UNIVERSITY OF SOUTHAMPTON

Faculty of Medicine, Health and Biological Sciences
School of Medicine
Division of Human Genetics

Do cytokine gene polymorphisms influence the development of primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma subsequent to *Helicobacter pylori* infection?

by

Helen North

Thesis for the degree of Doctor of Philosophy

December 2006

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
SCHOOL OF MEDICINE
DIVISION OF HUMAN GENETICS

Doctor of Philosophy

Do cytokine gene polymorphisms influence the development of primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma subsequent to *Helicobacter pylori* infection?

By Helen North

Infection with *Helicobacter pylori* has been associated with several upper gastrointestinal diseases, including primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma (GML). However, most individuals infected with *H. pylori* do not develop severe disease, strongly indicating that host factors are involved in disease development. GML develops from the acquired extra-nodal mucosa-associated lymphoid tissue that develops following chronic inflammation in response to *H. pylori* infection. In addition, the topography of inflammation is associated with clinical outcome and GML is associated with pan-gastritis of the stomach. Cytokines play a crucial role in regulating inflammation, are essential for the development of acquired MALT and modulate gastric physiological responses following infection. Therefore, cytokines play a key role in all of the pre-clinical sequelae of GML. There is individual variation in the level of expression of cytokines and some of this variation is due to polymorphisms in cytokine genes. In this case-control association study, single nucleotide polymorphisms (SNPs) in cytokine genes were investigated, to determine if host cytokine gene polymorphisms influenced the development of GML following *H. pylori* infection. In addition, the presence of the t(11;18)(q21;q21) translocation in GML is associated with adverse clinical outcome, therefore these SNPs were assessed to determine if there was an association with the presence or absence of this translocation.

The majority of DNA for the case-control association study would be extracted from an archival, paraffin-embedded, formalin-fixed, gastric biopsy. Therefore, the first phase of this project was to select the most appropriate method for genotyping SNPs using DNA derived from this source of material. Two polymerase chain reaction (PCR) genotyping methods were compared, amplification refractory mutation system – PCR (ARMS-PCR) and TaqMan® 5′ nuclease assay for allelic discrimination (5' nuclease assay). Both methods accurately assigned genotypes, however a much higher percentage of samples was successfully genotyped using the 5' nuclease method (96.1% - 99.4%) compared to the ARMS-PCR method (80.5% - 90%). Therefore, the 5' nuclease method was subsequently used in the case-control association study.

Eighteen SNPs selected from 14 cytokine genes and their promoter regions were selected for the case-control association study; IL1A-889, IL1B-31, IL1B-511, IL1B+3953, IL1RN+2018, IL4-590, IL6-174, IL8-251, IL10-592, IL12B+1188, IL18-137, IL18-607, IFNG+874, IFNGR1-56, TGFB1-509, TNF-308, TNF-376, and LTA+252. The cytokine genotype and haplotype frequencies from 206 primary GML patients were compared with 568 uncomplicated *H. pylori* positive gastritis controls collected from three European populations, UK, Germany and the Netherlands.

None of the cytokine polymorphisms investigated was significantly associated with the development of GML subsequent to *H. pylori* infection in all three European populations. In addition, there was no trend towards significance in all three populations, to indicate that any of the SNPs investigated were associated with the development of disease. Therefore, the SNPs investigated do not have a major influence in the development of GML subsequent to *H. pylori* infection. This indicates that the pro-inflammatory cytokine polymorphisms that favour the development of distal gastric adenocarcinoma do not appear to have a major influence in the development of GML subsequent to *H. pylori* infection. This might indicate that the aetiology of *H. pylori* associated gastric adenocarcinoma and GML are different.

List of contents.

List of figures.

List of tables.

Acknowledgements.

I would very much like to thank my supervisors, Dr. Martin Howell, Dr. John Holloway and Dr. Adrian Bateman, without whose expertise, persistence and kindness, this thesis would never have been completed. I would also like to thank the Wessex Cancer Trust, who kindly funded this study. I had the great pleasure of collaborating with Professor Daphne de Jong from the Netherlands Cancer Institute, Amsterdam, Professor Andreas Neubauer from the Phillips University, Marburg, Germany and Dr. Andrew Wotherspoon from the Royal Marsden Hospital, Sutton, UK, who very kindly donated not only samples for this study, but also their enthusiasm and intellect. Under the guidance of Professor M-Q Du and Dr. H Liu at the Department of Histopathology, Addenbrooke's Hospital, Cambridge University, I spent two very enjoyable weeks analysing samples for the t(11;18)(q21;q21) translocation. Dr N Maniatis, Division of Human Genetics, University of Southampton, kindly provided invaluable help with the haplotype analysis of the IL1 gene region. Finally, I would like to express my deepest appreciation to my husband, Tim North and my very good friend Dr. Louise Lavender, who relentlessly helped me survive the creation, duration and completion of this thesis.

Abbreviations.

AF	allele frequency
APC	antigen presenting cell
API2	cellular inhibitor of apoptosis protein 2
ARMS-PCR	amplification refractory mutation system – polymerase chain reaction
BAFF	B-cell activating factor
BCL10	B-cell lymphoma 10, an adapter protein
BCR	B-cell receptor
bp	base pair
cagA	gene encoding the cytotoxin associated antigen
CagA	cytotoxin associated antigen
cagPAI	cag pathogenicity island
CARD	caspase recruitment domain
CARMA1	caspase recruitment domain, membrane-associated guanylate kinase protein 1
CD	cluster determinant
DCs	dendritic cells
DGM	duodenal gastric metaplasia
DLBCL	diffuse large B-cell lymphoma
DNA	deoxyribonucleic acid
dNTP	2'-Deoxynucleoside 5'-Triphosphate (dATP, dCTP, dGTP, dTTP)
EDTA	ethylenediaminetetraacetic acid
EMS	electrophoretic mobility shift assay
FRET	fluorescent resonance energy transfer
G6PD	glucose-6-phosphate dehydrogenase gene
GC	germinal centre
GF	genotype frequency
GML	primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma
HCl	hydrogen chloride
H_2O	water

H. pylori	*Helicobacter pylori*
H. felis	*Helicobacter felis*
IFN	interferon
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IL	interleukin
IL1ra	interleukin-1 receptor antagonist protein
IL1RN	interleukin-1 receptor antagonist gene
kDa	kilodalton
LD	linkage disequilibrium
Le	Lewis blood group
LEL	lymphoepithelial lesion
LOH	loss of heterozygousity
LPS	lipopolysaccharide
LTα	lymphotoxin-α
M	molar
MALT	mucosa associated lymphoid tissue
MALT1	mucosa-associated lymphoid tissue lymphoma translocation protein 1, a paracaspase
MGB	minor groove binding
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
ml	millilitre
mRNA	messenger RNA
MZ	marginal zone
NADPH	nicotinamide adenine dinucleotide phosphate
NFAT	nuclear factor for activated T-cells
NF-κB	nuclear factor – kappa B transcription factor
ng	nanograms
NHL	non-Hodgkin's lymphoma
NK cells	natural killer cells
NOD	nucleotide-binding oligomerisation domain receptors
OR	odds ratio
PBMC	peripheral blood mononuclear cells

PCR	polymerase chain reaction
ROI	reactive oxygen intermediates
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
SHM	somatic hypermutation
SNP	single nucleotide polymorphism
SOM	somatostatin
STAT	signal transducer and activator of transcription
Taq	*Thermus aquaticus* DNA polymerase
TCR	T-cell receptor
TGFβ	transforming growth factor-β
Th1	T helper-1 cell
Th2	T helper-2 cell
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRAF2	tumour necrosis factor associated factor 2
UTR	untranslated region
UV	ultraviolet
vacA	gene encoding the vacuolating cytotoxin
VacA	vacuolating cytotoxin
VNTR	variable number tandem repeat

Chapter 1: Introduction.

1.1. General introduction to *Helicobacter pylori* and associated gastric diseases.

Gastric colonisation with the Gram-negative bacteria *Helicobacter pylori* (*H. pylori*) is extremely common worldwide and, by some estimates, currently affects over half the world's population (Parsonnet 1998), but historically may have infected nearly all humans (Ghose 2002; reviewed in Blaser 1998). Essentially, all infected individuals will have chronic gastric inflammation, but for the vast majority this will be asymptomatic (Bode 1998). However for a minority, infection progresses into clinically significant gastric disease (reviewed in Parsonnet 1998; Blaser 1998). Approximately 20% of infected individuals will develop peptic ulcer disease in their lifetime and between 1-3 % will develop gastric malignancies. *H. pylori* infection is acknowledged to cause duodenal and gastric ulcers, distal gastric (antral and fundic) adenocarcinoma and primary gastric extra-nodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue. The predisposition among *H. pylori* infected individuals to develop either duodenal ulcers or gastric malignancy closely correlates with the topography of gastric inflammation. Antral predominant gastritis heightens the risk of duodenal ulcers, whereas pan-gastritis is a risk factor for gastric malignancy. Indeed, patients who develop duodenal ulcers have a reduced risk of developing gastric malignancy (Hansson 1996).

The clinical outcome following *H. pylori* infection is determined by a complex interaction of environmental influences and host genetic and bacterial virulence factors. To maintain prolonged colonization of the human gastric mucosa, *H. pylori* must avoid both innate and adaptive immune responses, and during its long co-existence with humans it has evolved strategies to achieve this and persist in the presence of mild inflammation of the gastric epithelium. Severe disease might reflect loss of this balance between colonisation and mild inflammation. The continuing antigen drive, *H. pylori* immune-escape mechanisms and a pathogenic immune response are involved in the development of gastric disease. This introduction will discuss infection with *H. pylori* and factors involved in the

development of gastric disease, in particular the development of primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma subsequent to *H. pylori* infection, which for this thesis will be abbreviated to GML. The aims of this study are to evaluate the influence of host cytokine gene polymorphisms on the development of GML following *H. pylori* infection and the presence of the translocation, t(11;18)(q21;q21), which is associated with adverse prognosis in GML patients.

1.2. Identification and prevalence of *H. pylori*.

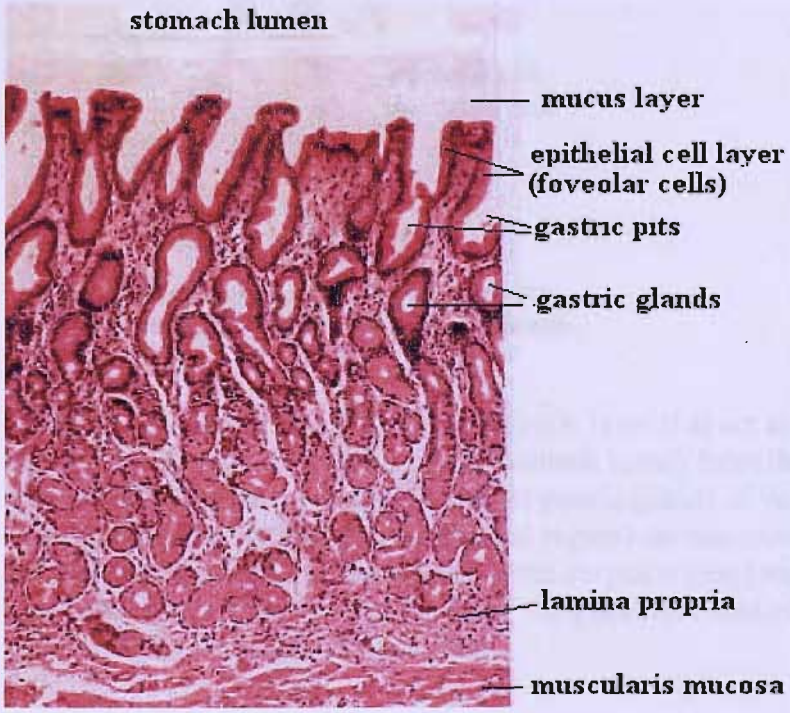
In 1984, Marshall and Warren published a paper describing a Gram-negative microaerophilic bacillus, now called *Helicobacter pylori*, that was identified in the antral mucosa of patients with chronic gastritis, duodenal or gastric ulcers.

H. pylori infection is almost always acquired in childhood (Mitchell 1992; Banatvala 1993; Cullen 1993) and usually persists for life unless specifically treated with antibiotics (Kuipers 1993). The mode of transmission of *H. pylori* infection is not known but person-to-person transmission, in particular from mother to child, seems to be the most likely route (Berkowicz 1987; Drumm 1990). The major risk factor for acquiring *H. pylori* infection is poor socioeconomic conditions during childhood (Correa 1990; Fiedorek 1991; Sitas 1991; Webb 1994; McCallion 1996). In developing countries the prevalence of infection can be 80% in children 10 years old (Perez-Perez 1990; Graham 1991; Holcombe 1992; Lindkvist 1996; Pelser 1997). In developed countries, while the overall prevalence of *H. pylori* in children is often only 10%, up to 50% of children living in poor socioeconomic circumstances may harbour the infection (Fiedorek 1991). The prevalence of *H. pylori* infection in developed countries ranges from 10% in children to 60% in 60 year olds. This increasing prevalence of *H. pylori* infection with increasing age seems to be attributable to a cohort effect. Poorer living conditions in the first half of the twentieth century, in particular, the greater density of children per household and less developed sanitation systems, is believed to be responsible for this cohort effect (Drumm 1990; Sitas 1991; Mitchell 1992; Banatvala 1993).

1.3. *H. pylori* colonisation of the stomach.

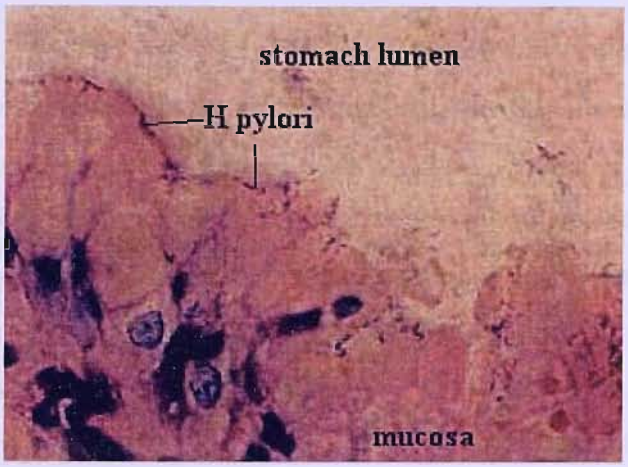
In order to colonise the stomach, the first barrier *H. pylori* must overcome is the acidic pH in the lumen of the stomach. *H. pylori* initially colonise the antral region of the stomach (fig. 1.1, 1.2 and 1.3) where the pH is higher compared to the acid-secreting corpus region. To survive, *H. pylori* produce a potent urease to split urea into ammonia and carbon dioxide, which effectively buffer the surrounding microenvironment and the bacterial cytosol (Weeks 2000). The bacteria possess flagella, which enable corkscrew motility through the mucin layer that protects the gastric epithelium, to reach the more neutral pH below the mucus. The bacteria also produce mucinase to digest the mucins. In the antral region of the stomach, the bacteria are found within the mucin layer and on the gastric epithelium surface, as well as within the upper regions of the gastric glands. Specific *H. pylori* adhesins anchor the bacteria to the mucins as well as mediating close interaction with the epithelium surface (Evans 2000). The presence of specific adhesins and the corresponding host ligand affects *H. pylori*'s ability to colonise microenvironments of the stomach (reviewed in Peek 2005).

Fig. 1.1. Normal mucosa layer of the gastric wall.



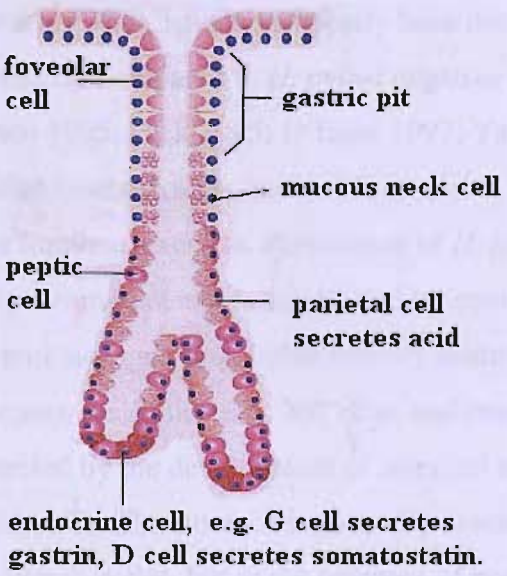
Normal mucosa from the antral region of the stomach. Adapted from Fengoglio-Preiser 1997.

Fig. 1.2. *H. pylori* adherent to gastric epithelial cells.



H. pylori associated with the epithelia cell surface of the stomach wall. Adapted from Fengoglio-Preiser 1997.

Fig. 1.3. Diverse cell types within the gastric mucosa.

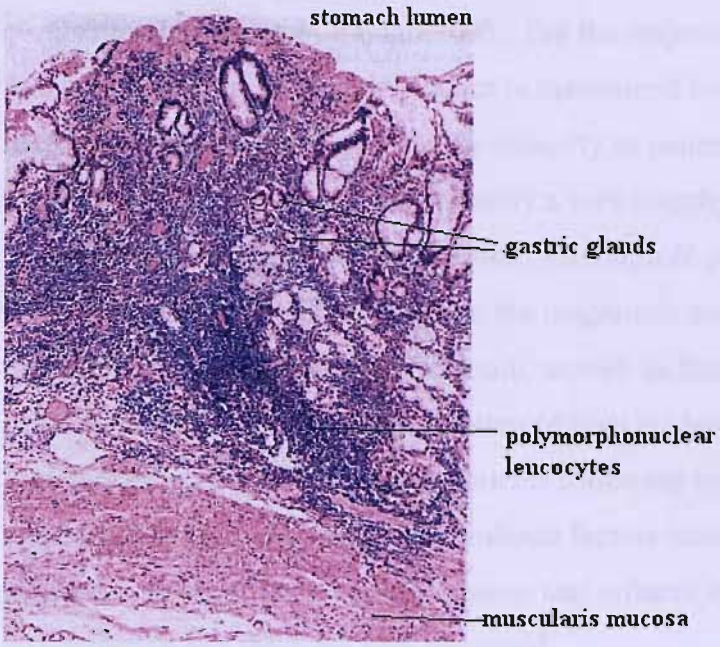


Foveolar cells (epithelial cells) form a single layer that act as an impermeable barrier, separating the contents of the stomach lumen from the layers of the gastric wall. The mucosal layer is rich in gastric glands of various types. Mucus cells (predominantly located in the antral region) secrete mucus, whereas parietal cells (predominantly located in the corpus region) secrete acid. All glands have endocrine cells at the pit of the gland that control secretion. Adapted from Fengoglio-Preiser 1997.

