Choi, I. J., Lu, H., Ogiwara, H., Graham, D. Y. & Yamaoka, Y. (2008). Regulation of IL-18 in *Helicobacter pylori* infection. *J Immunol* **180**, 1207-1216.

Yamazaki, S., Iyoda, T., Tarbell, K., Olson, K., Velinzon, K., Inaba, K. & Steinman, R. M. (2003). Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* **198**, 235-247.

Yang, D., Chertov, O., Bykovskaia, S. N. & other authors (1999). Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* **286**, 525-528.

Yang, D., Biragyn, A., Kwak, L. W. & Oppenheim, J. J. (2002). Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol* **23**, 291-296.

Ye, P., Rodriguez, F. H., Kanaly, S. & other authors (2001). Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* **194**, 519-527.

Yokoyama, K., Higashi, H., Ishikawa, S. & other authors (2005). Functional antagonism between *Helicobacter pylori* CagA and vacuolating toxin VacA in control of the NFAT signaling pathway in gastric epithelial cells. *Proc Natl Acad Sci U S A* **102**, 9661-9666.

Zagari, R. M., Pozzato, P., Martuzzi, C., Fuccio, L., Martinelli, G., Roda, E. & Bazzoli, F. (2005). 13C-urea breath test to assess *Helicobacter pylori* bacterial load. *Helicobacter* **10**, 615-619.

Zaiou, M., Nizet, V. & Gallo, R. L. (2003). Antimicrobial and protease inhibitory functions of the human cathelicidin (hCAP18/LL-37) prosequence. *J Invest Dermatol* **120**, 810-816.

Zambon, C. F., Navaglia, F., Basso, D., Rugge, M. & Plebani, M. (2003). *Helicobacter pylori babA2, cagA*, and s1 *vacA* genes work synergistically in causing intestinal metaplasia. *J Clin Pathol* **56**, 287-291.

Zambon, C. F., Basso, D., Navaglia, F. & other authors (2005). Pro- and anti-inflammatory cytokines gene polymorphisms and *Helicobacter pylori* infection: interactions influence outcome. *Cytokine* **29**, 141-152.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389-395.

Zasloff, M. (2006). Inducing endogenous antimicrobial peptides to battle infections. *Proc Natl Acad Sci U S A* **103**, 8913-8914.

Zasloff, M. (2007). Antimicrobial peptides, innate immunity, and the normally sterile urinary tract. *J Am Soc Nephrol* **18**, 2810-2816.

Zhang, Q. B., Nakashabendi, I. M., Mokhashi, M. S., Dawodu, J. B., Gemmell, C. G. & Russell, R. I. (1996). Association of cytotoxin production and neutrophil activation by strains of *Helicobacter pylori* isolated from patients with peptic ulceration and chronic gastritis. *Gut* **38**, 841-845.

Zhang, Z., Zheng, Q., Chen, X., Xiao, S., Liu, W. & Lu, H. (2008). The *Helicobacter pylori* duodenal ulcer promoting gene, *dupA* in China. *BMC Gastroenterol* **8**, 49.

Zhao, W., Wu, W. & Xu, X. (2007). Oral vaccination with liposome-encapsulated recombinant fusion peptide of urease B epitope and cholera toxin B subunit affords prophylactic and therapeutic effects against *H. pylori* infection in BALB/c mice. *Vaccine* **25**, 7664-7673.

Zheng, S. G., Wang, J., Wang, P., Gray, J. D. & Horwitz, D. A. (2007). IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. *J Immunol* **178**, 2018-2027.

Zhou, Y., Toh, M. L., Zrioual, S. & Miossec, P. (2007). IL-17A versus IL-17F induced intracellular signal transduction pathways and modulation by IL-17RA and IL-17RC RNA interference in AGS gastric adenocarcinoma cells. *Cytokine* **38**, 157-164.

Zhu, B. D., Feng, Y., Huang, N., Wu, Q. & Wang, B. Y. (2003). Mycobacterium bovis bacille Calmette-Guerin (BCG) enhances human beta-defensin-1 gene transcription in human pulmonary gland epithelial cells. *Acta Pharmacol Sin* **24**, 907-912.

Zhu, J. & Paul, W. E. (2010). Heterogeneity and plasticity of T helper cells. *Cell Res* **20**, 4-12.

Ziegler, S. F. (2006). FOXP3: of mice and men. *Annu Rev Immunol* **24**, 209-226.

Zorn, E., Nelson, E. A., Mohseni, M. & other authors (2006). IL-2 regulates *FOXP3* expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells *in vivo*. *Blood* **108**, 1571-1579.

Zou, W. (2006). Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* **6**, 295-307.

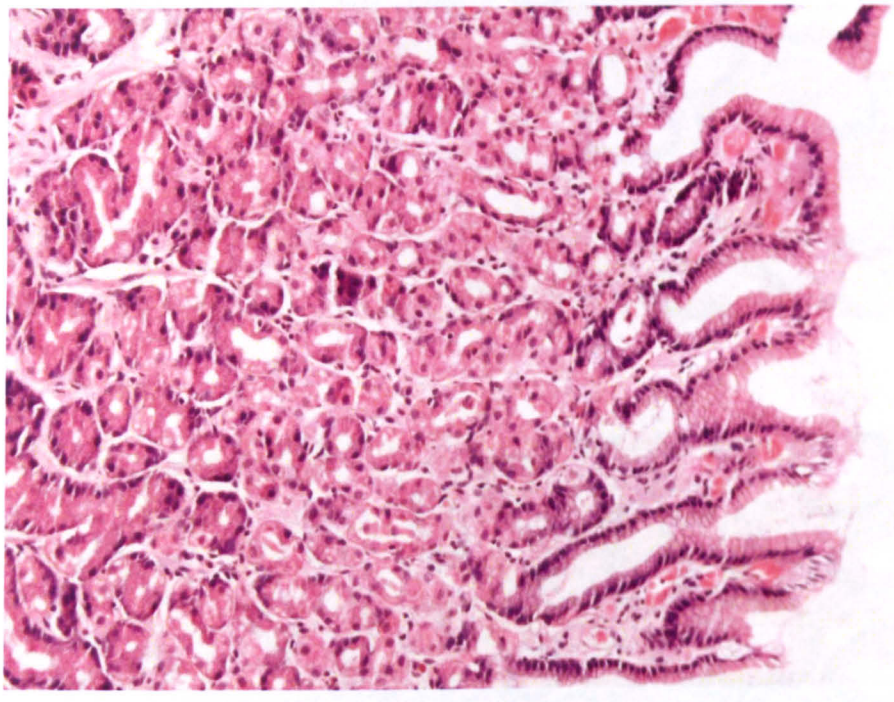
Zucca, E., Bertoni, F., Roggero, E., Bosshard, G., Cazzaniga, G., Pedrinis, E., Biondi, A. & Cavalli, F. (1998). Molecular analysis of the progression from Helicobacter pylori-associated chronic gastritis to mucosa-associated lymphoid-tissue lymphoma of the stomach. *N Engl J Med* **338**, 804-810.

Chapter 11

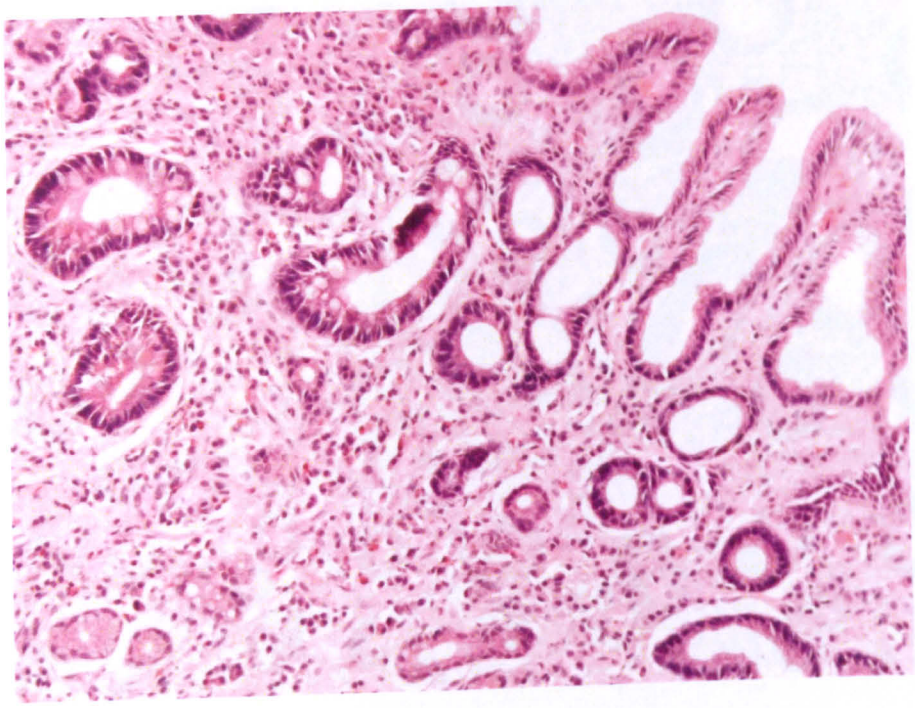
11. APPENDIX

11.1 Examples of histological findings in the *H. pylori* infected gastric mucosa

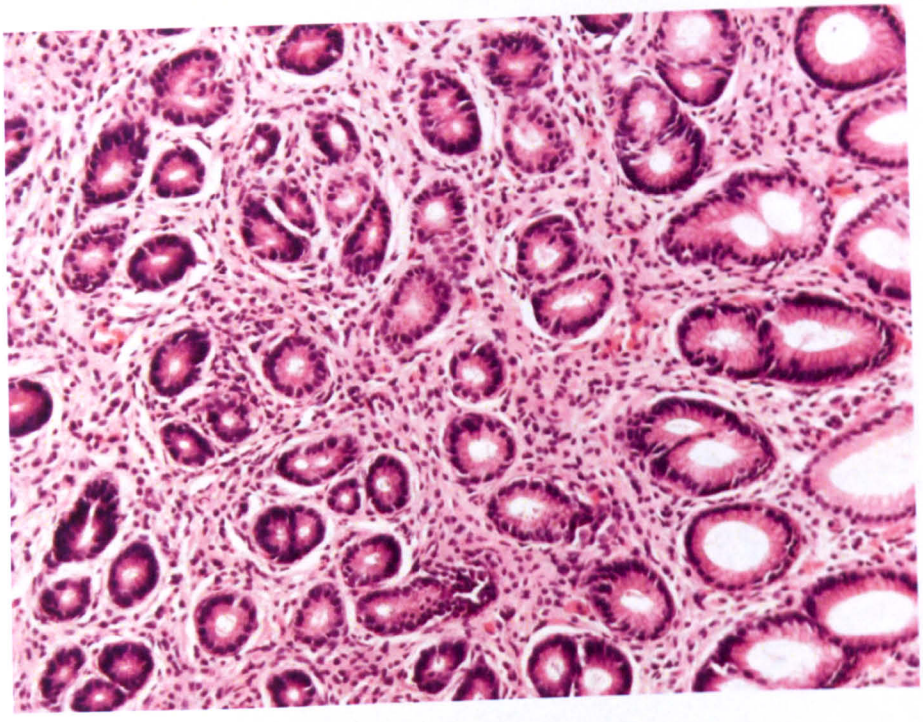
A.