1.4. Immune response to *H. pylori.*

The presence of *H. pylori* initiates an acute immune response that is characterised by an influx of neutrophils into the lamina propria (fig. 1.4). The innate immune response is activated by a range of stimuli, including the Toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain receptors (NOD), which recognise pathogen-associated molecular patterns. These stimuli activate epithelial cytokine and chemokine release. The release of the chemokine, interleukin-8 (IL-8) by epithelial cells plays an important part in the initial immune response to *H. pylori*, as it is strongly chemotactic for neutrophils (Crabtree 1993). The influx of neutrophils, which in-turn can produce IL-12 in response to bacterial products, contribute to the expansion of the inflammatory cascade (Trinchieri 2003). Activation of macrophages results in the release of cytokines such as tumour necrosis factor-α (TNFα), IL-1, IL-6 and IL-12. Raised levels of interferon-γ (IFNγ), TNFα, IL-1β, IL-6, IL-8, and IL-12, but not IL-4, have consistently been detected in antral biopsies of *H. pylori* positive compared to *H. pylori* negative individuals (Crabtree 1993; Kartunnen 1995; Peek 1995; D'Elios 1997; Yamaoka 1997; Bamford 1998). This initial local cytokine 'milieu' is crucial in driving the subsequent specific adaptive immune response. Persistence of *H. pylori* infection results in chronic gastric inflammation and is manifested by continuous epithelial cell cytokine and chemokine signalling, infiltration by neutrophils, macrophages, lymphocytes, dendritic cells, NK cells, and mast cells. Chronic inflammation is characterised by the development of acquired mucosa associated lymphoid tissue (MALT). The stomach is normally devoid of organised lymphoid tissue such as lymph nodes, but in the presence of persistent infection, such as *H. pylori*, acquired MALT develops. The acquired MALT is organised into distinct B-cell areas surrounded by other haematopoietic cells such as T-cells and dendritic cells and is similar to the distinct T and B-cell areas that are seen in secondary lymphoid organs, such as lymph nodes and Peyer's patches. Cytokines, such as lymphotoxin-α (LTα), chemokines, adhesion molecules and their ligands are instrumental in the development of the acquired MALT (Pasparakis 1996).

Fig. 1.4. Acute gastritis.



Acute gastritis of the gastric mucosa showing the influx of polymorphonuclear leucocytes. Adapted from Fengoglio-Preiser 1997.

Infection with *H. pylori* culminates in a specific adaptive immune response, with the generation of local and systemic antibodies. *H. pylori* infected individuals, both asymptomatic and with ulcers, exhibit a high frequency of immunoglobulin-M (IgM) and IgA secreting plasma cells (Mattsson 1998). *H. pylori* proteins are processed and presented to CD4+ T-cells via MHC class II molecules and CD4+ T-cells specific to *H. pylori* antigens such as CagA, urease, *H. pylori* heat shock protein 60 and VacA have been identified. CD8+ T-cells represent a minority of lymphoid cells infiltrating the gastric mucosa in *H. pylori* infected individuals (Bamford 1998). However, despite the initiation of a specific adaptive immune response, the infection persists and unless treated specifically with antibiotics, *H. pylori* will continue to colonise the stomach for life. *H. pylori* appear to have achieved in the majority of individuals a balance in which the immune system is stimulated sufficiently to cause inflammation and epithelial damage at the site of infection, but without the elimination of the bacteria by the immune response. Mild inflammation presumably supports *H. pylori* survival in the nutrient-poor

environment between the epithelium and the mucus layer. To persist, *H. pylori* may be able to evade or even modulate the immune response to prevent elimination (reviewed in Baldari 2005). For the majority of *H. pylori* infected individuals this asymptomatic balance is maintained for many decades or the lifetime of the host. However, for the minority of patients who progress from infection to clinical disease it is normally a very lengthy process and usually occurs after several decades of infection. Although *H. pylori* causes acute and chronic inflammation in the stomach, the magnitude and topography of the inflammation varies from strain to strain, as well as from host to host, which is believed to arise due to genetic variation of both the bacterium and the host. Differences in clinical outcome in patients following infection are clearly related to this variation. Bacterial virulence factors have been identified, which have been associated with gastric disease and influencing the level of inflammation and these are now discussed.

1.5. *H. pylori* virulence factors.

Genetic studies have shown *H. pylori* strains are enormously diverse and certain bacterial virulence factors have been associated with both the level of inflammation as well as gastric disease. However, the identification of *H. pylori* genes predictive of the clinical outcome following infection remains to be achieved (Yamaoka 2002; Lehours 2004; Lu 2005).

1.5.1. *H. pylori* genome.

H. pylori has a circular genome of approximately 1.67 megabases (Tomb 1997) which encodes systems for motility, scavenging for iron, for DNA restriction and modification, a minimal set of metabolic genes, genes for many potential adhesins, lipoproteins and outer membrane proteins, as well as mechanisms for antigenic variation. *H. pylori* isolates from unrelated individuals have a significant amount of variation at the nucleotide level across the genome. Indeed, within an individual, the bacterial population is not entirely clonal, reflecting *H. pylori*'s particularly high mutation rate and very high recombinational frequency (Suerbaum 1998; Bjorkholm 2001). Thus, each host is colonized not by a single clone but rather by a cloud of usually closely related organisms and genetic drift occurs within the *H. pylori* populations during the

lifelong colonisation of the host (Kuipers 2000; Israel 2001). *H. pylori* is also highly competent for uptake of DNA from other *H. pylori* strains. However, comparison of the genomic sequence of two independent clinical isolates showed the overall genomic organization, gene order and predicted protein sequences were very similar with only 7% of the genes being strain-specific, and over half of these were clustered in a single hypervariable region called the plasticity zone (Alm 1999). Israel *et al* (2001) compared 15 clinical *H. pylori* isolates and showed 22% of genes were variable and strain specific. Overall, this indicates that there are conserved core genes that represent the majority of the *H. pylori* genome, which encode for metabolic and cellular processes, and a minority of strain specific genes (Salama 2000).

1.5.2. Type I and II strains.

H. pylori strains have been divided into two main groups, type I and type II strains (Xiang 1995). Type I strains possess a cag pathogenicity island (cagPAI), approximately 40kb chromosomal DNA sequence, that consists of between 27-31 genes including a type IV secretory apparatus (Censini 1996; Salama 2000). Type I strains are also characterised by the production of the vacuolating cytotoxin (VacA) and the cytotoxin associated antigen (CagA). Type II strains do not possess the cagPAI and produce little VacA. Type I strains are more virulent, are associated with strong gastric inflammation and more severe pathologies than type II strains (Gerhard 1999; Backert 2004). Serological studies of patients with duodenal ulcers and gastric malignancy show that they are significantly more often infected with type I *H. pylori* strains.

1.5.2.1. The cagPAI and the type IV Secretion Machinery.

The cagPAI contains many genes including a type IV secretion system, which in other bacteria is specialized in transferring macromolecules, such as DNA and proteins, across the bacterial membrane into host cells (reviewed in Censini 2001). The secretory system forms a needle-like structure spanning the inner and outer membranes of the bacterium. On contact with host cells the secretion system is inserted into the plasma membrane and permits transfer of bacterial protein(s) to the cytoplasm, where they modulate host cell functions. In *H. pylori* the type IV system has been shown to inject the CagA protein into

epithelial cells, both *in vitro* (Segal 1999; Odenbreit 2000), and *in vivo* (Yamazaki 2003). In addition, the cagPAI leads to secretion of cytokines and chemokines such as IL-8 by epithelial cells (Sharma 1998; Foryst-Ludwig 2000; Fischer 2001). Through transformation the entire cagPAI may be restored or lost between strains infecting the stomach (Kersulyte 1999).

1.5.2.2. CagA protein.

The strain-specific *H. pylori* gene, cagA, is located on the border of the cagPAI chromosomal region (Covacci 1993; Tummuru 1993). Presence of the cagA gene in *H. pylori* strains has been associated with an increased risk of dyspepsia (Loffield 2001), peptic ulcer disease (Cover 1990; Crabtree 1991b; Nomura 2002a) and gastric adenocarcinoma (Blaser 1995; Nomura 2002b). No homologs are known for cagA in other *Helicobacter* species or in other bacteria, suggesting that it reflects a human gastric-specific gene. The CagA protein is extremely antigenic, and antibodies to CagA are readily detected in the serum from *H. pylori* positive patients.

The exact function of CagA is unknown, but it is thought to be the first bacterial protein to be translocated into host epithelial cells by the type IV secretory apparatus and has been shown to interact with a number of host signalling molecules (Asahi 2000; Odenbreit 2000; Stein 2000). After translocation, the 128-145kDa CagA protein, which contains a variable number of tyrosine-phosphorylation sites, can be phosphorylated by host cell enzymes including members of the Src family of kinases (Segal 1996 and 1999; Odenbreit 2000; Aras 2003a; Azuma 2004). CagA recruitment of Src kinase results in the de-phosphorylation of host cell proteins (Selbach 2002; Higashi 2002; Tsutsumi 2003; Higashi 2004). Thus, CagA interacts with several of the major signal-transduction pathways present in epithelial cells and subsequently influences host cellular functions.

In individual strains, parts of the cagPAI, including the cagA gene, may be deleted. In addition, the cagA gene shows phylogeographic variation with Eastern, Western, and hybrid genotypes (Azuma 2004). DNA sequences encoding the tyrosine-phosphorylation motifs are variable in number and flanked by repetitive elements, allowing their deletion or duplication, which affects the phenotype of the injected CagA protein (Aras 2003a). The East-

Asian CagA specific sequence confers stronger SHP-2 binding activity than Western CagA specific sequence (Higashi 2002). Thus, the association of cagA with increased gastric inflammation and more severe pathologies may be dependent on cagA variants, as well as influenced by other genes within the cagPAI (Atherton 2002; Argent 2004; Azuma 2004).

1.5.2.3. VacA protein.

The vacA gene is distant to the cagPAI and encodes for the 139kDa VacA protein (Cover 1994). As with the cagA gene, no close homologs of vacA exist in other *Helicobacter* species or in other bacteria, which suggests its importance in the specific relationship of *H. pylori* with the human stomach. The active form of the VacA protein induces extensive cytoplasmic vacuolation in epithelial cells that leads to epithelial cell death (Telford 1994). Although all strains have the vacA gene only 40% of strains cause vacuolation of the epithelium. Vacuolation of the epithelium is dependent on the insertion of VacA into cell membranes to form anion-selective channels which allow nutrients and urea to pass from the epithelial cell into the extracellular space, thereby providing nutrients for the bacteria (Szabo 1999; and reviewed in: Montecucco 2003).

The vacA gene is naturally polymorphic. The two most polymorphic regions are the signal region (type s1a, s1b, s1c or s2, encodes the signal peptide and the N terminus of the mature protein) and the mid region (type m1 or m2, which encodes part of the p58 domain and is important in binding to specific epithelial cell surface receptors). All combinations of the signal and mid region occur naturally, although s2/m1 is rare (Letley 1999). *In vitro* studies using HeLa cells showed s1/m1 strains caused more extensive vacuolation than s1/m2 strains and the s2/m2 strain was non-vacuolating. Thus, the s1m1 strain is the most toxic form of VacA.

VacA has several specific effects that may contribute to *H. pylori* persistence in the gastric niche. Not only does it form pores and vacuoles in epithelial cell membranes, allowing egress of anions and urea, and thereby increasing epithelial cell turn-over (Cover 2003), VacA may also induce loosening of epithelial tight junctions, potentially allowing nutrients to cross the mucosal barrier to the gastric luminal niche of *H. pylori* (Papini 1998; Szabo

1999; Tombola 2001). This may also allow virulence factors past the epithelial layer to the underlying lamina propria.

VacA may not only target epithelial cells, but can affect other cell types including cells of the immune system, potentially inducing immune suppression. VacA blocks phagosome maturation in macrophages enabling *H. pylori* to survive phagocytosis (Zheng 2003), and selectively inhibits antigen presentation in B-cells by its effects on antigen processing compartments (Molinari 1998). VacA may also be immunosuppressive by direct action on T-cells. VacA inhibits the activation and proliferation of T-cells *in vitro.* These effects may result from the specific receptors VacA binds to on the cell surface, initiating signal transduction pathways, and/or effects on endosomal vesicles (Gerbert 2003; Boncristiano 2003; Sundrud 2004). Therefore the actions of VacA benefit *H. pylori* persistence, not only by epithelial cell damage to aid nutrient delivery from the gastric mucosa, but also by potentially modulation of the immune response.

In several Western populations, patients infected with VacA s2/m2 strains have been shown to have a much lower risk of peptic ulceration and gastric adenocarcinoma than patients infected with s1/m1 and s1/m2 strains (Kidd 1999). The s1 strain is found in >90% of patients with duodenal ulcers, whereas the s2 type is isolated from less than 10% ((Kidd 1999; Letley 2003). The s1m1 strain is detected in 89% of patients with gastric adenocarcinoma, compared to only 40% of *H. pylori* gastritis patients (Kidd 1999; Miehlke 2000). This increases to 95% in gastric adenocarcinoma patients from Japan, which might explain the high frequency of gastric cancer in this country (Zhou 2004). In contrast to this, most clinical isolates express less vacuolating or non-vacuolating forms (Atherton 1995).

1.5.3. *H. pylori* adhesins.

1.5.3.1. The Lewis b blood group antigen and BabA2 protein.

Once below the mucus, adherence of *H. pylori* to mucins and the gastric epithelial layer involves specific *H. pylori* outer membrane molecules binding to host receptors. Several epithelial structures have been implicated in adhesion, but the best-studied receptor is the Lewis blood group antigens. The bacterial gene babA encodes for an outer membrane protein (BabA) and a particular

allele, babA2, which encodes the BabA2 protein binds to the Lewis b (Le^b) blood group antigen and mediates attachment of *H. pylori* to the epithelial surface. In 1993, Boren *et al* reported that *H. pylori* bound to receptors on gastric epithelial cells that were Le^b antigen and H antigen (blood group O) positive. Since blood group A with Le^b, and B with Le^b did not bind *H. pylori*, blood group A and B individuals are less susceptible to infection. This may explain earlier observations that blood group O persons have a higher incidence of ulcers than group A and B individuals.

1.5.3.2. The *H. pylori* adhesins SabA and OipA.

SabA is an *H. pylori* outer membrane adhesin, which binds to sialyl-Lewis x antigens, which are up-regulated during chronic inflammation and found on epithelial cells and neutrophils (Mahdavi 2002). Thus, during chronic inflammation increased expression of sialy-Le^x antigens may allow greater colonisation of SabA+ *H. pylori* strains.

The *H. pylori* outer membrane protein, OipA, has been associated as a virulence factor and its expression on the surface of *H. pylori* influences the release of the chemokine IL-8 from epithelial cells *in vitro* (Yamaoka 2000 and 2004). Therefore, expression of the oipA gene may influence the severity of acute inflammation following *H. pylori* infection due to enhanced IL-8 secretion.

Expression of many outer membrane proteins, including SabA and OipA, is regulated by dinucleotide repeats in the coding regions of the respective genes. If the 'on' signal sequence is present within the gene then the protein is expressed, whereas if the 'off' sequence within the genome is present, little protein is expressed.

1.5.4. *H. pylori* neutrophil activating protein.

H. pylori infected mucosa result in an influx of neutrophils and mononuclear cells. Recruitment of these cells is due to different factors including the chemokine IL-8. *H. pylori* produces neutrophil activating protein (NAP), which is directly chemoattractant to neutrophils and monocytes (Evans 1995), and is probably one of the mechanisms responsible for the mass influx of these cells to the gastric mucosa, as it is extremely inflammatory (Dundon

2002). Neutrophil infiltration is likely to contribute to epithelial damage as recruitment activates phagocyte NADPH oxidase to produce reactive oxygen and nitrogen intermediates (ROI and RNI) (Satin 2000).

1.5.5. Linkage of virulence genes in *H. pylori* strains.

The presence of the cagA gene and the cagPAI (type I strains) is significantly associated with the presence of the babA2 allele, s1m1 alleles of vacA, and also oipA 'on' genotype. This contrasts with cagA negative strains (type II strains), 90% of which are also negative for the babA2 allele, have s2/m2 vacA alleles and have the oipA 'off' genotype. Therefore, these *H. pylori* genes are in linkage with each other. When co-expressed, the CagA, s1m1 VacA, and BabA2 proteins synergistically worsen inflammation and are associated with a higher risk of developing intestinal metaplasia in several European populations (Atherton 1995; Zambon 2003). German patients infected with *H. pylori* babA2+ strains were significantly associated with duodenal ulcers (P=0.0002) (Gerhard 1999) and gastric adenocarcinoma (P=0.03) (Rad 2002), compared with babA2- strains. In addition 'triple positive' type I strains (cagA+, vacAs1, babA2+) showed a highly significant correlation with duodenal ulcers (P=0.2x10^{-5}) and adenocarcinoma (P=0.014). This has recently been confirmed by a study of patients from Germany, Portugal and Finland (Olfat 2005). However the same group did not identify an association with babA2+ strains in Swedish duodenal ulcer patients. However, the *in vitro* assays did reveal high adhesion properties correlated with the presence of duodenal ulcers. In addition, the presence of babA2 and cagA, although present in >80% of Japanese *H. pylori* strains, did not correlate with clinical outcome (Mizushima 2001). Thus, although adherence appears to be important in clinical outcome, this may be achieved by different adhesins and host receptors.

Thus, there is clear evidence of bacterial genetic virulence factors that are associated with heightened gastric inflammation and disease development. In addition, several *H. pylori* constituents essential for colonization or virulence also have the capacity to manipulate the immune response.

1.6. *H. pylori* evasion of the immune response.

If a microbe is to persist in a vertebrate host, its biggest challenge is to avoid clearance by the immune system. Transient *H. pylori* colonization has been documented in both primates and humans (Dubois 1996; Perez-Perez 2003) implying that colonisation did not inevitably follow infection. However, eradication of the organism is believed to be a rare event once colonisation has been established. Following *H. pylori* infection, there is rapid host recognition in the form of both innate and adaptive immune responses, including the generation of specific local and systemic antibodies (Peek 1995). The lifelong colonization of the stomach by the bacteria demonstrates the effectiveness of *H. pylori* strategies to evade host immunity. An important first step is to survive without tissue invasion, and the bulk of *H. pylori* reside in the gastric lumen, beyond the reach of most host immune effector mechanisms. However, *H. pylori* can establish intimate contact with the surface epithelium, and some *H. pylori* proteins cross the epithelial barrier (Mai 1992) and both innate and adaptive immune responses are activated (Peek 1995). Although it is not able to completely avoid immune activation, *H. pylori* have evolved potential mechanisms to reduce recognition by immune sensors, suppress activation of immune cells and evade immune effectors.

1.6.1. Evasion of innate immunity.

The innate immune response can be activated by TLRs and *H. pylori* have evolved to minimize such stimulation. TLR5 recognizes bacterial flagella such as those of *Salmonella typhimurium* but it is not stimulated by *H. pylori* flagella (Lee 2003; Gewirtz 2004). TLR9 recognizes the largely unmethylated DNA of most bacteria but the highly methylated *H. pylori* DNA minimizes recognition. Some pathogenic bacteria can modify the lipid A portion of their lipopolysaccharide (LPS) to prevent stimulating the innate immune response. *H. pylori* LPS is anergic compared to the LPS of other enteric bacteria due to lipid A core modifications (Tran 2004).

Although *H. pylori* can be effectively ingested by professional phagocytes (Odenbreit 2001), *H. pylori* type I strains (CagA+ and VacA+) are more resistant to killing than other Gram-negative bacteria (reviewed in Allen 2001). Several studies have shown that *H. pylori* bacteria phagocytosed within macrophage compartments can interrupt phagosome maturation allowing the bacteria to survive phagocytosis (Zheng 2003; Chaturvedi 2004; Bussiere 2005; Allen 2005). Oxidative stress resistance is one of the key properties that enable pathogenic bacteria to survive the toxic reactive oxygen and nitrogen species (ROI and RNI) released by the host. To manage oxidative stress and contribute to successful colonization, *H. pylori*, like many other bacteria, produce enzymes that remove ROI. *H. pylori* has several enzymes such as the MdaB protein, an NADPH quinone reductase (Wang 2004), alkyl hydroperoxide reductase, thiol-specific peroxidase and bacterioferritin comigratory protein (Wang 2005), enzymes that catalyse the reduction of hydrogen peroxide and organic hydroperoxides.