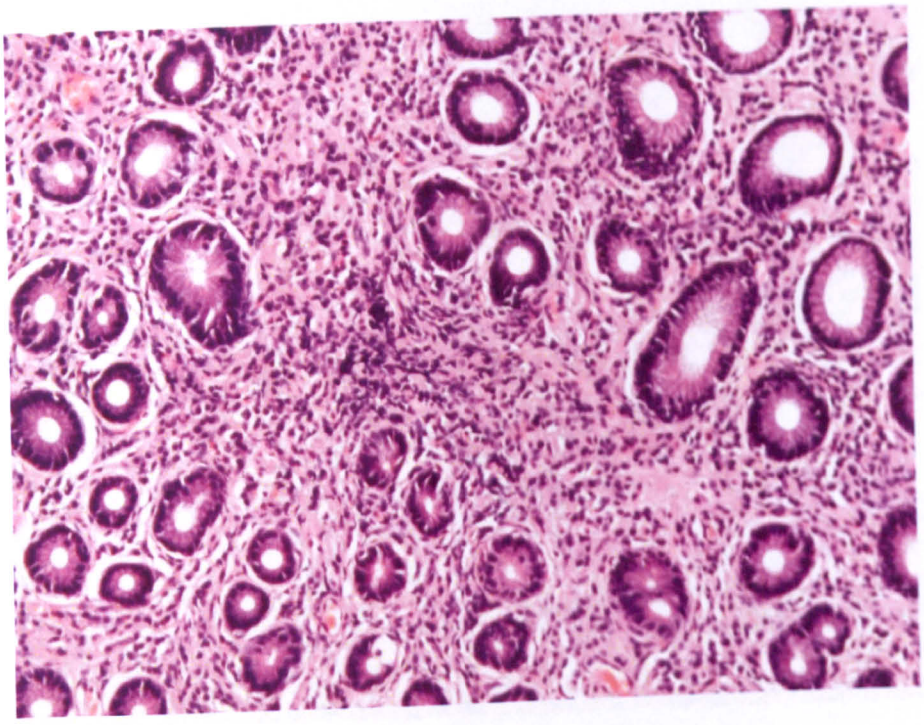
B.



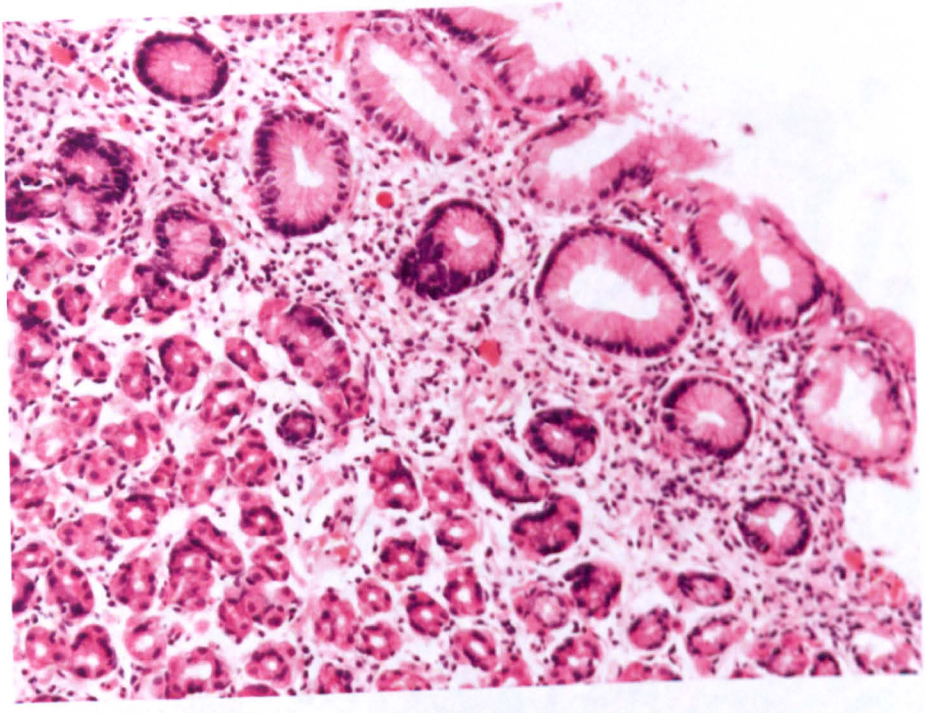
C.