However despite the above evasive mechanisms, *H. pylori* activate the nuclear factor – kappa B transcription factor (NF-κB) in epithelial cells apparently through recognition by NOD1. NOD1, an innate intracellular pathogen-recognition molecule, detects soluble components of Gram-negative bacterial peptidoglycan (Viala 2004). Although *H. pylori* are generally non-invasive, recognition by epithelial cells via NOD1 is dependent on the delivery of the peptidoglycan by the bacterial secretory type IV system, encoded in the cagPAI (Viala 2004). The resultant NF-κB induced pro-inflammatory cytokine expression is an important and continuing inflammatory stimulus.

1.6.2. Evasion of adaptive immunity.

H. pylori activates the adaptive immune system, as indicated by both humoral and cellular recognition of its antigens, although it may have evolved to substantially down-regulate and avoid adaptive immune effectors. Recognition by the adaptive immune system requires antigen presentation, and *H. pylori* interfere with both uptake and processing of antigens, partially through a VacA effect. VacA has been shown to interfere with antigen processing in B-

cells and subsequent presentation of peptides to T-cells (Molinari 1998). *H. pylori* also suppress T-cell proliferation, activation and induce selective T-cell apoptosis *in vitro* (Gebert 2003; Wang 2001, Boncristiano 2003; Gerhard 2005). The resulting defects in T-cell activation may prevent the adaptive immune response to be effective against *H. pylori*, allowing chronic colonization of the gastric niche.

Dendritic cells (DCs) may play a role in *H. pylori* potential to modulate or adapt in the presence of an immune response. C-type lectins, such as DC-SIGN, are DCs surface receptors that recognise carbohydrate structures and on binding, internalise pathogens for antigen processing and presentation to activate T- cells. DC-SIGN binds *H. pylori* strains expressing Lewis antigens (Le) (Appelmelk 2000; Bergman 2004). Studies have shown that Le+ *H. pylori* variants were able to bind to DC-SIGN and be presented on gastric DCs and this inhibited the development of a strong T helper 1 profile (Th1) (see section 1.7). In contrast, Le- variants escaped binding to DCs and a strong Th1 immune response was generated (Bergman 2004). Furthermore, the expression of Lewis antigens on *H. pylori,* as well as other surface antigens, can be switched on and off (phase-variable expression), allowing the bacteria to adapt in the presence of an immune response (Aras 2003b).

Whether the immune response is modulated by *H. pylori* and influences the clinical outcome remains to be determined. In addition to the bacterial virulence factors already discussed, inter-individual variation in the host immune response to *H. pylori* infection may also influence the clinical outcome.

1.7. Cytokines and the Th1 vs. Th2 immune response.

Cytokines modulate both the cellular and humoral arms of the immune response. Naïve CD4+ T helper cells (Th), after engagement of the T-cell receptor (TCR) by the appropriate peptide-MHC complex, can differentiate from an initial common state (Th0) into at least two functional types, Th1 and Th2, during an immune response (Mosmann 1986). These subtypes differ in the cytokines they secrete. Th1 cells secrete pro-inflammatory cytokines (IFNγ, IL-2, TNFα) that complement T-cell proliferation. This pathway is essentially cell-

mediated immunity, and will maintain a prolonged inflammatory response. Th2 cells secrete cytokines such as IL-4, -5, -13. The Th2 pathway is essentially an anti-inflammatory profile and initiates a humoral pathway, which promotes B-cell growth. Maintenance of the Th1/Th2 balance is crucial to the efficient functioning of the immune system.

The cytokines IL-12 and IL-4, acting through signal transducer and activator of transcription 4 (STAT4) and STAT6 respectively, are key determinants in the differentiation of T-cells to the Th1 or Th2 phenotype (reviewed in Murphy 2002). IL-12, and in synergy with IL-18, will polarize the differentiation of naïve T-cells to a Th1 profile secreting IFNγ and cell mediated responses (reviewed in Szabo 2003). Th1 cells express the transcription factor T-bet, which has a central role in Th1 development (Szabo 2000) and induces transcriptional competence of the IFNγ encoding gene (Mullen 2002). In contrast, the transcription factor GATA3 is crucial for inducing some of the key attributes of Th2 cells, in particular transcriptional competence of the cytokine cluster which includes the genes encoding IL-4, IL-5 and IL-13 (Zheng 1997).

Many studies indicate that the mucosal immune response to *H. pylori* infection has a predominant Th1 profile. Raised levels of IFNγ, IL-1β, TNFα, IL-6, IL-8 and IL-12 have consistently been detected in antral biopsies of *H. pylori* positive compared to negative individuals (Peek 1995). A Th1 profile, characterised by IFNγ and TNFα secreting effector cells, with high levels of IL-12, a Th1 stimulating cytokine, in the gastric mucosa contribute to this polarization of the T-cell response (D'Elios 1997). *H. pylori* specific activated T-cells from human biopsies have consistently been shown to express IFNγ but not IL-4 or IL-5 (Karttunen 1995; D'Elios 1997; Bamford 1998). In addition, increased levels of IL-18, which acts synergistically with IL-12 to induce a Th1 profile have been identified in *H. pylori* infected mucosa (Tomita 2001).

The immune response, and in particular, the severity of the gastric inflammation is believed to be a key factor in *H. pylori* pathogenesis and there are clearly distinct differences in the level and topography of inflammation between individuals in response to *H. pylori* infection. This variation may be dependent on the Th1/Th2 balance of the immune response (reviewed in Del Giudice 2001). Although it is difficult to assess whether the Th1 response

contributes to tissue injury in patients and thus pre-clinical sequelae, animal models of *H. pylori* infection clearly show that infection itself does not elicit gastric inflammation. Mice lacking an adaptive immune response show minimal pathology after infection with *H. pylori* or *H. felis*. The contribution of TNFα and IFNγ to the induction of gastric inflammation and protection from *H. pylori* infection has been studied in knockout mice (Yamamoto 2004). TNFα -/- or IFNγ -/- knockout mice had higher numbers of *H. pylori* bacteria colonising the stomach than wildtype mice, indicating that these two cytokines are important in controlling *Helicobacter* colonisation. *H. pylori* infection in IFNγ deficient mice led to decreased levels of gastric inflammation and atrophy compared with wild-type mice, and *in vivo* neutralisation of IFNγ in mice infected with *H. felis*, similarly reduced the severity of gastritis. However, TNFα -/- mice had the same degree of inflammation as wild type mice. Thus, in mice, gastric inflammation and atrophic changes are abrogated in the absence of the key Th1 cytokine IFNγ and can be induced by IFNγ infusion, even in the absence of *Helicobacter* (Smythies 2000). Indeed mice with a predominant Th1 response develop more gastric inflammation during *Helicobacter* colonization than those with a Th2 response (Fox 2000). Experiments that involved T-cell transfer between mice showed that these effects were dependent on Th1 cells (Mohammadi 1997). These studies indicate that although a Th1 response will minimise *H. pylori* colonisation, it has a key role in the level of inflammation and is likely to be pathogenic if uncontrolled.

A Th1 response needs to be modulated by a Th2 response and the anti-inflammatory effects of transforming growth factor-β (TGFβ) and IL-10. TGFβ is constitutively expressed in gastric mucosa and is a negative regulator of Th1 type immune responses. TGFβ null mice spontaneously develop gastritis and inflammatory bowel disease. In *H. felis* infected mice gastric pathologies are significantly more severe in IL-10-/- knockout mice compared to wild-type mice (Chen 2001; Ismail 2003). In addition, a more severe gastric inflammation is seen in IL-4 deficient *Helicobacter* infected mice compared to IFNγ deficient mice (Smythies 2000).

Whether re-directing the immune response towards a predominant Th2 response against *H. pylori* would provide protective immunity and reduce

pathogenic consequences is unknown (reviewed in Del Guidice 2001). However, certain strains of mice (C57/BL6) infected with *H. felis* that mount a polarized Th1 type response develop extensive gastric inflammation, whereas genetically distinct mice (BALB/c) that respond to infection with a Th2 type response develop only minimal gastritis. In humans, peptic ulceration is rare during immune suppression with cyclosporin A or pregnancy, a Th2-predominant state. Furthermore, it has been suggested that the relative scarcity of *H. pylori* associated disease in Africa despite high *H. pylori* prevalence (the "African enigma") may be due to predominant Th2 responses to *H. pylori* among black Africans (Fox 2000; Blaser 1993). This may result from co-infection with helminths generating a predominant Th2 immune response (Whary 2004 and 2005).

1.8. Immune response in development of disease

Therefore, a critical question is whether the level and type of immune response following *H. pylori* infection influences disease outcome? A strong Th1 response may minimise colonisation of *H. pylori,* but if excessive may be pathogenic leading to tissue destruction, atrophy and intestinal metaplasia. Gastric biopsies from *H. pylori* positive and negative patients had a significant correlation between in situ secretion of IFNγ and TNFα with the severity of gastritis, *H. pylori* density and epithelial cell apoptosis (Lehmann 2002). In acute inflammation rapid mobilisation of polymorphonuclear cells is essential, but in chronic infections such as *H. pylori*, persistent activation of cytokines and immune cells is likely to be detrimental. A Th1 type response would enhanced activation of macrophages and polymorphonuclear cells that would lead to greater tissue destruction through the production of reactive oxygen radicals and nitric oxide Activated neutrophils release highly toxic oxygen reactive species, which can cause a wide range of DNA damage including double strand breaks.

In addition to their role in the level and type of inflammation, gastric mucosal cytokines appear to be important in disease pathogenesis by modulating gastric physiological responses. Infection with *H. pylori* is associated with increased gastrin and decreased mucosal somatostatin secretion, changes which are reversed following *H. pylori* eradication. The

resultant hypergastrinaemia associated with infection appears to be due to cytokine action on the endocrine cells releasing these hormones. Cytokines, such as IFNγ, IL-1β and TNFα, influence the release of gastrin from G cells, whereas IL-4 influences somatostatin secretion from D cells (Zavros 2005).

1.8.1. Gastric acid secretion.

The predisposition among *H. pylori* infected individuals to develop duodenal ulcers or gastric malignancy closely correlates with the topography of gastric inflammation. Antral predominant gastritis heightens the risk of duodenal ulcers whereas pan-gastritis is a risk factor for gastric adenocarcinoma and GML. Gastric acid secretion is crucial in determining the anatomical distribution of *H. pylori* infection (Uemura 2001), and gastric acid secretion is itself modulated by cytokines. High acid production by parietal cells in the corpus mucosa probably protects it from colonisation, and the mucus secreting antral region is the site of *H. pylori* infection initially. Antral *H. pylori* gastritis is associated with up-regulation of gastrin, the acid stimulatory hormone, secreted from G-cells within the glands of the gastric mucosa, and down regulation of somatostatin (SOM), an inhibitory gastrointestinal hormone, normally secreted from D-cells located within the gastric mucosa. This results in a net increase in acid secretion. The amount of acid produced is then dependent on the health of the corpus and the parietal cell mass. In hosts with a lower acid secretory capacity, *H. pylori* is able to colonise a wider niche than is possible in higher acid producing hosts. Colonisation of a wider niche leads to further reduction in acid production as the corpus becomes hyperplastic and eventually atrophic with the loss of acid secreting glands.

In patients with duodenal ulcers, the *H. pylori* gastritis is usually restricted to the antral region with only very low-grade corpus *H. pylori* gastritis. These patients retain normal (or high) acid secretion and have a large parietal cell mass that is relatively *H. pylori* free. The high acid production is associated with duodenal gastric metaplasia (DGM), a protective mechanism against the continual delivery of increased acid to the duodenum. The presence of DGM in the duodenum allows *H. pylori* to colonise this region, further weakening the mucosa here, which together

with high levels of acid, results in the development of ulcers. These patients very rarely develop gastric adenocarcinoma (Hansson 1996). This contrasts with patients with extensive corpus gastritis, who are more likely to develop hypochlorhydria and gastric atrophy, which are precursors for the development of gastric adenocarcinoma (Correa 1976; Reviewed in Meining 2001).

1.8.2. The role of cytokines in the development of gastric adenocarcinoma.

The level of gastric acid secretion is regulated by cytokines. Cytokines, such as IL-1β, TNFα, IFNγ and IL-4 affect the hormone secretory cells of the stomach, which subsequently regulate gastric acid production (Zavros 2003). Treatment of mice with IFNγ, a Th1 cytokine, was sufficient to induce gastritis with concomitant increase in gastrin secretion and decrease somatostatin levels, both of which are hallmarks of *H. pylori* gastric pathogenesis. In contrast, the Th2 profile cytokine, IL-4, increased somatostatin levels and effectively suppressed gastrin expression and secretion. Zavros *et al* (2003) demonstrated that pre-treatment with IL-4 prevented the development of gastritis in infected wild-type but not in somatostatin null mice. Immunofluorescence showed the presence of IL-4 receptors on the gastric somatostatin secreting cells. In addition, IL-4 stimulated somatostatin release from primary D cell cultures. Treatment of mice chronically infected with *H. felis* for two months with the somatostatin analogue octreotide resolved inflammation. Therefore, these experiments identified a mechanism by which IL-4 resolves inflammation in the stomach by stimulating the release of somatostatin from gastric D cells.

IL-1β is up-regulated in the presence of *H. pylori* and is a pro-inflammatory cytokine, important in initiating and amplifying the immune response to infection. IL-1β is also the most powerful known inhibitor of gastric acid secretion. El-Omar *et al* (2000) have studied several polymorphisms in cytokine genes in relation to gastric adenocarcinoma. Polymorphisms within the IL1 gene cluster on chromosome 2q13-14 (IL1B-31C and IL1RN*2 alleles) that are believed to enhance production and functional activity of IL-1β were associated with an increased risk of both

hypochlorhydria and gastric adenocarcinoma (El Omar 2000). In addition, gene polymorphisms that are believed to affect the expression levels of TNFα, IL-4, IL-6 and IL-10 cytokines were investigated in a US group of gastrointestinal cancer patients (El-Omar 2003). Genotypes believed to enhance TNFα production (TNF-308A allele) and a haplotype believed to confer low expression of the anti-inflammatory cytokine IL-10 (IL10 -1082A, -819T, -592A haplotype) were associated with more than a doubling risk of developing gastric adenocarcinoma. Indeed, carriage of multiple pro-inflammatory polymorphisms of TNF, IL1B, IL1RN, and IL10 conferred greater risk (OR of 2.8 for one, 5.4 for two, 27.3 for three or four high risk genotypes). This indicated that, in addition to acid secretion as previously discussed, a genetic pro-inflammatory cytokine profile increases the risk of distal gastric adenocarcinoma in response to *H. pylori* infection. In addition, these polymorphisms were not associated with other upper gastrointestinal cancers, such as squamous cell carcinoma and adenocarcinoma of the oesophagus and cardia gastric adenocarcinoma.

This introduction has so far discussed the influence of *H. pylori* and the host immune response in the development of gastritis, peptic ulcers and gastric adenocarcinoma. The remainder of this introduction will discuss the development of GML subsequent to *H. pylori* infection.

1.9. Primary gastric extra-nodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (GML).

1.9.1. GML and association with *H. pylori* infection.

Development of GML is closely associated with *H. pylori* infection. Patients are significantly more likely than matched controls to have evidence of previous *H. pylori* infection (Parsonnet 1994) and the presence of the organism can be identified in the gastric mucosa in 92% of patients with GML (Wotherspoon 1991). In addition, low-grade lymphomas have been shown to regress following eradication of *H. pylori* (Wotherspoon 1993). Indeed, eradication with antibiotics results in approximately 70% of low-grade GML

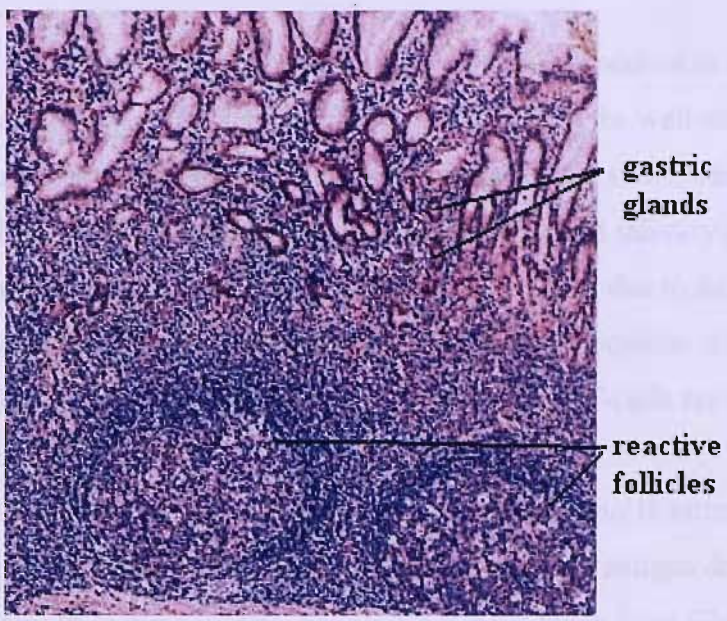
regressing. Eradication of *H. pylori* infection is by triple therapy comprising an acid reducing agent (omeprazole or lansoprazole) and two antimicrobial drugs (clarythromycin and amoxycillin or metronidazole), given for one or two weeks. Eradication is usually achieved after one or two courses of treatment.

1.9.2. Diagnosis of GML.

Compared to the other gastric diseases already discussed, GML is rare, with only 0.71 cases/100 000 per year (Severson 1990). Invariably the lymphoma occurs in adults (median age 61 years), with a male to female ratio of 1:1.2. GML is a neoplastic clonal B-cell population with the immunophenotype CD79a+, CD20+, CD5-, CD10-, CD23-, CD43-/+, and CD11c-/+. The neoplastic B-cells usually express surface IgM but not surface IgD. Rearrangement and somatic hypermutation of the immunoglobulin genes has occurred and the cells express the marginal zone cell associated antigens CD21 and CD35. Thus, the normal equivalent of these tumour cells is believed to be marginal zone B-cells (Suarez 2006). Marginal zone B-cells are found within the marginal zone of lymphoid organs such as the spleen, lymph nodes and MALT with infection. In MALT lymphoma the marginal zone is expanded and surrounds the germinal centre and the neoplastic cells extend into the adjacent mucosa and invade the glandular epithelium.

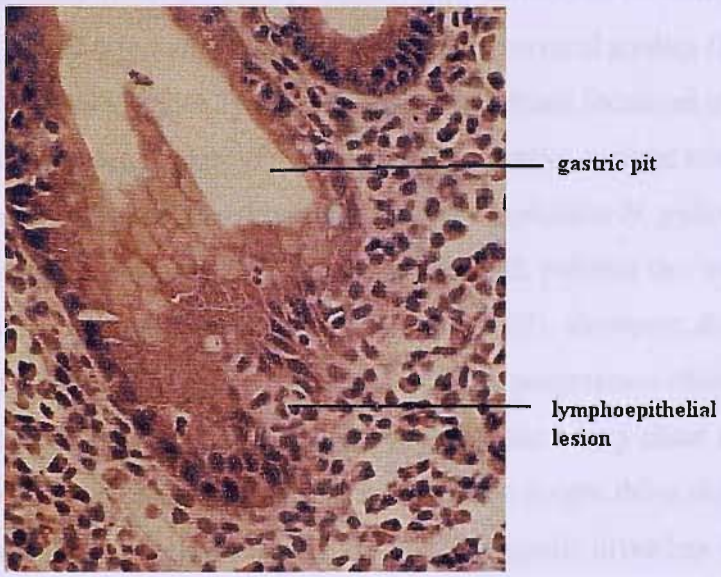
Low-grade lymphoma is diagnosed by the presence of a dense lymphoid infiltrate of centrocyte like cells in the lamina propria of the gastric mucosa (fig. 1.5), multiple lymphoepithelial lesions (LEL are defined as aggregates of three or more marginal zone cells with distortion or destruction of the epithelium) (fig. 1.6), loss of gastric glands by the lymphoid infiltrate and moderate atypia. Diagnosis is based on the histology and immunophenotype from a gastric biopsy. In the gastric mucosa the tumour cells are present with a heterogeneous population of T and B-cells. The tumour infiltrate is located in the marginal zone of reactive B-cell follicles of MALT tissue and extends into the interfollicular region. Tumour cells also infiltrate the gastric epithelium and may over run the germinal centres (called follicular colonisation).

Fig. 1.5. Gastric extra-nodal marginal zone B-cell (MALT) lymphoma.



The tumour cells surround reactive follicles and infiltrate the mucosa. Adapted from Jaffe 2001.

Fig. 1.6. GML characterised by the presence of LELs.



Lymphoepithelial lesion. Adapted from Jaffe 2001.

1.9.3. The immune response to *H. pylori* infection and the development of GML.

GML develops from the acquired MALT produced in response to *H. pylori* infection. MALT lymphomas rarely arise in the well-developed MALT of the gastro-intestinal tract e.g. Peyer's patches of the small intestine, and are usually seen in the stomach, thyroid gland, orbit and salivary gland where the original MALT is poorly developed, but is acquired due to an infection. GML always contain reactive T lymphocytes in close association with the malignant B-cells, and both non-malignant CD4+ and CD8+ T-cells are seen mixed with the tumour B-cells.

It seems that the immune response to *H. pylori* is intimately involved in the development of GML. Development of GML is antigen driven, requiring T-cell help. In *in vitro* studies, neoplastic B-cells taken from GML patients proliferated in response to strain specific *H. pylori* (Hussell 1993). This was also dependent on the presence of autologous T-cells, which needed to be in contact for the neoplastic B-cells to proliferate and this was abrogated if antibodies to CD40 ligand were present (Hussell 1996). Although the tumour B-cells were *H. pylori* sensitive their immunoglobulin specificity was shown to be autoimmune (Greiner 1998). Indeed, auto-antibodies probably as a consequence of *H. pylori* infection have been detected by several studies (Negrini 1996; Crabtree 1993; Faller 1997). GML tend to remain localised in the stomach and this may be dependent on the need for continuous antigen stimulus and/or help provided by the acquired lymphoid tissue, especially *H. pylori* specific tumour infiltrating T-cells. In 70% of low-grade GML patients the lymphoma regresses following *H. pylori* eradication (Isaacson 2005). However, the presence of malignant B-cells can still be detected using polymerase chain reaction to identify the presence of clonal immunoglobulin heavy chain (IgH) genes. It is believed that the *H. pylori* specific T-cells no longer drive the proliferation of the malignant B-cells, once the *H. pylori* antigenic drive has been removed. Therefore, malignant B-cells may be in a dormant phase, but are still polymerase chain reaction positive for clonal IgH genes. Re-infection with *H. pylori* tends to result in a relapse of the lymphoma.

1.9.4. Identification of translocations in GML patients.

Certain genomic abnormalities have been detected in GML cases and although their exact role in the development of disease is not known they are reviewed next.

The t(11;18)(q21;q21) is the most common translocation detected in low-grade GML. This translocation results in the fusion of the amino terminal of the API2 gene (chromosome 11q21) to the carboxyl terminal of the MALT1 gene (chromosome 18q21) and generates a fusion product (Dierlamm 1999). The API2 gene is believed to be an apoptosis inhibitor and inhibits caspases 3,7 and 9. MALT1 is involved in the antigen receptor activation of the transcription factor complex, NF-κB. Wild-type API2 or MALT1 are incapable of activating NF-κB, however, the fusion product is a potent activator (Uren 2000; Lucas 2001). Approximately 30% of patients with low-grade GML do not respond to *H. pylori* eradication as a therapy to initiate lymphoma regression. H. Liu *et al* (2001) assessed whether the presence of the t(11;18)(q21;q21) translocation predicted lymphoma resistance to *H. pylori* eradication. They screened 10 responsive and 12 non-responsive low-grade GML patients to *H. pylori* eradication treatment. Nine of the 12 non-responders had the translocation, whereas this translocation was not detected in any of the responders. Therefore, t(11;18)(q21;q21) translocation-positive GML patients correspond to cases that do not respond to *H. pylori* eradication (Liu 2002a). When GML invades deeper layers of the gastric wall and disseminates to local lymph nodes and distal sites, the tumour loses its dependency on *H. pylori* specific T-cells and no longer responds to *H. pylori* eradication. Therefore, the t(11;18)(q21;q21) translocation may support increased B-cell survival and decreased dependency on autologous T-cell help, thereby enabling metastasis. The presence of this translocation can therefore be used as a diagnostic marker for adverse clinical features of low-grade GML. In addition, the presence of the t(11;18)(q21;q21) translocation is significantly associated with infection by *H. pylori* CagA positive strains. Fourteen out of fifteen (93%) t(11;18)(q21;q21) translocation-positive GML patients were infected with CagA positive strains compared to 14/27 (51.9%) translocation-negative cases ($P< 0.01$) (Ye 2003). This translocation has only been detected in MALT lymphomas, and predominantly from lymphomas

arising at sites of the lung and stomach (Ye 2003). In addition, in t(11;18)(q21;q21) translocation-positive cases no other chromosomal abnormalities have been identified.

Two other translocations have been identified in MALT lymphomas, t(1;14)(p22;q32) and t(14;18)(q32;q21) translocations. The t(1;14)(p22;q32) translocation occurs in around 1-2% of *H. pylori* associated GML cases and causes the deregulation of BCL10 (chromosome1p22). BCL10 specifically relays the antigen receptor signalling to the NF-κB pathway (Ruland 2001). The t(1;14) and t(11;18) translocations in GML cases appear to be mutually exclusive. The t(14;18)(q32;q21) translocation involving the MALT1 gene has been described in MALT lymphomas arising in the lung and eye (Streubel 2003), although this translocation may be rare in lymphomas arising in the stomach.

1.10. Cytokines: A role in the development of GML?

Gastric inflammation and acquired MALT are a prerequisite for the development of GML. Therefore, factors involved in the initiation and regulation of the inflammatory response may confer susceptibility to or protection from this lymphoma. As already discussed, cytokines play a crucial role in regulating inflammation, are essential for the development of acquired MALT and modulate gastric physiological responses following infection. In addition, the topography of inflammation is associated with clinical outcome and GML is associated with pan-gastritis. Cytokines play a key role in all of these pre-clinical sequelae and therefore, are potential candidates for correlating with the development of this disease. There is individual variation in the level of expression of cytokines and some of this variation is due to polymorphisms in cytokine genes. Inter-individual differences in the level of cytokine expression may lead to differences in the modulation of an immune response to an infection.

1.10.1. Polymorphisms in cytokine genes.

The inflammatory response may be genetically programmed with some people having a very vigorous response and others a more measured response to

the same stimulus. Indeed, Sorensen *et al* (1988) demonstrated that adopted individuals carried a greater risk of death from infectious causes that was equivalent to that of their natural, rather than adoptive parents. Thus, they established that death in adults from infectious causes had a genetic background and the genetic variation known to exist in the immune system may have a role.

Cytokine genes and their receptors are polymorphic, although the exon sequences are usually highly conserved (notable exceptions include TGFB1 and TNFRII (p75) genes). The majority of known polymorphisms are found in the upstream promoter sequences, in introns and in 3' untranslated regions (UTR) which in many cases results in differential *in vitro* expression of the respective pro- or anti-inflammatory cytokine e.g. TNFα (Wilson 1997), IL-1β (El-Omar 2000; Rad 2004) and IL-10 (Turner 1997; Eskdale 1998; Rad 2004). However *in vivo*, the situation is certainly more complex and may be influenced by both the stimulus and the cell type (Kroeger 2000; Allen 1999; Gibson 2001). Polymorphisms in the human IL1A, IL1B, IL1RN (IL-1 receptor antagonist), IL4, IL6, IL8, IL10, IL12, IL18, IFNG, TGFB1, LTA and TNF genes have all been reported to influence cytokine expression. For example, IL-10 is an anti-inflammatory cytokine that inhibits transcription and production of IL-12, TNF, major histocompatibility complex (MHC) expression, co-stimulatory molecules and reduced production of ROI and RNI by phagocytes. There are many polymorphisms within the promoter region of the IL10 gene, which are believed to contribute to the inter-individual variability in IL-10 expression levels. A study looking at environmental and genetic effects on IL-10 in monozygotic and dizygotic twins showed that 50% of the variation in level of expression was due to a genetic component (Reuss 2002). Studies of the IL10 gene have described at least 49 polymorphisms, only one of which causes an amino acid substitution in the IL-10 protein. However, some of these polymorphisms influence the level of protein expression. Polymorphisms within the 5'and 3' regulatory sequences may affect transcription by altering the sequence at sites where transcription factors bind, whereas intronic polymorphisms may affect mRNA splicing and rate of mRNA degradation. Several studies have looked at individual single nucleotide polymorphisms (SNPs), or haplotypes within the IL10 gene region and correlated this with expression levels. For example, SNPs at positions –1082, -819, -592 of the IL10 gene form three well-conserved haplotypes, GCC,

ACC and ATA. For the majority of *in vitro* studies the GCC haplotype is associated with 'high expression' and ATA haplotype is associated with the 'low expression' forms of IL-10. However, other polymorphic sites may in addition affect expression of IL-10 (Bidwell 1999 and 2001; Haukim 2002).

Some of these polymorphisms, considered to alter the rate of gene transcription, are thought to influence inflammatory processes in response to infectious diseases and in support of this, many of these polymorphisms show associations with susceptibility to a number of immune-mediated diseases (reviewed in: Bidwell 1999 and 2001; Haukim 2002). Currently, it is unknown if the polymorphisms associated with differential cytokine expression and the overall cytokine genetic profile of an individual results in qualitative or quantitative programming of the inflammatory response. However, there are plenty of examples in the literature that support this hypothesis. For example, associations between polymorphisms in the promoter of the TNF gene and rheumatoid arthritis (Brinkman 1997), cerebral malaria (McGuire 1994; McGuire 1999; Knight 1999) and IL10 promoter polymorphisms with systemic lupus erythematosus (Lazarus 1997; Gibson 2001) and pathogenesis to AIDS (Shin 2000). Recent reports have linked cytokine gene polymorphisms with influencing gastric inflammation following *H. pylori* infection (Rad 2004) and subsequent development of gastric adenocarcinoma (El-Omar 2000). In addition, a number of studies have reported associations between TNFα and/or lymphotoxin-α (LTα) and certain cancers, including chronic lymphocytic leukaemia (Demeter 1997), non-Hodgkin's lymphoma (NHL) (Warzocha 1998; Juszczynski 2002; Spink 2006) and breast cancer (Chouchane 1997). In cutaneous malignant melanoma, IL10 genotypes associated with low IL-10 expression *in vitro* are associated with disease susceptibility (Howell 2001).

H. pylori infection is characterised by a strong neutrophil and lymphocyte infiltration. The T helper response towards *H. pylori* is generally considered to be Th1 phenotype, leading to a cell-mediated immune response. This Th1 response may contribute to cancer development as down regulation of the Th1 response in mice with concurrent helminth infection was shown to protect against atrophy, intestinal metaplasia and invasive gastric carcinoma (Fox 2000). IL-12 is the key cytokine in directing the immune response towards a Th1 phenotype, which acts synergistically with IL-18, whereas IL-4 plays a

key role in the differentiation of T-cells into Th2 cells that promote a humoral response. Important cytokines characterising Th1 mediated immune responses are IFNγ, TNFα, IL-1β and IL-8, all being up-regulated during chronic *H. pylori* infection. IL-10 and TGFβ, which are highly expressed in the *H. pylori* infected stomach (Karttunen 1997), are important regulatory cytokines influencing the balance of the cell- mediated immune response.

For the study presented in this thesis, SNPs associated with differential expression of candidate pro- and anti-inflammatory cytokines, particularly cytokines with altered expression as a result of the immune response to *H. pylori*, were investigated in primary gastric extra- nodal marginal zone B-cell (MALT) lymphoma patients (GML) and a large *H. pylori* infected patient control group with uncomplicated chronic gastritis from three European populations. The aim of the study was to determine the influence, if any, of polymorphisms in cytokine genes on the development of GML subsequent to *H. pylori* infection. These results will be of interest in comparison with the IL-1β/IL-1 receptor antagonist results associated with gastric adenocarcinoma reported by El-Omar *et al* (2000; 2003; Furuta 2002).

Eighteen SNPs were selected from 14 cytokine genes and their promoter regions. The selection of the SNPs was based on one or more of the following criteria: the cytokine has an altered expression level in response to *H. pylori* infection; there is evidence of the SNP affecting expression levels *in vivo* and/or *in vitro*; there is evidence that the SNP is associated with an inflammatory disease. Based on these criteria, five SNPs within the IL1 gene cluster (chromosome 2q12-14) were selected; IL1A-889 (C/T), IL1B-31 (T/C), IL1B-511(C/T), IL1B+3953 (C/T) and IL1RN+2018 (T/C). Three SNPs within the TNF region (chromosome 6p21.3) were selected; TNF-308 (G/A), TNF-376 (G/A), and LTA+252(A/G). Finally, a further ten SNPs from cytokines or their receptors that modulate T helper lymphocyte responses were selected; IL4-590 (C/T), IL6-174 (G/C), IL8-251 (T/A), IL10-592 (C/A), IL12B+1188 (A/C), IL18-137 (G/C), IL18-607 (C/A), IFNG+874 (A/T), IFNGR1-56 (T/C) and TGFB1-509 (C/T).

1.10.2. The selected cytokine SNPs:

1.10.2.1. IL1 gene region (IL1A, IL1B IL1RN):

IL1A, IL1B and IL1RN are three related genes located within the IL1 gene cluster on chromosome 2q, which encode the pro-inflammatory cytokines IL-1α, IL-1β, as well as their endogenous receptor antagonist, IL-1ra, respectively. IL-1β is a pro-inflammatory cytokine and a potent inhibitor of gastric acid production. IL-1β is up-regulated in the presence of *H. pylori* and is important in initiating and amplifying the immune response to this infection. It exhibits stable expression differences among individuals and these inter-individual differences are associated with polymorphisms in the genes of the IL1 region. IL1B-31, -511 and +3953 are three SNPs located within the IL1B gene and its promoter. The IL1B-511T allele, which is in complete linkage disequilibrium (LD) with the IL1B-31C allele, is associated with increased IL-1β secretion from peripheral blood mononuclear cells (PBMC) (Hurme 1998). The –31 polymorphism involves a TATA sequence which is abolished in the C allele. El-Omar (2000) assessed *in vitro* DNA-protein binding of this polymorphism using electrophoretic mobility shift analysis (EMS). LPS stimulation of PBMC indicated that one or more proteins, presumably transcription factors, interact with IL1B-31T allele but not the –31C allele. In addition, no differences in binding activity were detected between the two alleles of the IL1B–511 polymorphism. The –31 polymorphism may be a functional polymorphism, as the T allele showed an increase in protein binding compared to the –31C allele in the EMS assay and therefore, any association of the –511 polymorphism is due to LD with the –31 SNP.

The functional impact of the IL1+3953 SNP was studied by analysing the *in vitro* stimulated PBMC IL-1β response. PBMC with 1 or 2 copies of the IL1B+3953T allele resulted in a 2- to 4-fold increase in IL-1β production when stimulated with LPS compared to PBMC that have two copies of allele C. This indicates that the IL1+3953 polymorphism may result in quantitative differences in expression levels (Pociot 1992).

The IL1 receptor antagonist gene (IL1RN) has a penta-allelic 86bp variable number of tandem repeat region (VNTR) in intron 2, of which allele 2

(IL1RN*2) was previously associated with enhanced IL-1β secretion (Hurme 1998; Santilla 1998; Hwang 2002). IL1RN+2018C allele, located in exon 2, is in near complete linkage disequilibrium (>90%) with IL1RN*2 allele (Smith 2004) and is associated with decreased levels of IL-1ra protein (Andus 1997), as well as associated with enhanced IL-1β production *in vitro* (Santilla 1998).

The IL1B-31C/IL1-511T/ IL1RN*2 haplotype has been associated with increased IL-1β expression, more severe inflammation, an increased prevalence of intestinal metaplasia and gastric atrophy in *H. pylori* chronic gastritis patients (Rad 2004). In addition, the IL1B-31C/ IL1RN*2 haplotype has been detected in several European populations to be associated with a significant increase in the risk of developing chronic hypochlorhydria and gastric adenocarcinoma following *H. pylori* infection (El-Omar 2000; Machado 2001; Furuta 2002; Zambon 2002).

A functional polymorphism has been identified in the promoter region of the IL1A gene, a C to T polymorphism at position –889. Individuals with the –889TT genotype had significantly increased levels of IL1A mRNA levels and increased protein levels in their plasma compared to individuals with the –889CC genotype. Furthermore, the T allele at –889 creates a consensus sequence for the transcription factor skn-1. Possession of IL1-889T allele was associated with early onset, juvenile rheumatoid arthritis with iridocyclitis (P=0.002, OR=6.2) (McDowell 1995) and early onset Alzheimer's disease (Dominici 2002).

1.10.2.2. IL4 gene:

The cytokine IL-4 mediates a variety of interactions among components of the immune system including inducing immature T-cells to assume a Th2 phenotype and to repress Th1-inducing signals.

A single nucleotide polymorphism, IL4-590, in the promoter of the multi-functional cytokine IL-4 affects the binding of nuclear factor for activated T-cells (NFAT), a key transcriptional activator of IL-4 in T-cells. This regulatory polymorphism influences the balance of cytokine signalling in the immune system (Rockman 2003). When Th2 cells are stimulated by antigen presenting cells (APC), signal transduction cascades lead to the

dephosphorylation and nuclear translocation of pre-existing cytosolic NFAT, as well as the transcriptional up-regulation of the AP-1 group of transcription factors. Co-operative binding of NFAT and AP1 to the IL4 promoter activates its transcription. The human IL4 promoter contains six invariant binding sites for NFAT, but because of a polymorphism at position –590 from the transcriptional start site, the –590T allele contains an experimentally verified seventh binding site. Transfection of allelic reporter constructs into cultured T-cells has shown that the presence of this seventh NFAT binding site has a synergistic affect on transcription rate. IL4–590T allele results in more than 3-fold greater expression than –590C. The influence of the site on IL-4 expression has also been confirmed *in vivo*. The additional NFAT binding site at –590T creates a hair-trigger for IL4 transcription. The resulting sensitivity is advantageous when a Th2 response is required, as when individuals are confronted with extracellular pathogens such as intestinal helminths. On the other hand, –590C is favoured when a Th1 response is required, as when individuals are confronted with intracellular pathogens, such as tuberculosis or leprosy, or when they face CXCR4 binding viruses.