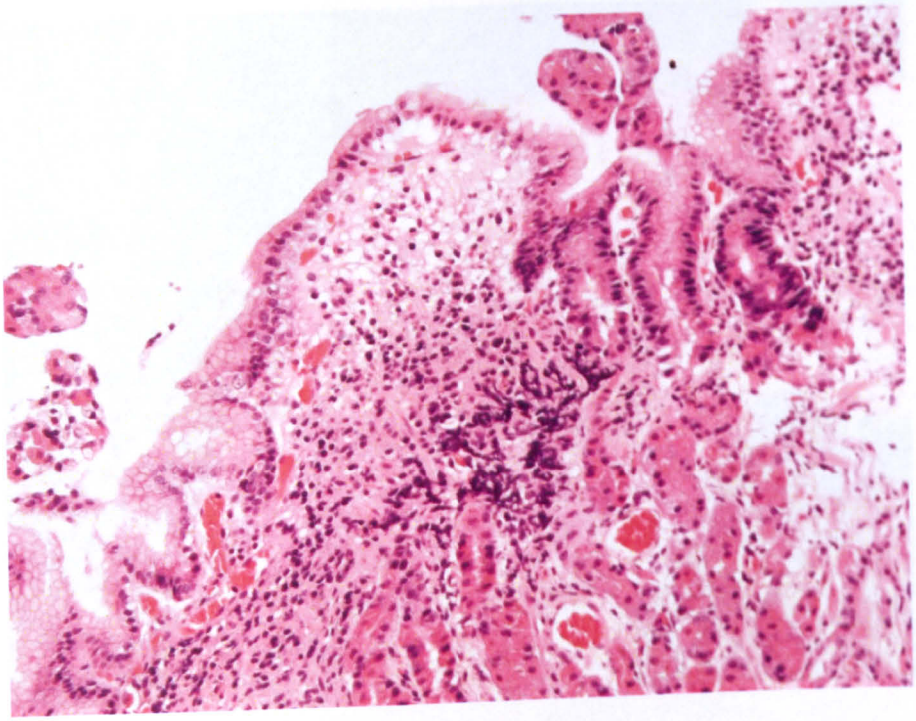
D.



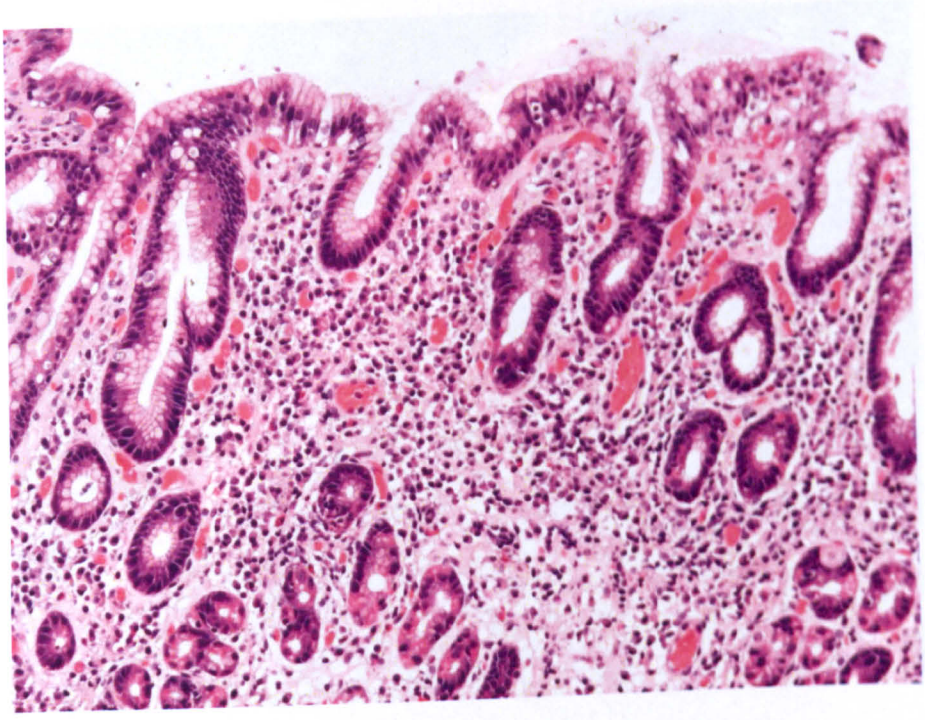
E.



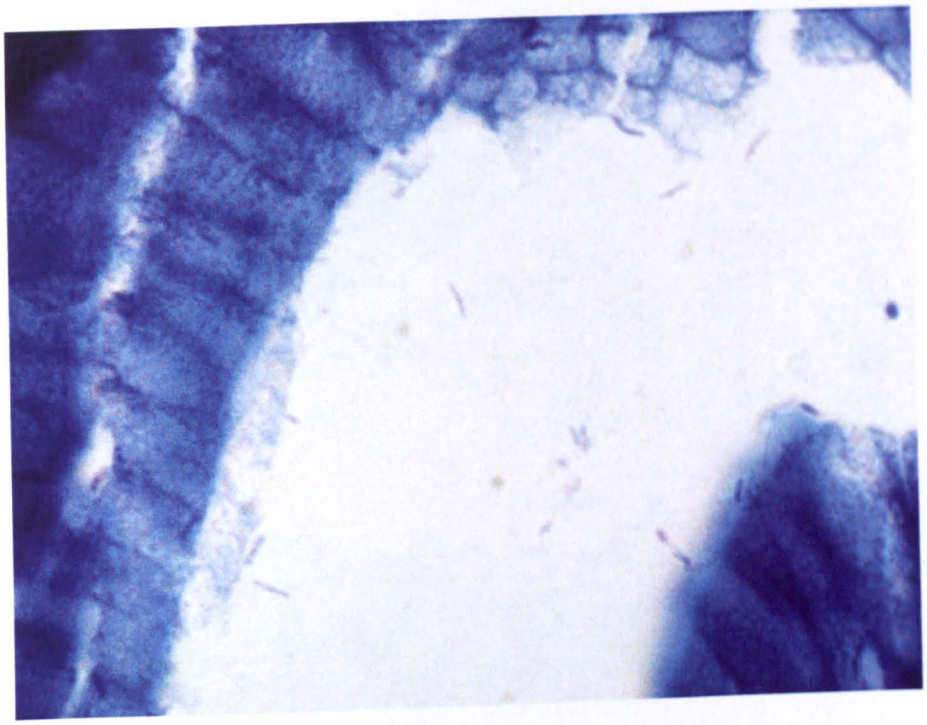
F.