1.10.2.3. IL6 gene:

IL-6 is a multi-functional cytokine with diverse functions including stimulation of the acute phase response following infection or injury, differentiation and activation of macrophages and T-cells and growth and terminal differentiation of B-cells. IL-6 is not constitutively expressed but is induced in response to several inflammatory stimuli such as IL-1, TNFα and LPS (reviewed in Terry 2000). In IL-6 knockout mice, the T-cell dependent antibody response is dramatically compromised in response to infection. Circulating levels of IL-6 are largely regulated at the level of expression due to the rapid clearance of this cytokine. Experiments in Hela cells indicate that the –180 to –123 promoter region is crucial for transcription in response to cytokines such as IL-1 and TNFα. A polymorphism at position –174 G to C appears to affect IL6 transcription. A change from -174G to C creates a potential NF-1 transcription binding site which, in Hela cells, leads to repression of gene expression. In a luciferase reporter vector transiently transfected into HeLa

cells, the -174C construct showed 0.624(+/-0.15)-fold lower expression than the -174G construct. After stimulation with LPS or IL-1, expression from the -174C construct did not significantly change after 24 hours, whereas expression from the -174G construct increased by 2.35(+/-0.10)- and 3.60(+/-0.26)-fold, respectively, compared with the unstimulated level. Plasma levels of IL-6 were also measured in 102 healthy individuals, and the C allele was found to be associated with significantly lower levels of plasma IL-6. These results suggest that there is a genetically determined difference in the degree of the IL-6 response to stressful stimuli between individuals (Fishman 1998). Furthermore, systemic-onset juvenile chronic arthritis was associated with a reduced incidence of the potentially protective IL6-174CC genotype in young patients (Fishman 1998).

1.10.2.4. IL8 gene:

H. pylori infection is characterised by a marked neutrophil infiltration and elevated gastric mucosal IL-8 protein levels have been consistently associated with active gastritis. The chemokine IL-8 is pro-inflammatory, primarily mediating the activation and migration of neutrophils into tissue from peripheral blood. Sequencing a 1.4kb promoter region directly upstream of the transcription start site of the IL8 gene in 36 healthy individuals identified a single polymorphism at position –251 (Hull 2000). Furthermore, expression levels of IL-8 from 50 healthy individuals indicated that the IL8-251A allele was associated with increased IL-8 production following LPS stimulation of PBMC compared to the IL8-251T allele (P=0.07). In addition, in 117 case-parent triad families, the presence of the A allele was significantly associated with susceptibility to severe bronchiolitis disease after infection with respiratory syncytial virus (RSV) (P=0.004) (Hull 2000). This indicates that susceptibility to severe disease may be a consequence of increased IL-8 production after infection with RSV.

1.10.2.5. IL10 gene:

IL-10 is an anti-inflammatory cytokine that down-regulates TNFα, IL-12, MHC class II molecules and co-stimulatory molecules as well as the

production of reactive oxygen and nitrogen intermediates, particularly on phagocytic cells and antigen presenting cells (APC) (reviewed in Trinchieri 2001). In part through inhibition of IL-12 and co-stimulatory molecule expression on APC, IL-10 has an overall immunosuppressive effect on the generation of Th1 responses. In addition, IL-10 profoundly affects the bactericidal activity of phagocytic cells, allowing intracellular survival of pathogens such as *Mycobacterium tuberculosis* and *Leishmania major*. Furthermore, IL-10 is essential to protect infected animals from severe inflammatory pathology. IL-10 knockout mice succumb within 1-2 weeks of infection to a systemic inflammatory response with enhanced production of IL-12, INFγ and TNFα.

As previously discussed, several polymorphisms have been identified in the IL10 gene and its promoter. Carriers of the IL10-1082G/-819C/-592C (GCC haplotype) had higher mucosal IL10 mRNA levels than ATA haplotype carriers and were associated with colonisation of more virulent cagA+ strains in *H. pylori* infected patients with chronic gastritis (Rad 2004). Carriers homozygous for the IL10 'low haplotype' ATA (-1082A/-819T, -592A) had significantly increased risks of non-cardia gastric adenocarcinoma (OR=2.5, 95% CI= 1.1-5.7) (El-Omar 2003). However, it should be noted that the -819 and –592 are in complete LD (Turner 1997).

1.10.2.6. IL12 genes:

Various factors influence the decision of a naïve T-cell to function either as a Th1- or Th2-cell, including the presence of IL-12 or IL-4 at the time of initial CD4+ T-cell activation. IL-12 is mainly produced by antigen presenting cells in response to certain bacterial and parasitic infections. IL-12 binding to its receptor, results in signal transduction cascade and nuclear translocation of the signal transducer and activation of transcription 4 (STAT4) to induce IFNγ production. IFNγ also rapidly induces the expression of T-bet, a Th1 specific transcription factor, that is thought to be a master switch controlling Th1 differentiation. IL-12 is a pro-inflammatory cytokine composed of two disulfide-linked chains, p40 and p35, encoded by IL12B and IL12A genes, respectively and located on different chromosomes (5q31-33 and 3p12-3q13.2).

The co-expression of both subunits is required to generate a biologically active heterodimer (p70), which appears to be predominantly regulated at the level of IL12B transcription (Gubler 1991). This cytokine plays a key role in the promotion of a Th1 immune response in the gastrointestinal mucosa. Enhanced IL12 mRNA levels have been reported in *H. pylori* infected individuals (Karttunen 1997; Bauditz 1999). The functional polymorphism at position +1188 (A/C) in the 3'UTR in exon 8 of the IL12B gene has been shown to be biologically relevant (Seegers 2002). Presence of the C allele was associated with increased IL-12 p70 secretion by stimulated monocytes. Furthermore, this polymorphism has been associated with disease susceptibility to several chronic inflammatory, autoimmune and infectious diseases such as insulin-dependent diabetes mellitus (Morahan 2001), multiple sclerosis (van Veen 2001) psoriasis (Tsunemi 2002) and cerebral malaria (Morahan 2002).

1.10.2.7. IL18 gene:

IL-18 is a pro-inflammatory cytokine mainly produced by activated macrophages and in synergy with IL-12 can induce IFNγ, particularly from T-cells and NK cells. Two polymorphisms have been identified in the promoter region of IL18 that have been shown to affect transcription. A change from C to A at position –607 disrupts a potential cAMP-responsive element binding site. In addition, a change at position –137 from G to C changes the H4TF-1 nuclear factor binding site (Giedraitis 2001). Expression analysis by reverse transcritase PCR showed individuals homozygous for –607C and –137G had higher levels of IL18 mRNA compared to other genotypes (P=0.17). Although this is not statistically significant, in reporter assays this haplotype also showed higher transcriptional activity (Giedraitis 2001).

1.10.2.8. IFNG gene:

A polymorphism at position +874(T/A) in the first intron of the IFNG gene has been reported (Pravica 2000). Allele T of this SNP shows an absolute correlation with allele 2 of a penta-allelic CA microsatellite polymorphism, which has been associated with high IFNγ production from PBMC (Pravica 1999). Furthermore, the +874 polymorphism in intron 1 coincides with a

putative NF-κB binding site (the consensus NF-κB binding sequence is GGGANTYYCC), which may have functional consequences for the transcription of the IFNG gene. The +874T allele creates the sequence AATCTC and EMS studies show NF-κB binding to this sequence (Pravica 2000).

1.10.2.9. IFNGR1 gene:

The release of IFNγ enhances anti-bacterial immunity. It binds to the IFNγ receptor (IFNGR), which comprises IFNGR1 and IFNGR2 chains. Binding activates signalling via Janus kinases (JAK) and activates STAT1. STAT1 homodimers enter the nucleus and bind to IFNγ activated site (GAS) promoter sequences and stimulate gene transcription of genes encoding inducible nitric oxide synthase (iNOS), an enzyme that generates NO radicals and phagocyte (NADPH) oxidase, which generates oxygen radicals. IFNγ functions predominantly on macrophages and induces a microbicidal state against pathogens.

IFNGR1 encodes the chain 1 of the IFNγ receptor. A genome wide screen of *H. pylori* positive families (Senegalese population) found an association with a 90cM region of chromosome 6 around marker D651009 (located next to the IFNGR1 gene). A multipoint LOD score of 3.1 was obtained at IFNGR1. Sequencing of the IFNGR1 gene revealed three polymorphisms, including the IFNGR1-56 T/C SNP, which was found to be associated with high antibody concentrations to *H. pylori*. The inclusion of this polymorphism in the linkage analysis raised the LOD score to 4.2. Thus, the IFNGR-56 C to T polymorphism was associated with *H. pylori* infection (Thye 2003). Furthermore, this SNP was shown to influence transcriptional activity in reporter gene assays (Rosenzweig 2004).

1.10.2.10. TGFB1 gene:

TGFβ1 negatively regulates Th1 cell development (Letterio1998; Wahl 1994) and TGFβ1 knockout mice spontaneously develop gastritis and inflammatory bowel disease (Hahm 2002; Monteleone 2001). In a study of 170 pairs of either monozygotic or dizygotic twins the heritable estimate for TGFB1

was 0.54 (95% CI=0.39-0.66) (Grainger 1999). Sequencing of the promoter region identified two polymorphisms in strong LD, -800 and –509 SNPs. Furthermore, the –509C to T polymorphism was significantly associated with TGFβ1 plasma concentration, with the -509T allele associated with higher concentrations of TGFβ1 (Grainger 1999).

1.10.2.11. TNF/LTA gene region:

Secreted or membrane bound TNFα and secreted LTα binds to TNFR55 and TNFR75 receptors to activate the TNF pathway. TNFα is a potent cytokine with a range of pro-inflammatory activities. It is produced by monocytes and macrophages although other cells such as T and B-cells can produce significant amounts. The TNF and LTA genes lie in the major histocompatibility complex on chromosome 6. TNFα has potent biological actions, and its production is tightly regulated. The level of TNFα is controlled at the transcription level by LPS and IFNγ, as well as post-transcriptional regulation due to RNA stability and translation efficiency. In response to LPS stimulation of macrophages, TNF transcription increases 3-fold, TNF mRNA increases 50- to 100-fold and protein secretion increases by a factor of 10,000-fold (Beutler 1989). Several polymorphisms have been reported in the TNF gene region and a complex pattern of polymorphisms is seen that is associated with secretion levels (reviewed in Hajeer 2001). The majority of expression studies have focused on the G/A polymorphism at position –308. In some *in vitro* studies the A allele was associated with heightened TNFα secretion. However, other studies have not found a correlation (reviewed in Allen 1999). However, the presence of high or low TNFα plasma levels is associated with disease. Inheritance of a 'low TNFα secretion' phenotype (TNF-308G) was associated with a ten-fold increased risk of fatal outcome from meningococcal disease (OR=8.9, 95% CI=1.8-45), while high IL-10 production was associated with a twenty-fold increased risk of fatality (OR=19.5, 95% CI=2.3-165) and inheritance of both conferred greatest risk (Westendorp 1997). Fatal cerebral malaria is associated with high circulating levels of TNFα, which correlates with inheritance of 'high TNFα expression' phenotype (TNF-308A; TNF-376A) (McGuire 1994; Knight 1999). Systemic release of TNFα and LTα has been found to be associated with

the severity of NHL (Warzocha 1998) and TNF/LTA haplotypes associated with development of NHL (Spink 2006). Warzocha *et al* (1998) showed patients with high circulating levels of TNFα and LTα, and high TNFα plasma levels were associated with poorer disease outcome. In 273 lymphoma patients the TNF-308A allele was significantly associated with higher plasma levels of TNFα at diagnosis. Presence of two or more TNF/LTA 'high producing' alleles (TNF-308A and LTA+252G) was significantly associated with higher rate of relapse and progression of disease (P=0.007).

Wilson *et al* (1997) have shown the TNF-308 polymorphism has a direct effect on TNF gene regulation in reporter gene assays and conclude that the TNF-308A allele may be responsible for the high TNFα phenotype and its associations with more severe disease in infections such as malaria and leishmaniasis. The TNF-308A allele in B-cell lines has higher constitutive and inducible levels of TNFα expression than presence of the TNF-308G allele. The TNF-308G allele creates a 10bp sequence homologous to the consensus binding site for activator protein 2 (AP-2), which is disrupted in the TNF-308A allele. Functional assays demonstrated that AP-2 binding represses the activity of the TNF promoter in Jurkat cell line, indicating that the TNF-308 SNP affects gene expression.

Finally, carriers of TNF-308A allele were significantly associated with an increased risk of non-cardia gastric adenocarcinoma (OR=2.2, 95% CI= 1.4-3.7) (El-Omar 2003).

Monocytes and macrophages are a major source of TNFα. Knight *et al* (1999) identified a SNP in the TNF promoter at position –376 from the transcriptional start site, that was protected from DNAse I digestion in a well-differentiated monocyte cell line. EMS assays revealed that the pattern of protein binding to this site was different between the two alleles (-376G to A). An additional 95kDa protein binds to the TNF promoter when the –376A allele is present, which is abolished when the –376G allele is present. This DNA binding protein was identified as OCT-1, a ubiquitous transcription factor with the consensus binding sequence ATGCAAAT. OCT-1 is known to interact with a broad range of transcription factors. Therefore, the –376 polymorphism as

well as altering the promoter sequence, may also be responsible for influencing the structural conformation of the region. Indeed, binding of OCT-1 to the -376A region is predicted to cause this region of DNA to adopt a higher degree of curvature. In reporter gene assays the –376A SNP showed a 35% increase in TNF expression compared to the wild-type, -376G (P=0.002). Furthermore, to determine if the –376 polymorphism exerts a functional effect *in vivo,* Knight *et al* examined the relationship of this SNP with clinical outcome of cerebral malarial infection. TNF has a pivotal role in human malaria, acting both to suppress parasitic growth as well as being implicated in the pathogenesis of cerebral malaria. Individuals with the –376A allele were increased in the cerebral malaria group compared to the ethnic-, age-, sex- and residency-matched control group with mild malaria in two different population, a Gambian study (OR=4.3, 95% CI=1.5-12.8, P=0.008) and a Kenyan study (OR=4.6, 95% CI=1.3-15.7, P=0.016) (total individuals in the two studies was 2,192 cases). Thus, the rare TNF-376A allele influences the transcriptional activity of the TNF gene.

Soluble LTα and TNFα bind the same receptors and share many biological and structural characteristics. However, there is a membrane form of LTα, when co-expressed with another protein LTβ, it forms a heteromeric cell surface, membrane bound molecule, tethered by the transmembrane region of LTβ. LTβ is not functional by itself but the LTαβ form binds to the receptor LTβR. A different function for LTαβ (referred to here as the LT pathway) has emerged that is distinct from TNF functions. To ensure T and B-cells encounter antigens the immune system has developed highly structured environments such as the lymph nodes and marginal zone of the spleen. The LT pathway can trigger the development of secondary lymphoid organs and is involved in the formation of ectopic lymphoid structures (organised lymphocytic aggregates that form at sites of chronic inflammation). Signalling through the membrane bound LT pathway is crucial in the maintenance of the organisation and architecture of the secondary lymphoid organs. Cell surface bound LT is expressed by activated lymphocytes and a subset of resting B-cells, whereas, the receptor is expressed mainly by dendritic cells, monocytes and non-

haematopoietic cells. Furthermore, inhibitors of the LT pathway reduce disease in a wide range of autoimmune diseases.

A polymorphism at position +252 in the first intron of the LTA gene has been identified. Presence of the lower frequency G allele has been shown to result in raised LTα concentrations in stimulated PBMC (Messer 1991; Knight 2003). This SNP is within a phorbol ester responsive DNA element and has affinity for the transcription factor family, AP-1, jun and c-fos.

The TNF-308A and LTA+252G alleles are in LD and both are thought to result in 'high producer' TNFα and LTα expression, respectively. In addition, these alleles are in LD with the HLA-A1, -B8, -DR3, -DQ2 haplotype. This haplotype is associated with autoimmunity as well as rapid progression of HIV infection to AIDS.

1.11. Case-control association studies.

Complex diseases do not exhibit classical Mendelian patterns of inheritance and it is harder to identify causal genes due to several factors. Disease development is likely to be influenced by multiple genetic as well as environmental factors. Currently, allelic association studies provide the most powerful method for locating genes of small effect contributing to complex diseases (Daniels 1998). Identification of a positive association of a genetic marker with a disease in several populations indicates the allele may be a causal gene or is in linkage disequilibrium with the causal gene. However, careful consideration must be given to the power and design of the study. Selection of the phenotype of cases and controls is critical to the success of identifying causal genes.

Finally, a summary of factors that may be involved in the progression to GML following *H. pylori* infection is shown in figure 1.7.

Fig. 1.7. Summary of bacterial and host factors that influence development of GML.

H. pylori infection in the stomach

- Influenced by host genetic factors e.g. blood group antigens.
- Influenced by bacterial genetic factors e.g. babA2, sabA.
- Stimulates innate and adaptive immune response.
- Acute inflammation
- Chronic inflammation
- Acquired MALT
- *H. pylori* specific antibody and T-cells generated.
- *H. pylori* evades the immune response by adapting to its environment

Degree and location of inflammation is influenced by host and bacterial genetic factors:

- Bacterial genes (cagPAI, cagA, s1m1vacA, mechanisms to modulate the immune response).
- Host genes (acid production, Th1-Th2 balance, ?cytokine genes).
- Environmental (smoking, diet).

Low grade GML develops:

- A neoplastic clonal, autoimmune B-cell.
- Dependent on presence of *H. pylori.*
- Dependent on presence of *H. pylori* specific intratumoural T- cells.
- Responds favourably to *H. pylori* eradication.
- Translocations t(11;18) or t(1;14)

H. pylori independent GML:

- Resistant to *H. pylori* eradication
- Metastasis

Aims.

The immune response to *H. pylori* infection is intimately involved with the development of primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma (GML). Cytokines, which initiate and regulate the inflammatory response, may influence the development of this disease. Single nucleotide polymorphisms (SNPs) within cytokine genes, that are believed to affect cytokine expression and the inter-individual variation in inflammatory responses, are potential candidates for influencing the development of this disease. Therefore, a case-control association study compared the cytokine genotype and haplotype frequencies from primary GML patients and uncomplicated *H. pylori* positive gastritis controls collected from three European caucasoid populations, UK, Germany and the Netherlands.

The majority of DNA for the GML patient groups and *H. pylori* gastritis control groups would be extracted from an archival, paraffin-embedded, formalin-fixed, gastric biopsy. Therefore, the first phase of this project was to select the most appropriate method for genotyping SNPs using DNA derived from this source of material. A comparison of two polymerase chain reaction (PCR) genotyping methods, amplification refractory mutation system – PCR (ARMS-PCR) and TaqMan® 5′ nuclease assay for allelic discrimination (5' nuclease assay), was performed to determine the most appropriate method for genotyping. For this method comparison, four SNPs located in cytokine gene promoter regions were selected, IL1B-511, IL4-590, IL6-174, and TNF-308. For this comparison, DNA extracted from 335 archival gastric biopsies was genotyped using the 5' nuclease method and a proportion of these samples were genotyped using the ARMS-PCR method. The percentage of samples successfully genotyped for these four SNPs, as well as the accuracy and ease of genotyping was assessed for these two methods.