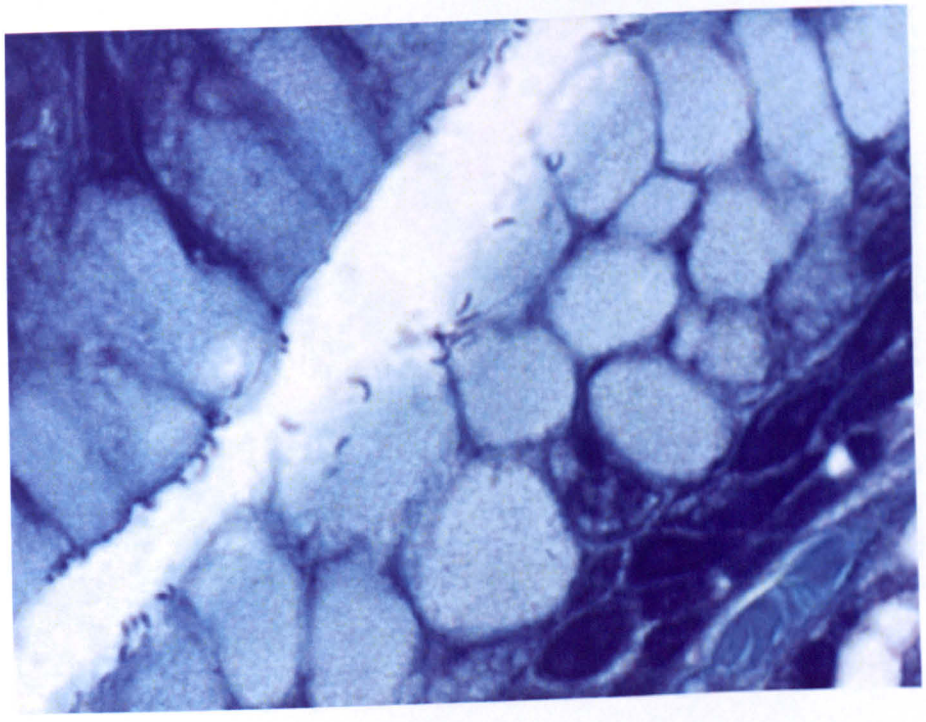
G.



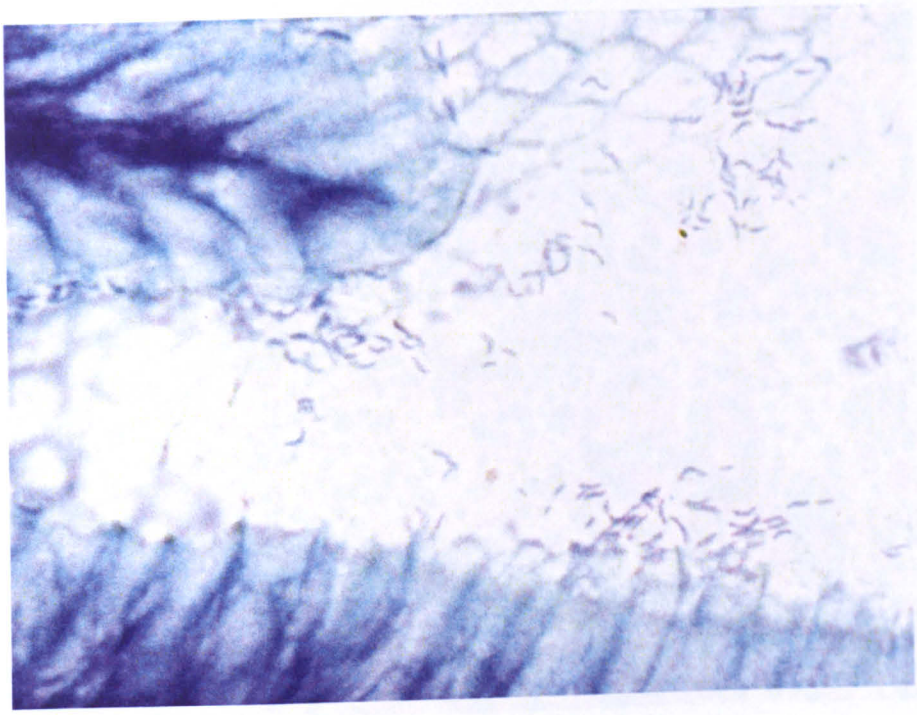
H.



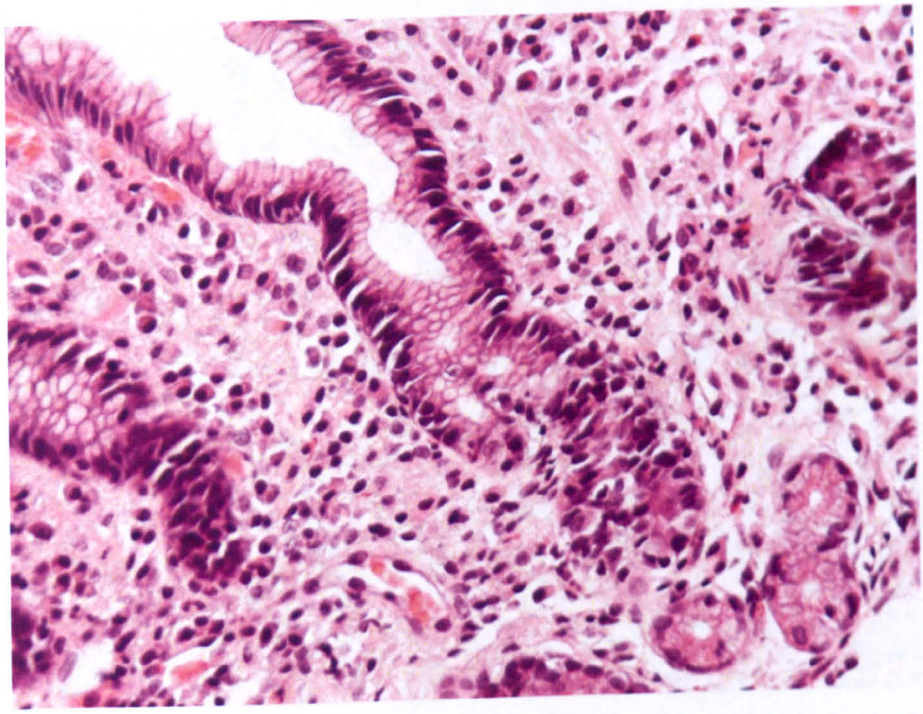
I.



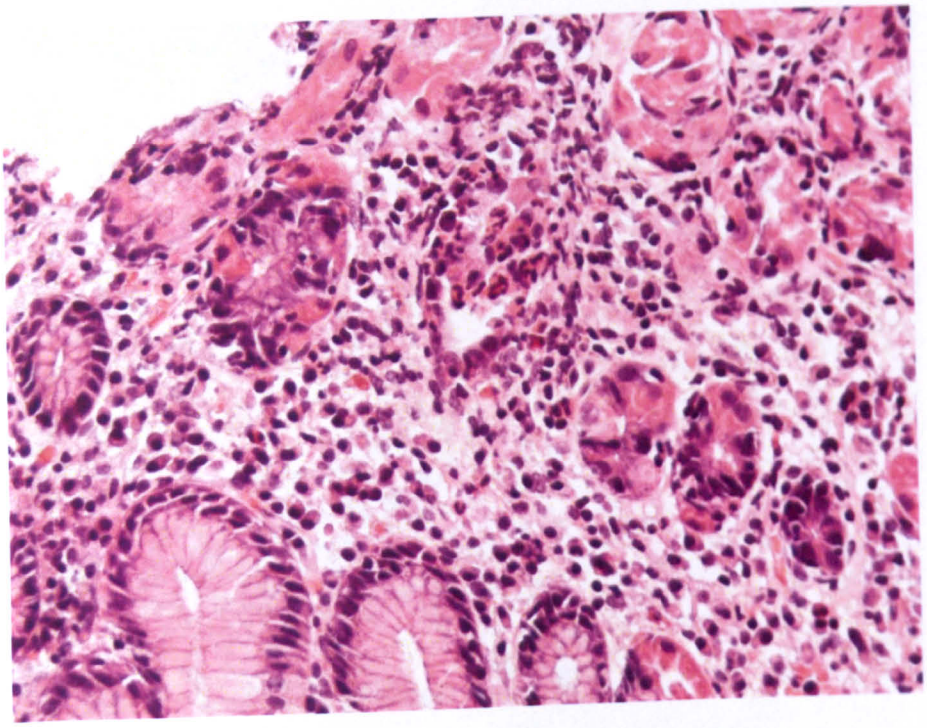
J.



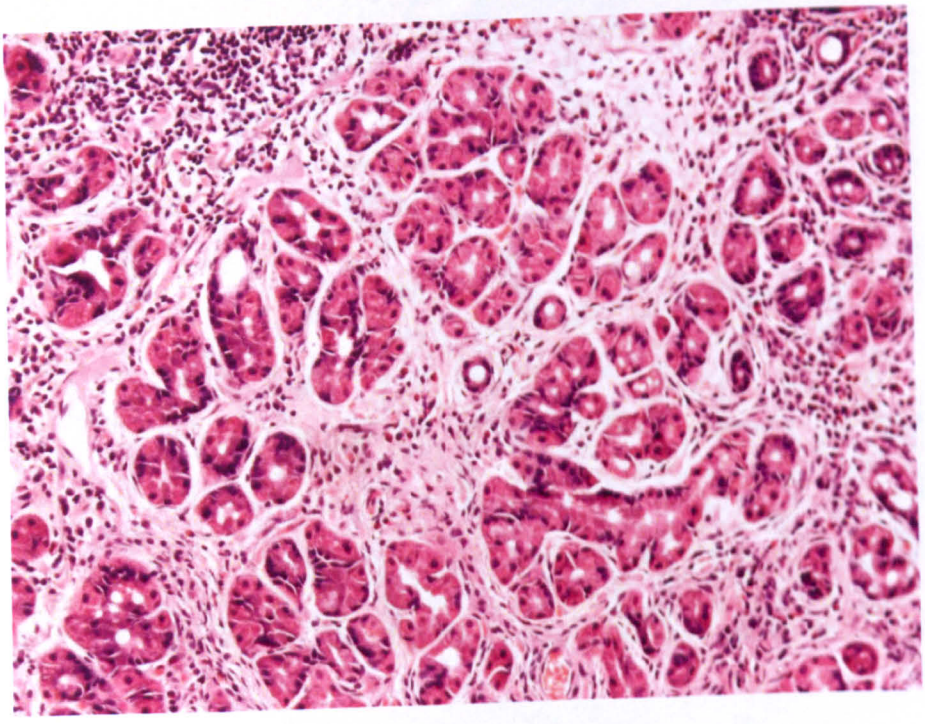
K.