Following selection of the most suitable genotyping method, the second stage of this project was to evaluate the hypothesis:

Are known SNPs in cytokine genes associated with the development of primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma subsequent to *Helicobacter pylori* infection?

A case-control association study compared the cytokine genotype and haplotype frequencies from 206 primary GML patients and 568 uncomplicated *H. pylori* positive gastritis controls collected from three populations, UK, Germany and the Netherlands. Eighteen SNPs selected from 14 cytokine genes and their promoter regions were selected for the case-control association study; IL1A-889, IL1B-31, IL1B-511, IL1B+3953, IL1RN+2018, IL4-590, IL6-174, IL8-251, IL10-592, IL12+1188, IL18-137, IL18-607, IFNG+874, IFNGR1-56, TGFB1-509, TNF-308, TNF-376, and LTA+252.

The presence of the t(11;18)(q21;q21) translocation in GML patients is associated with adverse clinical outcome. It is not known if cytokine polymorphisms are associated with the presence or absence of this translocation. Therefore, the identification of the t(11;18)(q21;q21) translocation in the GML cases from each population was determined to evaluate the hypothesis:

Are cytokine polymorphisms associated with the presence or absence of the t(11;18)(q21;q21) translocation in GML patients subsequent to *H. pylori* infection?

2.1. General materials.

2.1.1. Reagents.

All reagents used for the manipulation of DNA, RNA and for PCR amplification were of analytical reagent grade.

Reagent	Source
Acetone, >99.5%	Sigma Aldrich
Acrylamide: Bisacrylamide solution, 40% (19:1)	Sigma Aldrich
Agarose	Helena Biosciences
Ammonium persulphate, >98%	Sigma Aldrich
Bromophenol Blue (3'3''5'5''tetrabromophenolsulfonethalein)	Sigma Aldrich
Cresol Red sodium salt, dye content 95%	Sigma Aldrich
2'-Deoxynucleoside 5'-Triphosphate (100mM each of dATP, dCTP, dGTP, dTTP)	Amersham Pharmacia
Ethylenediaminetetraacetic acid (EDTA), 0.5M	Sigma Aldrich
Ethanol, Absolute 96-100%	Sigma Aldrich
Ethidium Bromide, 100mg tablets	Sigma Aldrich
Formamide (Hi-Di™)	Applied Biosystems
Isopropanol, >99%	Sigma Aldrich
Mineral oil	Sigma Aldrich
Nonidet P40®	Merck
pBR322/Hae III digest (diluted to 50µg/200µl in H_2O)	Sigma Aldrich
PCR reagents:	
AmpliTaq Gold (5U/µl)	Applied Biosystems
PCR Buffer II, x10	Applied Biosystems
Magnesium chloride, 25mM	Applied Biosystems
qPCR Mastermix, x2 (contains 1unit hotstart Taq DNA polymerase, dNTP mix, 5mM $MgCl_2$, PCR buffer and ROX as a passive reference).	Eurogentec
Proteinase K, lyophilised powder	Sigma Aldrich

Sucrose, 99%	Sigma Aldrich
TAE, x50 concentrate (Ultrapure 2.0M Tris-Acetate and 100mM Na_2EDTA)	National Diagnostics
TBE, x10 concentrate (Ultrapure 0.89M Tris- Borate pH 8.3 and 20mM Na_2EDTA)	National Diagnostics
TEMED (N,N,N^1,N^1-Tetramethylethylenediamine)	Sigma Aldrich
Tris-HCl, 1M pH 8	Sigma Aldrich
Tween 20®	Merck
Xylene, 99%	Merck

2.1.2. Solutions.

Ammonium persulphate, 20% solution:

0.2g dissolved in 1ml deionised H_2O. For each polyacrylamide gel the ammonium persulphate was freshly prepared.

Electrophoresis loading dye, x6 concentrate:

4g Sucrose, 0.025g Bromophenol Blue and 10mls x6 TBE.

Ethidium bromide solution, 10mg/ml:

One 100mg ethidium bromide tablet was dissolved in 10ml deionised H_2O.

Paraffin lysis buffer:

100mM Tris-HCl (pH 8), 4mM EDTA, 0.45% Nonidet P40® and 0.45% Tween 20®. The lysis buffer was filter-sterilised using 0.2μM pore filters.

Proteinase K solution, 10mg/ml:

100mg lyophilised proteinase K powder was dissolved at room temperature in 10ml TE buffer. The solution was filter-sterilised using 0.2μM pore filters.

1x TAE electrophoresis buffer:

TAE x50 concentrate diluted in deionised H_2O.

0.5x TBE electrophoresis buffer:

TBE x10 concentrate diluted in deionised H_2O.

Tris-EDTA (TE) buffer:

10mM Tris-HCl and 1mM EDTA, pH8. The TE buffer was filter-sterilised using 0.2μM pore filters.

The water used for PCR reactions was prepared using a Millipore Milli-Q Water purification system.

2.2. General methods.

Basic molecular techniques were performed as described in Sambrook *et al* (1989).

2.2.1. Electrophoresis using 6% polyacrylamide gels (6%).

The following reagents were mixed in the order listed below in a 20ml universal (Sterilin):

Reagent	Volume
H_2O	7.0ml
TBE x1 concentrate	10.0ml
Acrylamide/Bisacrylamide	3.0ml
20% Ammonium persulphate	150µl
TEMED	22.5µl

The gel mix was poured between two glass plates (18x16cm) (Hoefer Scientific instruments) and a comb inserted to form the wells of the gel. This was allowed to set for 30 minutes. The gel was placed in a vertical slab gel electrophoresis tank (Hoefer Scientific Instruments) with 0.5x TBE as a running buffer. Ten µl of the PCR product was mixed with 2µl x6 loading dye and loaded onto the gel. PCR products were run in parallel with the molecular weight marker pBR322/Hae III digest and electrophoresed for one hour at 240 volts. Gels were stained with a solution of 0.5µg/ml ethidium bromide in 0.5x TBE for five minutes. The PCR products were visualised using an UV transilluminator and photo imager (Syngene).

2.2.2. Electrophoresis using 2% agarose gels.

10g agarose powder was dissolved in 500ml TAE x1 concentrate by heating on a hot plate (Bibby Stuart) and stirred continuously with a magnetic stirrer. When the agarose was completely dissolved 27.5µl of a 10mg/ml ethidium bromide solution was added to obtain a final concentration of 0.5µg/ml. The agarose solution was cooled to approximately 50°C at which

point it was carefully poured onto a horizontal tray and combs inserted to form the wells of the gel (Flowgen). The gel was allowed to set for 40 minutes, after which the combs were removed and the gel placed in a horizontal slab gel electrophoresis tank (Flowgen). Ten µl volumes of the PCR products were loaded onto the gel and run for 25 minutes at 170 volts using x1 TAE as an electrophoresis running buffer. To determine the size of the amplicons the molecular weight marker pBR322/Hae III digest was run in parallel on the gel. PCR amplicons were visualised using an UV transilluminator and photo imager (Syngene).

2.2.3. DNA extraction from archival, paraffin-embedded tissue biopsies.

The blade and blockholder of a microtome (Leitz) was thoroughly cleaned using xylene and acetone prior to cutting sections of tissue from each paraffin-embedded gastric biopsy. The biopsy surface was trimmed initially and this tissue was discarded. Five 10µm sections were cut and transferred to a 1.5ml DNase free microcentrifuge tube. Samples were processed in batches of thirty and in parallel with an empty (no template) 1.5ml microcentrifuge tube, which was subsequently used as a PCR negative control to determine the presence of any contamination.

The paraffin was dissolved in 1ml of xylene with gentle agitation for twenty minutes at room temperature. Following centrifugation at 15 000g for 5 minutes the supernatant was discarded. This procedure was repeated using 1ml 1:1 xylene: ethanol to remove the last traces of paraffin. The DNA was precipitated by two washes of 1ml absolute ethanol, gentle agitation at room temperature for 2 minutes, followed by centrifugation at 15 000g for 5 minutes, at which point the supernatant was discarded. The last traces of ethanol were removed by drying the samples at 55°C in a dri-block (Techne). Samples were incubated overnight at 55°C with 100µl sterile paraffin lysis buffer and 6.7µl of 10mg/ml proteinase K. After incubation the Proteinase K was inactivated by heating the samples to >95°C for 5 minutes in the dri-block, followed by centrifugation for 5 minutes at 15 000g. The supernatant was transferred into a 0.6ml DNase free microcentrifuge tube. The quality and quantity of the extracted DNA

present in the supernatant was assessed using the Gene control PCR (section 2.2.4.) and the RNase P assay (section 2.2.5.).

2.2.4. Assessment of the DNA quality using the gene control PCR.

The quality of extracted DNA was assessed by a single multiplex generic PCR using primers that amplify 100bp, 200bp, 300bp, and 400bp size products (van Dongen 2003). Every fifth sample extracted, as well as the no template controls, was assessed using this method. This assay indicated the size of the DNA fragments available for PCR amplification. Using 0.2ml thin walled PCR tubes (Biohit), the quality of a DNA sample was assessed using the following 50µl PCR reaction:

Reagent	Volume/ reaction	Final concentration
H_2O	30.3µl	
PCR Buffer II, x10	5.0µl	x1
dNTP mix (1.25mM)	8.0µl	0.2mM
$MgCl_2$ (25mM)	4.0µl	2.0mM
Each primer (Oswel DNA Services)	(total volume 2µl)	2.5pmol each primer
AmpliTaq Gold (5U/µl)	0.2µl	1 unit
DNA	0.5µl	Approx. 50-100ng

PCR tubes were placed in a PCR machine (DNA engine thermal cycler, MJ Research, GRI) and the DNA amplified using the following cycling conditions, 10 min at 94°C, followed by 35 cycles of, 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, then followed by 10 min at 72°C. PCR products were separated by electrophoresis using a 6% polyacrylamide gel (section 2.2.1.). The presence of 100bp, 200bp, 300bp and 400bp amplicons indicated the size of amplifiable DNA.

2.2.5. Assessment of DNA quality and concentration using the RNase P assay.

DNA quality and concentration was further determined using a commercial kit, RNase P assay (Applied Biosystems) and real time quantitative PCR. The RNase P assay consists of oligonucleotide primers and an oligonucleotide probe fluorescently labelled with the fluorophore 6-FAM and with TAMRA as a quencher. The primers amplify a target genomic DNA region that contains the human RNase P gene, a single copy gene. The probe is specific for the gene and the assay can be used to quantify copies of the gene in a DNA sample when compared to a DNA control of known concentration (supplied with the RNase P assay, Applied Biosystems). The purpose of this assay was to determine the optimal concentration of DNA for subsequent genotyping. This assay was also used to standardise the concentration of amplifiable DNA in all samples to improve genotyping calling.

2.2.5.1. The RNase P assay.

The human genomic DNA standard of known concentration (RNase P assay) was tested in duplicate at the following concentrations, 2.5ng, 5ng, 10ng, and 20ng in order to generate a standard curve. DNA samples were initially diluted at a ratio of 1:5 with TE buffer and tested in parallel with the DNA standard. Five µl PCR reactions were set up as follows using a 384-well TaqMan® optical microplate (Applied Biosystems):

Reagent	Volume/ reaction	Final concentration
qPCR Mastermix, x2	2.5µl	x1
RNase P probe and primer mix, x20 (Applied Biosystems)	0.25µl	200nM probe 900nM each primer
H_2O	1.25µl	
DNA**	1µl	Approx.2-20ng

** extracted DNA sample, or DNA standard of known concentration (RNase P assay), or H_2O (PCR negative control).

The 384-well plates were sealed with optical film (Applied Biosystems), centrifuged at 3000g for 1 minute and placed onto the 7900HT SDS instrument (Applied Biosystems) for real time quantitative PCR using the following PCR cycling conditions, 95°C for 10 minutes, followed by 45 cycles of, 95°C for 15 seconds and 60°C for 1 minute. Following PCR amplification a standard curve was generated for the 2.5ng, 5ng, 10ng and 20ng concentrations of the DNA standard using the 7900HT SDS instrument software. From the standard curve the concentration of amplifiable DNA in all samples was determined.

2.2.6. TaqMan® 5′ nuclease allelic discrimination (5' nuclease assay).

TaqMan® 5' nuclease allelic discrimination assays (5' nuclease assay) (Heid 1996) were developed for genotyping single nucleotide polymorphisms in DNA derived from paraffin-embedded, formalin-fixed, tissue biopsies. The 5' nuclease assay is a single tube PCR assay consisting of a forward and reverse oligonucleotide primer to amplify the target region and two fluorescent labelled oligonucleotide probes to detect the two alleles of a single nucleotide polymorphism (SNP). The two oligonucleotide probes are labelled with a different reporter fluorophore at the 5' end, one labelled with VIC and the other labelled with 6-FAM, as well as a quencher dye located at the 3' end of the probes. Alleles that differ in sequence by a single nucleotide can be detected using this method.

2.2.6.1. Design of probes and primers for the 5' nuclease assay.

2.2.6.1.1. Design of primers and probes for the 5'nuclease assays using Primer Express™ software.

Oligonucleotide primers and TaqMan® MGB (minor groove binding) probes were designed for 5' nuclease assays using the software Primer Express™ v2.0 (Applied Biosystems). Genomic DNA sequences were obtained from GenBank and the location of the SNP identified. Approximately, 150bp sequence upstream and down stream of the SNP was assessed and a BLAST® search performed (Basic local alignment search tool) (http://www.ncbi.nlm.nih.gov/BLAST) of this 300bp sequence. This indicated the uniqueness of the sequence compared to other regions of the human

genome, the presence of repetitive elements as well as the location of other polymorphisms within the 300bp sequence. Using the Primer Express™ software and the 300bp sequence two probes were designed to detect the two alleles of the SNP using the following criteria as defined by the manufacturer, Applied Biosystems. Both MGB probes must anneal to the same DNA strand and have the following characteristics:

1.	Probe length must be as short as possible and between 13-25 bases in size.
2.	Avoid deoxyguanosine triphosphate (G) at the 5' end of the probe, as this will quench the reporter fluorescence.
3.	The difference between the melting temperatures (Tm) of the two probes must be <1°C, and the optimal range of the probe Tm is 66-67°C.
4.	Location of the SNP is ideally in the middle or towards the 3' end of the probe.
5.	Avoid runs of a repeat nucleotide.
6.	%GC content: 30-80%.

Following the design of the probes, primers were designed using the following criteria as defined by the manufacturer, Applied Biosystems:

1.	The overall amplicon length should be as short as possible but between 50-150bp.
2.	Primers must be as close to the probes as possible without overlapping.
3.	Primer Tm: 58-60°C, optimal 59°C.
4.	Primer length: approx 20 nucleotides.
5.	Avoid GC clamp at 3' ends of primers.
6.	%GC content: 30-80%

Finally, following the design of suitable probes and primers the secondary structure and the possibility of primer-dimer formation were determined. The probes and primers were designed to work using the following PCR cycling conditions, 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 62°C for 1 minute. The following probes and primers were

designed using this method to identify SNPs in DNA derived from archival tissue biopsies and the 5' nuclease assay. The GenBank gene sequence used to design the probes and primers as well as the SNP reference sequence are provided in parenthesis. All probes and primer sequences are written in the 5' to 3' orientation and bold italic indicates the site of the polymorphism:

IL1B-511 (SNP reference sequence 16944; Genbank accession number X04500) (expected amplicon size 66bp)
Forward primer GGTCTCTACCTTGGGTGGTGTT
Reverse primer TCCTCAGAGGCTCCTGCAAT
6-FAM MGB probe detects the T allele TCTGCCTC***A***GGAGC
VIC MGB probe detects the C allele TGCCTC***G***GGAGCT

IL1RN+2018 (SNP reference sequence 419598; Genbank accession numbers U65590 and AY196903) (expected amplicon size 79bp)
Forward primer GACCTTCTATCTGAGGAACAACCAA
Reverse primer CAACCACTCACCTTCTAAATTGACA
6-FAM MGB probe detects the C allele TAGTTGC***C***GGATACTT
VIC MGB probe detects the T allele CTAGTTGC***T***GGATACTT

IL4-590 (SNP reference sequence 2243250; Genbank accession number M23442) (expected amplicon size 80bp)
Forward primer CCTGTCCTTCTCAAAACACTAAACTTG
Reverse primer GCAGAATAACAGGCAGACTCTCCTA
6-FAM MGB probe detects the T allele AGAACATTGT***T***CCCCAGTG
VIC MGB probe detects the C allele AGAACATTGT***C***CCCCAGT

IL6-174 (SNP reference sequence 1800795; Genbank accession number V00081) (expected amplicon size 80bp)
Forward primer GCTGCACTTTTCCCCCTAGTT
Reverse primer GCTGATTGGAAACCTTATTAAGATTGT
6-FAM MGB probe detects the C allele CTTTAGCAT***G***GCAAGAC
VIC MGB probe detects the G allele CTTTAGCAT***C***GCAAGAC

IL8-251 (SNP reference sequence 4073; Genbank accession number M23130) (expected amplicon size 87bp)

Forward primer TGTCACATGGTCTATGATAAAGTTATCTAGA

Reverse primer TTTAAAATACTGAAGCTCCACAATTTG

6-FAM MGB probe detects the A allele AGCATACA***A***TTGATAATT

VIC MGB probe detects the T allele AGCATACA***T***TTGATAATT

TNF-308 (SNP reference sequence 1800629; Genbank accession number M16441) (expected amplicon size 66bp)

Forward primer GAAATGGAGGCAATAGGTTTTGA

Reverse primer GTAGGACCCTGGAGGCTGACC

6-FAM MGB probe detects the G allele CCGTCC***C***CATGCC

VIC MGB probe detects the A allele CCGTCC***T***CATGCC

2.2.6.1.2. Assays-by-Design™ Service (Applied Biosystems).

During the course of this study the Assays-by-Design™ Service from Applied Biosystems became available for the design of probes and primers for 5' nuclease assays. To use this service a genomic DNA sequence of 300 nucleotides containing the SNP and marked with any other known polymorphisms was submitted to Applied Biosystems for oligonucleotide primers and MGB probes to be designed. Genomic DNA sequences were obtained from GenBank and the location of the SNP identified. Approximately 150bp sequence upstream and down stream of the SNP was assessed by performing a BLAST® search, which indicated the uniqueness of the sequence compared to other regions of the human genome, the presence of repetitive elements as well as the location of other polymorphisms within the 300bp sequence. The 300bp sequence with the above information was then submitted to Applied Biosystems for oligonucleotide primers and MGB probes to be designed. The following probes and primers were designed using this service. All probes and primer sequences are written in the 5' to 3' orientation, and bold italic indicates the polymorphic nucleotide. The SNP reference sequence and the Genbank sequence accession number are provided in parenthesis:

IL1A-889 (SNP reference sequence 1800589; Genbank accession number X03833) (expected amplicon size 81bp)

Forward primer ATTATAAAAGCTGAGAAATTCTTTAATAATAGTAACC

Reverse primer GGAAGGCATGGATTTTTACATATGA

6-FAM MGB probe detects the T allele AGGCAACA***T***CATTG

VIC MGB probe detects the C allele CAACA***C***CATTGAAGG

IL1B-31 (SNP reference sequence 1143627; Genbank accession number X04500) (expected amplicon size 77bp)

Forward primer AGAAATTTCTCAGCCTCCTACTTCTG

Reverse primer AGGTTTGGTATCTGCCAGTTTCTC

6-FAM MGB probe detects the T allele TTGAAAGC***T***ATAAAAAC

VIC MGB probe detects the C allele TTTGAAAGC***C***ATAAAA

IL1B+3953 (SNP reference sequence 1143634; Genbank accession number X04500) (expected amplicon size 74bp)

Forward primer CCTAAACAACATGTGCTCCACATT

Reverse primer ATCGTGCACATAAGCCTCGTTA

6-FAM MGB probe detects the C allele TGTGTC***G***AAGAAGAT

VIC MGB probe detects the T allele TCCCATGTGTC***A***AAGA

IL10-592 (SNP reference sequence 1800872; Genbank accession number X78437) (expected amplicon size 87bp)

Forward primer GGTAAAGGAGCCTGGAACACATC

Reverse primer CCCTTCCATTTTACTTTCCAGAGA

6-FAM MGB probe detects the A allele CGCCTGT***A***CTGTAGGA

VIC MGB probe detects the C allele CGCCTGT***C***CTGTAGG

IL12+1188 (SNP reference sequence 17875322; Genbank accession numberAY008847) (expected amplicon size 81bp)

Forward primer GGATCACAATGATATCTTTGCTGTATTT

Reverse primer CTATACATAAATTAGCTGATTGTTTCAATGAG

6-FAM MGB probe detects the C allele TTTAGCATC***G***AACTATAC

VIC MGB probe detects the A allele CATTTAGCATC***T***AACTATAC

IL18-137 (SNP reference sequence 187238; Genbank accession number AB015961) (expected amplicon size 73bp)

Forward primer GGCACAGAGCCCCAACTTT

Reverse primer CGAGTACTTCTTTTAATGTAATATCACTATTTTCA

6-FAM MGB probe detects the C allele CGGAAGAAAA***C***ATTT

VIC MGB probe detects the G allele CGGAAGAAAA***G***ATTT

IL18-607 (SNP reference sequence 1946518; Genbank accession number AB015961) (expected amplicon size 74bp)

Forward primer CCCAAGCTTACTTTCTGTTGCA

Reverse primer AAGCCACACGGATACCATCATT

6-FAM MGB probe detects the A allele AAGTGTAAAAATTATTA***A***ATAAA

VIC MGB probe detects the C allele AAAAATTATTA***C***ATAAAATTCT

IFNG+874 (SNP reference sequence 2430561; Genbank accession number J00219) (expected amplicon size 74bp)

Forward primer ACATTCACAATTGATTTTATTCTTACAACA

Reverse primer GTGCGAGTGTGTGTGTGTGTGT

6-FAM MGB probe detects the T allele TGTGTGTG***A***GATTTG

VIC MGB probe detects the A allele TGTGTGTG***T***GATTTGA

IFNGR1-56 (SNP reference sequence 17550899; Genbank accession number U19241) (expected amplicon size 60bp)

Forward primer GGTGACGGAAGTGACGTAAGG

Reverse primer CGCCTGCGGGACCAG

6-FAM MGB probe detects the T allele CCAGC***A***CTGCCCT

VIC MGB probe detects the C allele CCAGC***G***CTGCCCT

TGFB1-509 (SNP reference sequence 1800469; Genbank accession number AC011462) (expected amplicon size 84bp)

Forward primer AATTCTTACAGGTGTCTGCCTCCT

Reverse primer GCCTCCGGAGGGTGTCA

6-FAM MGB probe detects the T allele CATCC***T***TCAGGTGTC

VIC MGB probe detects the C allele CCATCC***C***TCAGGTGT

TNF-376 (SNP reference sequence 1800750; Genbank accession number M16441) (expected amplicon size 78bp)

Forward primer CCCCTCCCAGTTCTAGTTCTATCTT

Reverse primer GGGACCAGGTCTGTGGTCTG

6-FAM MGB probe detects the G allele TTTCCTTCTAA***C***TTCC

VIC MGB probe detects the A allele TTCCTTCTAA***T***TTCC

Assay-by-design (ABD) TNF-308

(SNP reference sequence 1800629; Genbank accession number M16441)

(expected amplicon size 63bp)

Forward primer CCAAAAGAAATGGAGGCAATAGGTT

Reverse primer GGACCCTGGAGGCTGAAC

6-FAM MGB probe detects the A allele CCCGTCC***T***CATGCC

VIC MGB probe detects the G allele CCCGTCC***C***CATGCC

LTA+252 (SNP reference sequence 909253; Genbank accession number X02911) (expected amplicon size 77bp)

Forward primer CAGTCTCATTGTCTCTGTCACACATT

Reverse primer ACAGAGAGAGACAGGAAGGGAACA

6-FAM MGB probe detects the G allele CCATG***G***TTCCTCTC

VIC MGB probe detects the A allele CTGCCATG***A***TTCC

2.2.6.2. SNP genotyping using the 5' nuclease assays.

For each 5' nuclease assay, DNA samples, positive and negative controls and approximately 5% of duplicate samples were genotyped within a single 384-well plate. If available three positive controls were tested for each possible genotype i.e. three controls homozygous for allele 1, three heterozygous controls and three controls homozygous for allele 2, for each SNP. Positive controls were DNA samples kindly provided by Dr. WM Howell. The DNA positive controls were previously extracted from a peripheral blood sample from bone marrow donors, which had been genotyped using the amplification refractory mutation system – PCR (ARMS-PCR) method (Howell 2003). Samples were genotyped using 2-10ng of amplifiable DNA in a 10µl PCR volume, as determined by the RNase P assay (section 2.2.5.1.). The

concentrations of probes, primers and PCR reagents were according to the manufacturers guidelines for the 7900HT SDS instrument (Applied Biosystems). Using a 384-well TaqMan® optical microplate (Applied Biosystems), the following protocol was used:

Reagent	Volume/ reaction	Final concentration
qPCR Mastermix, x2	5µl	x1
VIC labelled MGB probe (10µM) (Applied Biosystems)	0.2µl	200nM
6-FAM labelled MGB probe (10µM) (Applied Biosystems)	0.2µl	200nM
Forward primer (10µM) (Applied Biosystems)	0.9µl	900nM
Reverse primer (10µM) (Applied Biosystems)	0.9µl	900nM
Standardised concentration of DNA**	2µl	2-10ng
H_2O	0.8µl	

** Either extracted DNA sample, DNA positive control, or H_2O as a negative control.

Plates were sealed with optical cover film (Applied Biosystems), centrifuged at 3000g for 1 minute and placed on the 7900HT SDS instrument (Applied Biosystems). Samples were amplified using one of the following two cycling programs:

1. Assays designed using Primer Express™ (section 2.2.6.1.1.); 95°C for 10 minutes, followed by 45 cycles of, 95°C for 15 seconds and 62°C for 1 minute.
2. Assays-by-Design™ service (section 2.2.6.1.2.); 95°C for 10 minutes, followed by 45 cycles of, 95°C for 15 seconds and 60°C for 1 minute.

Following amplification, the fluorescence in each well of the plate was recorded using the end-point program of the 7900HT SDS instrument from

which the software generated an allelic discrimination scatterplot. Clustering of the data points into three distinct groups and assessment of the controls and hidden duplicates enabled the genotypes of the samples to be determined. The accuracy of PCR amplification from these assays was also confirmed by visualisation of the expected PCR amplicon using 6% polyacrylamide gel electrophoresis and staining with ethidium bromide (section 2.2.1) for three of the DNA samples for each assay.

2.2.7. SNP genotyping using ARMS-PCR.

Primers for genotyping SNPs in DNA derived from peripheral blood and archival tissue biopsies using the ARMS-PCR method had previously been designed and optimised for the following polymorphisms (Howell 2003); TNF-308, IL1B-511, IL6-174 and IL4-590. Samples were genotyped using 2-10ng/µl amplifiable DNA in a 10µl PCR volume. Each DNA sample was tested in two separate PCR reactions to determine the genotype. The two separate PCR reactions for each DNA sample were set-up as follows:

1. PCR reaction 1 consisted of a primer mix containing a forward primer specific for allele 1 and a common reverse primer.
2. PCR reaction 2 consisted of a primer mix containing a forward primer specific for allele 2 and the common reverse primer.

Five µl of allele 1 specific primer mix (2µM) was aliquoted into a well of a 96-well plate (AB gene) and 5µl of allele 2 specific primer mix (2µM) was aliquoted into a paired well of the 96-well plate (AB gene). The final working concentration of the primer mixes in a 10µl PCR was 1µM. A PCR reaction mix was prepared using the following reagents:

Reagent	Volume	Final concentration (10µl PCR)
PCR Buffer II, x10	50µl	x1
dNTP mix (2mM)	50µl	200µM
$MgCl_2$ (25mM)	50µl	2.5 mM
AmpliTaq Gold (5U/µl)	2.5µl	0.25 units
60% (w/v) sterile sucrose/cresol red solution	75µl	

To the two paired-wells of the 96-well plate containing the primer mixes, 4.5µl of the above PCR reaction mix was added. Finally, 0.5µl (2-10ng/µl) of DNA was added to each of the two wells. A negative control (0.5µl H_2O) containing no DNA template, as well as the same positive controls (0.5µl of DNA) used for the 5' nuclease assays were also tested in parallel to determine the accuracy of the primers and PCR conditions. Plates were sealed with cover film (AB gene), placed in a PCR machine (DNA engine thermal cycler, MJ Research, GRI) and samples amplified using the specific PCR cycling conditions optimised for each assay (sections 2.2.7.1. - 2.2.7.4.2.). Following PCR amplification, PCR amplicons were separated by 2% agarose gel electrophoresis and visualised using an UV transilluminator (section 2.2.2.).

2.2.7.1. IL1B-511 ARMS-PCR assay for archival biopsy derived DNA.

This assay determined the C/T SNP at position -511 in the promoter region of the IL1B gene. Primer sequences (Oswel DNA Services) are written in the 5' to 3' orientation, and bold italic indicates site of polymorphism:

IL1-511 common primer	AATGGGTACAATGAAGGGCCA
IL1-511 T allele specific primer	TGCAATTGACAGAGAGCTCC***T***
IL1-511 C allele specific primer	TGCAATTGACAGAGAGCTCC***C***

The following PCR cycling conditions were used: 10 minutes at 96 °C, followed by 10 cycles of, 20 seconds at 96 °C, 50 seconds at 64 °C, 40 seconds at 72 °C, followed by 20 cycles of, 20 seconds at 96 °C, 50 seconds at 60 °C, 40 seconds at 72 °C. The expected size of the PCR amplicon from the IL1B-511 assay was 103bp.

2.2.7.2. IL6-174 ARMS-PCR assay for archival biopsy derived DNA.

This assay determined the G/C SNP at position -174 in the promoter region of the IL6 gene. Primer sequences (Oswel DNA Services) are written in the 5' to 3' orientation, and bold italic indicates site of polymorphism:

IL6-174 common primer	TTTGTTGGAGGGTGAGGGTGG
IL6-174 G allele specific primer	TTCCCCCTAGTTGTGTCTTGC***G***
IL6-174 C allele specific primer	TTCCCCCTAGTTGTGTCTTGC***C***

The following PCR cycling conditions were used: 10 minutes at 96 °C, followed by 10 cycles of, 20 seconds at 96 °C, 50 seconds at 63.5 °C, 40 seconds at 72 °C, followed by 20 cycles of, 20 seconds at 96 °C, 50 seconds at 60 °C, 40 seconds at 72 °C. The expected size of the PCR amplicon from the IL6-174 assay was 108bp.

2.2.7.3. TNF-308 ARMS-PCR assay for archival biopsy derived DNA.

This assay determined the G/A SNP at position -308 in the promoter region of the TNF gene. Primer sequences (Oswel DNA Services) are written in the 5' to 3' orientation, and bold italic indicates site of polymorphism:

TNF-308 common primer	TCTCGGTTTCTTCTCCATCG
TNF-308 G allele specific primer	ATAGGTTTTGAGGGGCATG***G***
TNF-308 A allele specific primer	ATAGGTTTTGAGGGGCATG***A***

The following PCR cycling conditions were used: 10 minutes at 96 °C, followed by 10 cycles of, 15 seconds at 96 °C, 50 seconds at 65 °C, 40 seconds at 72 °C, followed by 20 cycles of, 10 seconds at 96 °C, 50 seconds at 60 °C, 40 seconds at 72 °C. The expected size of the PCR amplicon from the TNF-308 assay was 184bp.

2.2.7.4. IL4-590 ARMS-PCR assay for archival biopsy derived DNA.

IL4-590 genotyping by ARMS-PCR using biopsy derived DNA required an initial generic PCR (section 2.2.7.1.) to amplify the IL4 promoter region, followed by ARMS-PCR (section 2.2.7.4.2.) to detect the T/C SNP at position -590 of the IL4 gene promoter. Primer sequences (Oswel DNA Services) are written in the 5' to 3' orientation, and bold italic indicates site of polymorphism:

Generic IL4 promoter Primers:

IL4 generic primer L GTTGTAATGCAGTCCTCCTG

IL4 generic primer U ACTAGGCCTCACCTGATACG

IL4-590 specific primers:

IL4-590 common primer	AGTACAGGTGGCATCTTGGAAA
IL4-590 T specific primer	CTAAACTTGGGAGAACATTGT***T***
IL4-590 C specific primer	CTAAACTTGGGAGAACATTGT***C***

2.2.7.4.1. Generic PCR to amplify the IL4 promoter region.

The following protocol was used to amplify the DNA region containing the IL4 promoter region.

Reagent	Volume/ reaction	Final concentration
PCR Buffer II, x10	10µl	x1
$MgCl_2$ (25 mM)	8µl	2mM
dNTP mix (2mM)	16µl	300µM
60% Sucrose (w/v)	20µl	
H_2O	31.8µl	
50 pmoles/µl IL4-590 L primer	2.0 µl	100 pmoles
50 pmoles/µl IL4-590 U primer	2.0 µl	100 pmoles
DNA	10.0 µl	Approx.1 µg
AmpliTaq Gold (5U/µl)	0.2 µl	1 unit

Total PCR volume = 100µl per reaction.

In a 0.6ml DNase free PCR tube, 90µl of the above PCR reaction mix followed by 10µl of DNA (1µg) was added. This was covered with 100µl of mineral oil to prevent evaporation. In parallel, a negative control (H_2O as a substitute for the DNA template) was set up with each PCR run to check for contamination. PCR tubes were placed in an Hybaid Omnigene PCR machine to amplify the generic IL4 product using the following cycling parameters, 94 °C for 10 minutes, followed by 30 cycles of, 94 °C for 30 seconds, 61 °C for 1 minute and 72 °C for 90 seconds, followed by 72 °C for 8 minutes. Following amplification, the PCR amplicon was transferred to a fresh 0.6ml tube. Electrophoresis of PCR products was performed using 2% agarose gels (section 2.2.2.) to assess the success and specificity of amplification. The size of the expected generic IL4 PCR amplicon is 253bp.

2.2.7.4.2. IL4-590 ARMS-PCR.

Using the PCR amplicon generated from the generic IL4 promoter region PCR (section 2.2.7.4.1) as the template, the following PCR reaction was

prepared to genotype for the C/T SNP at position –590 of the IL4 promoter. A PCR reaction mix was prepared using the following reagents:

Reagent	Volume	Final concentration (10µl PCR)
PCR Buffer II, x10	50µl	x1
dNTP mix (2mM)	50µl	200µM
$MgCl_2$ (25mM)	35µl	1.7mM
AmpliTaq Gold (5U/µl)	2.5µl	0.25 units
60% (w/v) sucrose/cresol red solution	90µl	

Samples were amplified in a PCR machine (DNA engine thermal cycler, MJ Research,GRI) with the following cycling conditions: 96 °C for 10 minutes, followed by 5 cycles of, 96 °C for 15 seconds, 70 °C for 50 seconds, 72 °C for 40 seconds, and followed by 15 cycles of, 96 °C for 10 seconds, 65 °C for 50 seconds, 72 °C for 40 seconds. The expected amplicon is 131 base pairs.

2.2.8. Detection of the t(11;18)(q21;q21) translocation.

This work was performed at Cambridge University, Department of Histopathology, Addenbrooke's Hospital under the guidance of Professor M-Q Du and Dr. H Liu. The t(11;18)(q21;q21) translocation can be identified in a proportion of patients with MALT lymphoma and results from the fusion of the amino terminal of the API2 gene (Apoptosis inhibitor gene) to the carboxyl terminal of the MALT1 gene and generates a chimeric fusion product. The presence of the translocation can be identified from an archival paraffin-embedded, formalin-fixed tissue biopsy of the malignancy by detecting the chimeric fusion product using cDNA generated from RNA extracted from the biopsy.

2.2.8.1. RNA extraction from paraffin-embedded tissues.

Using a microtome as previously described (section 2.2.3) four 10µM sections of biopsy tissue were cut and transferred into a

DNase/RNase free 1.5ml microcentrifuge tube. In parallel, an empty paraffin wax block was also cut after every fourth biopsy sample to act as a negative control between each batch of four biopsies. No more than four samples were processed at any one time to reduce the risk of contamination.

To remove the paraffin 1ml xylene was added and gently agitated at room temperature for 20 minutes. Following centrifugation at 15 000g for 5 minutes the supernatant was discarded. Three separate washes of 1ml absolute ethanol with gentle agitation for one minute, followed by centrifugation at 15 000g for 5 minutes and removal of the supernatant each time was performed. After the last centrifugation the sample was dried at 55°C in a dri-block (Techne). RNA was isolated using a commercial kit, Ambion Paraffin Block RNA Isolation Kit (AMS Biotechnology), according to the manufacturers instructions. Briefly, 100µl Proteinase K Digestion Buffer (Ambion) and 5µl Proteinase K Solution (20mg/ml) (Ambion) was added to each sample and incubated in a waterbath at 45°C for two hours. Following this incubation, tubes were briefly spun and 600µl RNA Extraction Buffer (Ambion) was added followed by pulse vortexing for 5-second bursts, 5 times. Samples were left to incubate at room temperature for 5 minutes. 700µl of the phenol phase of acid phenol chloroform (Ambion) was added and the samples pulse vortexed for 5 seconds, 5 times, and finally incubated at room temperature for 5 minutes. Samples were centrifuged at 15 000g for 5 minutes and the aqueous layer containing the RNA was carefully transferred to a DNase/RNase free 1.5ml microcentrifuge tube. One µl linear acrylamide (5mg/ml) (Ambion) was added. An equal volume of isopropanol to the aqueous phase (approximately 600µl) was added and mixed thoroughly by inversion and placed in a freezer at -20°C overnight for the RNA to precipitate out. The following morning samples were centrifuged at 4°C for 15 minutes at 15 000g. The supernatant was discarded and 500µl of cold 75% ethanol was added to the pellet and vortexed briefly to wash. Samples were spun at 4°C for 5 minutes at 15 000g, followed by removal of the supernatant. Samples were dried for 10 minutes at 55°C in the dri-block, and finally the RNA was resuspended in 20µl RNA Storage Solution (Ambion).