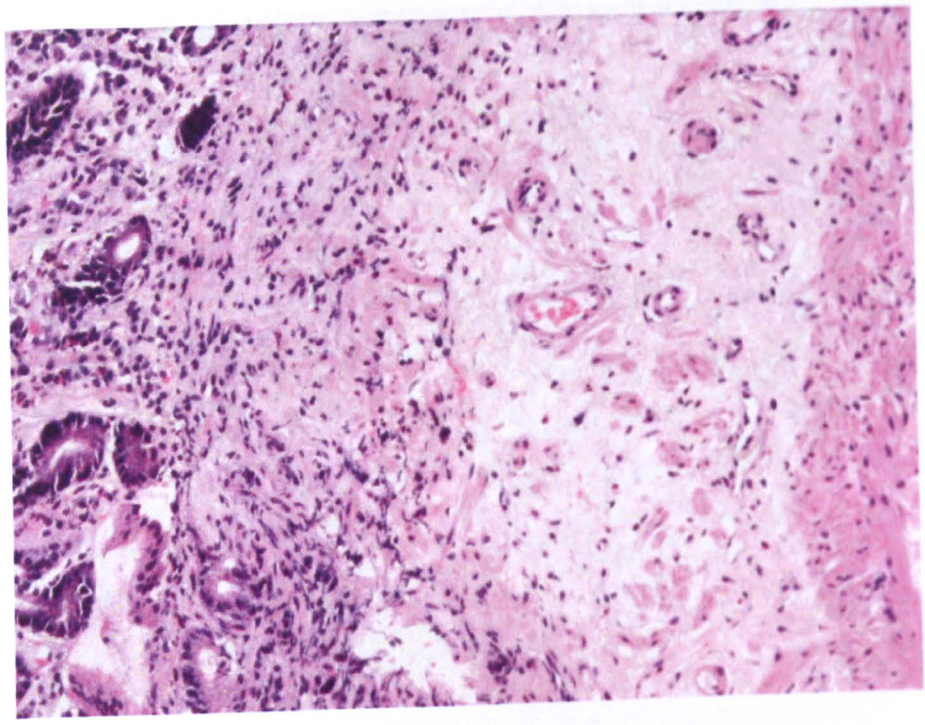
L.



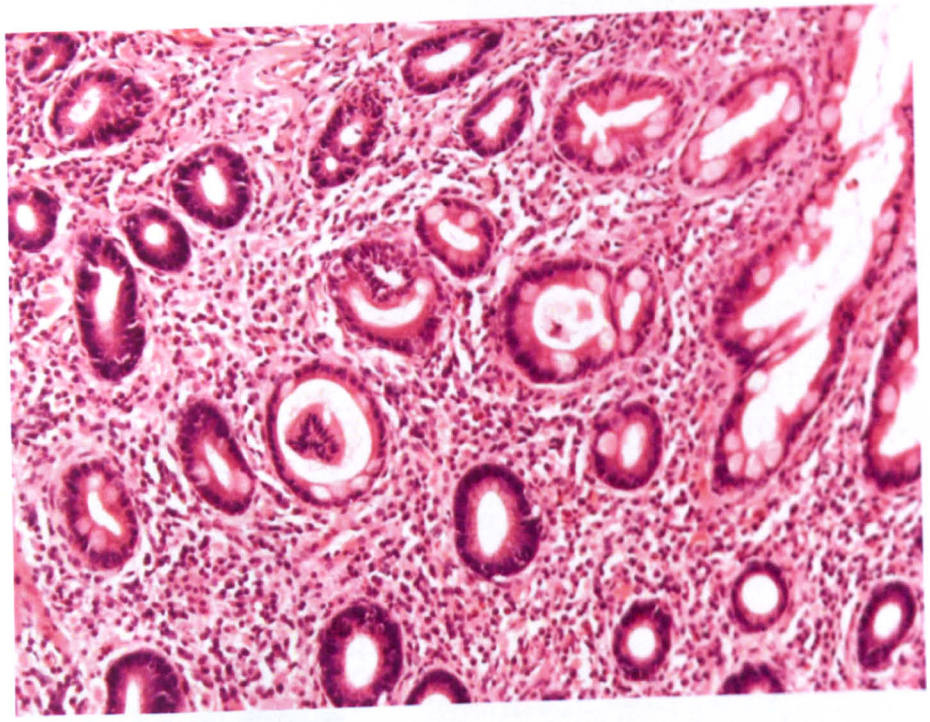
M.



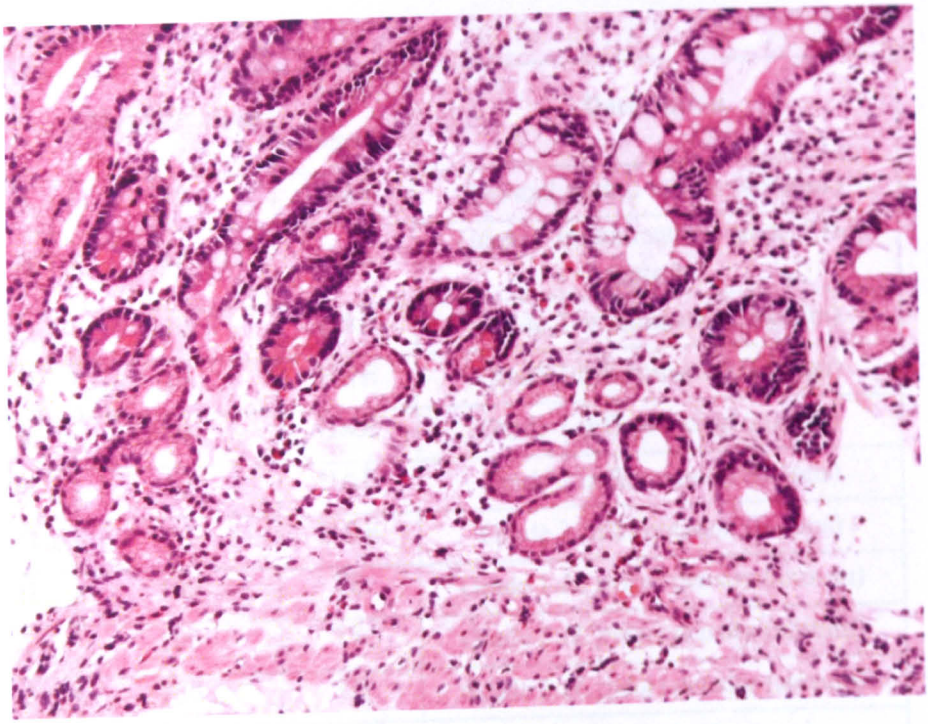
N.



O.



P.



Q.

	Magnification	Stain used	Description
A	10	H&E	Normal gastric mucosa (corpus)
B	10	H&E	Grade 1 inflammation, grade 1 IM (antrum)
C	10	H&E	Grade 2 inflammation (antrum)
D	10	H&E	Grade 3 inflammation (antrum)
E	10	H&E	Grade 1 inflammation (corpus)
F	10	H&E	Grade 2 inflammation (corpus)
G	10	H&E	Grade 3 Inflammation (corpus)
H	100	TB	Grade 1 *H. pylori* density (antrum)
I	100	TB	Grade 2 *H. pylori* density (antrum)
J	100	TB	Grade 3 *H. pylori* density (antrum)
K	20	H&E	Grade 2 activity (antrum)
L	20	H&E	Grade 2 activity (corpus)
M	10	H&E	Grade 1 atrophy (antrum)
N	20	H&E	Grade 3 atrophy (antrum)
O	10	H&E	Grade 2 IM (antrum)
P	10	H&E	Grade 3 IM and paneth cell metaplasia (antrum)
Q	10	TB	*H. heilmanni* (antrum)

Table 11.1: Details of example slides

Descriptions of the key histological findings are given for slides A-Q. Stains used include Heamotoxilin and eosin (H&E) for the visualisation of inflammation, activity, atrophy and intestinal metaplasia (IM), and Toluidine blue stain (TB) for the visualisation of *H. pylori*. Grading was base on the Sydney scoring system.

11.1 CALCULATING DNA CONCENTRATION AS GENOMES PER µL

The molecular weight (MW) of a molecule of DNA 1bp long is 678 daltons (average molecular mass).

For a DNA template (standard) S bp long, MW = $678 \times S$

Concentration of DNA template = C pg/µl

$$= \frac{C}{678 \times S} \text{ pmol/µl} \qquad \left[\text{pmol} = \frac{\text{pg}}{\text{MW}}\right]$$

$$= \frac{C}{678 \times S \times 10^{12}} \text{ mol/µl} \qquad [\text{mol} = 10^{12} \text{ pmol}]$$

$$= \frac{C \times \left(6 \times 10^{23}\right)}{678 \times S \times 10^{12}} \text{ molecules/µl} \qquad [1\text{mol} = 6 \times 10^{23} \text{ molecules}]$$

If the template only contains 1 copy of the target sequence per molecule (e.g. PCR product or plasmid), then the above equation also equates to copies/µl, i.e.:

$$\text{Concentration of DNA template} = \frac{C \times \left(6 \times 10^{23}\right)}{678 \times S \times 10^{12}} \text{ copies/µl}$$

For concentration in 'genomes' per µl, where there are n copies of the target gene per genome, divide above equation by n:

$$= \frac{C \times \left(6 \times 10^{23}\right)}{678 \times S \times 10^{12} \times n} \text{ genomes/µl}$$

This simplifies to:

$$= \frac{C}{S \times n} \times \frac{\left(6 \times 10^{23}\right)}{678 \times 10^{12}} \text{ genomes/µl}$$

$$= \frac{C}{S \times n} \times \left(8.85 \times 10^{8}\right) \text{ genomes/µl}$$

OR

$$\frac{\text{Template Concentration (pg/ul)}}{\text{Template Size (bp)} \times \text{Gene Copies per Genome}} \times \left(8.85 \times 10^{8}\right)$$