2.2.8.2. Assessment of RNA concentration.

The concentration of extracted RNA was assessed using a spectrophotometer (UV1101 Biotech Photometer, WPA). Absorbance readings were taken for each sample at wavelengths of 260nm and 280nm. The reading at 260nm allowed the calculation of the concentration of nucleic acid in the sample, while the ratio of the readings at 260nm and 280nm (A_{260}/A_{280}) provided an estimate of the purity of the nucleic acid. The concentration of RNA in the sample was then determined using the calculation, $BxCxDxA_{260}$ (ng/µl), where B is the dilution factor, C is the concentration constant (33 for RNA) and D is the factor to compensate for cuvette thickness. Pure RNA preparations have A_{260}/A_{280} ratios of 2.0. If there is protein contamination in the sample the ratio will be significantly less and accurate quantitation of the RNA concentration is not possible. RNA extracted from archival biopsy tissue usually has a ratio of between 1.3-1.6, therefore only an approximate RNA concentration was achieved.

2.2.8.3. Transcription of RNA into cDNA.

The total RNA extracted from the biopsy tissue was transcribed into cDNA using the commercial kit, Invitrogen™ First-Strand Synthesis system for reverse transcriptase-PCR. cDNA was generated using a mixture of gene specific primers comprising 1pmol each of the three MALT1 reverse primers and also the glucose-6-phosphate dehydrogenase (G6PD) reverse primer.

Primer name	Primer sequence (5'-3')
G6PD-898	CGAAGTGCATCTGGCTCC
Reverse 1, MALT1-p-AS1	CCAAGACTGCCTTTGACTCT
Reverse 2, MALT1-p-AS2	GGATTCAGAGACGCCATCAA
Reverse 3, MALT1-p-AS3	CAAAGGCTGGTCAGTTGTTT

The following was performed on ice in a 0.6ml DNase/RNase free microcentrifuge tube. One µg RNA was made up to a total volume of 8µl with DPEC (diethyl pyrocarbonate)-treated water. One µl of the gene specific primer mix (1µM concentration, kindly provided by Dr. H Liu) and 1µl dNTP mix (10mM each dATP, dCTP, dGTP, dTTP) was added to the

RNA. Samples were incubated in a waterbath at 65°C for 5 minutes then plunged into ice for one minute to denature the samples. In parallel, a 9µl reaction mix was prepared consisting of 2µl 10x RT buffer (200mM Tris-HCl (pH 8.4) and 500mM KCl) (Invitrogen), 4µl 25mM $MgCl_2$ (Invitrogen), 2µl 0.1M DTT (Invitrogen), 1µl RNaseOUT™ (Invitrogen). To the 9µl reaction mix, the 10µl volume containing 1µg denatured RNA was added. The samples were incubated in a waterbath at 50°C for two minutes and finally, 1µl Superscript™ II RT enzyme (50 units/µl) (Invitrogen) was added and incubated in a waterbath at 50°C for 50 minutes. The reaction was terminated by placing samples in a waterbath at 70°C for 15 minutes, followed by cooling on ice for 30 seconds. One µl RNase H enzyme (2 units/µl) (Invitrogen) was added to the samples and incubated in a waterbath at 37°C for 20 minutes to digest the RNA template. The resultant cDNA was stored at -20°C or used directly for PCR.

2.2.8.4. Detection of t(11;18)(q21;q21) translocation.

Primers previously designed by H Liu *et al* (2002a) to amplify a short segment of the fusion junction of the API2-MALT1 chimaeric product was used to identify the presence of the t(11;18)(q21;q21) translocation. Amplicon products were small and therefore suitable for cDNA generated from the fragmented RNA in archival biopsies. Three sets of primers were used, a common API2 forward primer that covered 93% of the known API2 breakpoints and three reverse primers, p-AS1, p-AS2 and p-AS3, that target all four variable breakpoints on the MALT1 gene. A separate set of primers that amplify 67bp and 151bp products of the G6PD gene was included as a control to validate RNA quality and reverse transcription-PCR efficiency. The primers were designed to span an exon junction to avoid amplification from genomic DNA.

For each cDNA sample, four individual PCRs were performed with each primer pair:

Primer name	Primer sequence (5'-3')	Amplicon size
Common Forward API2- p-s	GGAAGAGGAGAGAGAAAGAGCA	
Reverse 1 MALT1-p-AS1	CCAAGACTGCCTTTGACTCT	81bp
Reverse 2 MALT1-p-AS2	GGATTCAGAGACGCCATCAA	65bp 338bp
Reverse 3 MALT1-p-AS3	CAAAGGCTGGTCAGTTGTTT	71bp 98bp
G6PD Forward	ACGGCAACAGATACAAGAAC	
G6PD Reverse	CGAAGTGCATCTGGCTCC	67bp 151bp

A known t(11;18)(q21;q21) positive control (API2-MALT1 fusion cDNA sample), the paraffin extraction negative control (no template) and a PCR negative control (H_2O) were also tested in parallel with samples. The following 25µl PCR reactions were prepared:

Reagent	Volume/ Reaction	Final concentration (25µl PCR)
Platinum PCR buffer, x10 (Invitrogen)	2.5µl	X1
dNTP mix, 25mM (Invitrogen)	0.2µl	0.2mM
$MgCl_2$, 50mM (Invitrogen)	0.8µl	1.6 mM
Platinum Taq DNA polymerase (5U/µl)	0.2µl	1.0 unit
Forward primer 10µM	0.5µl	0.2µM
Reverse primer 10µM	0.5µl	0.2µM
cDNA	1µl	
H_2O	19.3µl	

The PCR was performed using a Thermohybaid PX2 engine and the following cycling parameters; 94°C for 3 minutes, followed by ten cycles of

94°C for 30 seconds, 66°C for 30 seconds (decreased by 1°C/cycle until 59°C), 72°C for 30 seconds. Followed by 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds and finally a ten-minute incubation at 72°C. Four µl of the PCR products were analysed in parallel with a 25bp molecular weight ladder (Bioline) by electrophoresis using polyacrylamide gels and staining with ethidium bromide (section 2.2.1).

The control samples were examined before an assessment was made for the presence of the translocation. If the 67bp product was clearly amplified from the G6PD PCR, and the positive control (known t(11;18)(q21;q21) cDNA sample), the paraffin extraction negative control (no template) and a PCR negative control (H_2O) were as expected, the presence of the t(11;18)(q21;q21) translocation could be determined. The presence of a prominent amplicon of expected size in any of the three PCR assays specific for the API2-MALT1 fusion product indicated the presence of the translocation. If no 67bp product was amplified from the G6PD PCR then it was determined that insufficient RNA or RNA quality was obtained from the biopsy sample and that the sample was unsuitable for the assessment of the presence of the translocation.

2.2.9. Sequencing.

The accuracy of the 5' nuclease assays and genotype calling was confirmed by sequencing a proportion of samples genotyped in each assay. Three samples (one genotyped as homozygous for allele 1, one genotyped as homozygous for allele 2 and one heterozygous example) were selected and the genotypes confirmed by sequencing. An initial PCR amplification of the target region was performed using oligonucleotide primers (section 2.2.9.3) followed by column purification of the PCR products using QIAquick PCR purification kit (Qiagen). The purified PCR template was then amplified using an unlabelled oligonucleotide sequencing primer in the presence of a mixture of 3'-fluorescently labelled dideoxynucleoside triphosphates (dye terminators) and deoxynucleoside triphosphates (Big Dye® Terminator v.1.1 cycle sequencing kit, Applied Biosystems) and analysed by capillary electrophoresis using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems).

2.2.9.1. Initial amplification of the target region for subsequent sequencing.

PCR amplification of the target region was performed using unlabelled oligonucleotide primers. Using 0.2ml thin walled PCR tubes (Biohit) the following 25µl PCRs were prepared:

Reagent	Volume/reaction	Final concentration
PCR Buffer II, x10	2.5µl	x1
dNTP mix (2mM)	2.5µl	200µM
$MgCl_2$ (25mM)	2µl	2mM
AmpliTaq Gold (5U/µl)	0.1µl	0.5U
Forward primer (10µM)	1µl	0.4µM
Reverse primer (10µM)	1µl	0.4µM
DNA (2-3ng/µl)	2µl	4-6ng
dH_2O	13.9µl	

Samples were amplified in a PCR machine (DNA engine thermal cycler, MJ Research, GRI) using the following cycling conditions, 94°C for 10 min, followed by 35 cycles of, 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, followed by 72°C for 4 minutes. The size, quality and quantity of the PCR amplicon were assessed using 2% agarose gel electrophoresis (section 2.2.2.) and comparison with the quantitative molecular weight marker, Hyperladder I (Bioline). Following assessment of PCR amplification, amplicons were column purified to remove unincorporated primers, dNTPs and buffers using QIAquick PCR purification kit (Qiagen) according to the manufacturers instructions. Products were recovered in 30µl of the elution buffer to give a final PCR product concentration of between 2-10ng/µl.

2.2.9.2. Sequencing of the amplified target region.

Ideally, another primer internal to the primers that amplified the target region is used for sequencing. In addition, sequencing should be performed in both the forward and reverse directions. However, due to the small, fragmented size of the DNA extracted from archival biopsy tissue this was not possible and the primers used to amplify the target region were also used for the sequencing PCR. Sequencing was performed using 2-10ng of amplified template (section 2.2.9.1.) in a 20µl PCR volume using Big Dye® Terminator v1.1 cycle sequencing reagents (Applied Biosystems). The following PCR reaction was prepared using 0.2ml thin-walled PCR tubes:

Reagent	Volume/reaction	Final concentration
Big Dye® reaction mix (x2.5)	8µl	x1
Sequencing primer (10µM)	0.32µl	3.2pmol
Template (2-10ng/µl)	1µl	2-10ng
dH_2O	10.68µl	

Samples underwent linear amplification using a PCR machine (DNA engine thermal cycler, MJ Research, GRI) with the following cycling conditions, 96°C for 10 min, followed by 25 cycles of, 96°C for 10 secs, 50°C for 5 secs, 60°C for 4 minutes. Following completion of the sequencing reaction, the products were purified to remove unincorporated dye terminators prior to capillary electrophoresis using alcohol precipitation with isopropanol. Samples were resuspended in 10µl formamide (Hi-Di™ Formamide, Applied Biosystems), transferred to a well of a 96-well plate (MicroAmp® reaction plate, Applied Biosystems) and denatured into single strands by heating to 94°C for 5 minutes in a PCR machine (DNA engine thermal cycler, MJ Research, GRI). Finally, the single strand sequencing products were analysed by electrophoresis using 36cm length capillaries containing POP-4™ polymer (Applied Biosystems) and using the ABI Prism® 3100 Genetic Analyzer (Applied Biosystems).

2.2.9.3. Sequencing primers.

All primers are written in the 5'-3' direction:

IL1A-889 forward sequencing primer: ACATTCATTTGCTAAGAGTCTG

IL1A-889 reverse sequencing primer: GTATGCGTAAGGCCTCAGCCAG

IL1B-511 forward sequencing primer: GTAACAGCACCTGGTCTTGCAG

IL1B-511 reverse sequencing primer: TTCCCACTTACAGATGGATAAATG

IL1B-31 forward sequencing primer: CCTAAGAAGCTTCCACCAATACTC

IL1B-31 reverse sequencing primer: GTCATACTTGAGCAATGAAGATTG

IL1B+3953 forward sequencing primer: TATGCTCAGGTGTCCTCCAAG

IL1B+3953 reverse sequencing primer: TATGGACCAGACATCACCAAGC

IL1RN+2018 forward sequencing primer: AGCACAAGGCTGGGCACATG

IL1RN+2018 reverse sequencing primer: ACATTGCACCTAGGGTTTGTG

IL4-590 forward sequencing primer: ACCTACCTTGCCAAGGGCTTC

IL4-590 reverse sequencing primer: GTACAGGTGGCATCTTGGAAAC

IL6-174 forward sequencing primer: GTAAAGGAAGAGTGGTTCTGC

IL6-174 reverse sequencing primer: GAGACTCTAATATTGAGACTCATG

IL8-251 forward sequencing primer: CATCCATGATCTTGTTCTAACACC

IL8-251 reverse sequencing primer: CAAATACGGAGTATGACGAAAG

IL10-592 forward sequencing primer: CACTTCCCCCAAGCACAGTTG

IL10-592 reverse sequencing primer: CTTAGGTCTCTGGGCCTTAGT

IL12+1188 forward sequencing primer: TAAAGACACAACGGAATAGACC

IL12+1188 reverse sequencing primer: GCATGAAGGCCCATGGCAAC

IL18-137 forward sequencing primer: GAAGATGCTTCTAATGGACTAAG

IL18-137 reverse sequencing primer: CTTTCCTAGGGCAATGGAAGTC

IL18-607 forward sequencing primer: CAGGAATAGAAAGTTTTAACACTG

IL18-607 reverse sequencing primer: GTGGAACAGGAGTCCATTTTC

TNF-308 forward sequencing primer: CCTCCCAGTTCTAGTTCTATC

TNF-308 reverse sequencing primer: GACACACAAGCATCAAGGATAC

TNF-376 forward sequencing primer: GGCCTCAGGACTCAACACAG

TNF-376 reverse sequencing primer: TGGGCCACTGACTGATTTGTG

LTA+252 forward sequencing primer: CTGCACCTGCTGCCTGGATC

LTA+252 reverse sequencing primer: AGATCGACAGAGAAGGGGAC

IFNG+874 forward sequencing primer:

TTCATTATTTGTTTAAAACTTAGCTG

IFNG+874 reverse sequencing primer: CAGGTTTCTATTACATCTACTG

IFNGR1-56 forward sequencing primer: TGCGGCTTCCCGGACTTGAC

IFNGR1-56 reverse sequencing primer: CTCACACCCTGCATGACAAG

TGFB1-509 forward sequencing primer: TCGCAGGGTGTTGAGTGACAG

TGFB1-509 reverse sequencing primer: CAGAACGGAAGGAGAGTCAG

Chapter 3: Comparison of two methods, ARMS-PCR and TaqMan® 5' Nuclease allelic discrimination assay, for genotyping DNA derived from archival tissue.

3.1. Introduction.

3.1.1. Polymorphism within the human genome.

Common types of sequence variation in the human genome include single nucleotide polymorphisms (SNPs), insertions or deletions of a few nucleotides, and variation in the repeat number of a sequence motif (variable number tandem repeat, VNTR). SNPs are the most common type of known sequence variation in the human genome. SNPs occur on average once every 250-1000bp in the human genome although the intensity of SNPs across regions is greatly variable (Miller 2005). Therefore, SNPs can be used as markers for mapping phenotypes and disease traits by linkage or association studies. A SNP may be in linkage disequilibrium with another polymorphism influencing the phenotype or may directly have a functional relevance in the phenotype. Selection of the method for SNP genotyping is dependent on the quality and quantity of DNA samples to be processed, as well as accuracy, speed and cost.

3.1.2. SNP genotyping DNA derived from archival biopsy tissue: A comparison of two methods.

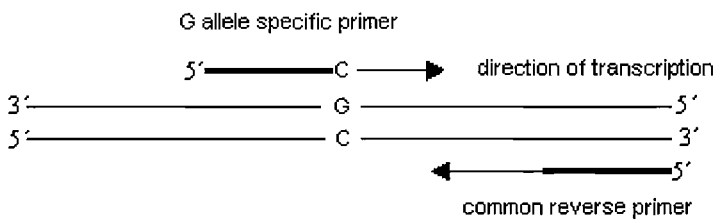
For the case-control association study presented in chapter 4, the source of DNA for the majority of cases and controls was from archival, paraffin-embedded, formalin-fixed, gastric biopsies. Therefore, the first phase of this project was to select the most appropriate method for genotyping SNPs using DNA derived from this source. Two polymerase chain reaction (PCR) methods for SNP genotyping, namely amplification refractory mutation system – PCR (ARMS-PCR) and TaqMan® 5′ nuclease allelic discrimination (5' nuclease assay), were compared to determine the most appropriate method for typing DNA derived from archival paraffin-embedded, formalin-fixed, gastric biopsies. The selected method would subsequently be used to genotype DNA for the case-control association study outlined in chapter four.

3.1.2.1. Description of the ARMS-PCR method.

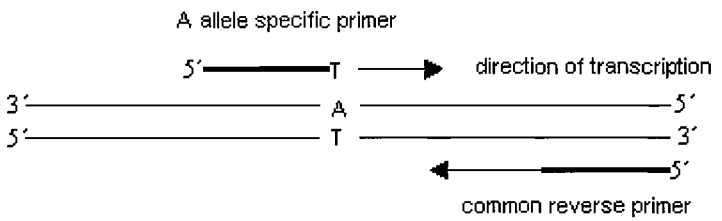
ARMS-PCR is a method suitable for detecting a single point mutation such as a SNP (Krausa 1993). Accurate genotyping is dependent on the optimisation of PCR conditions so that the enzyme, Taq DNA polymerase, will not amplify from oligonucleotide primers mismatched at the 3' end to the template DNA. Therefore, under strict PCR conditions, successful PCR amplification results if the primers are identical in sequence to the template DNA. If the exact target sequence is not present amplification does not occur or is significantly reduced. Target sequences that differ by a single nucleotide can be discriminated. Two separate PCR reactions are required (fig.3.1), the first contains primers specific for the wild type allele and the second contains primers that amplify template DNA containing the variant allele. Following strict PCR amplification, the presence or absence of PCR products of the correct amplicon size indicates the genotype of the DNA sample. Ideally, internal control primers that amplify a conserved region of the genome are also included to distinguish between a failed PCR reaction and a true negative reaction.

Fig.3.1 Diagram of ARMS-PCR.

Reaction 1: G allele specific primer set.



Reaction 2: A allele specific primer set.



Two separate PCR reactions are required to detect the presence of the two possible alleles of a G/A SNP. The specificity of the two reactions is dependent on strict PCR conditions to prevent mis-priming.

The success of this assay is dependent on primers and PCR conditions that will not mis-prime and amplify non-target sequences, whilst allowing efficient amplification of the target sequence. Extensive optimisation of PCR conditions is required to achieve this and involves determining the optimal concentration of magnesium ions and the temperature of the annealing phase in the PCR, as well as the DNA sequence. The type and position of the nucleotide mismatches between the primer and DNA template affect both the stability of the duplex and the efficiency of the Taq DNA polymerase (Kwok 1990). In addition, different optimal conditions are sometimes required depending on the source of DNA. For example, DNA derived from peripheral blood may require a lower magnesium concentration than samples derived from archival biopsy tissue. Finally, after successful optimisation the ARMS-PCR can be used for genotyping, although positive and negative (no DNA template) control samples must always be genotyped in parallel with unknown samples to ensure the assay is specific and sensitive. The PCR amplicons can be visualised by agarose gel electrophoresis and staining with ethidium bromide. The ARMS-PCR method is a low to medium throughput system and is therefore not ideal for processing large numbers of samples. However, the set up and running costs are minimal as no specialist equipment or reagents are required.

3.1.2.2. Description of the TaqMan® 5' nuclease allelic discrimination assay.

The 5' nuclease assay is a single tube PCR assay that uses different fluorescent labelled probes to detect the two alleles of a SNP (Heid 1996). Forward and reverse primers are designed that amplify the target region. Two oligonucleotide probes are also designed each labelled with a different reporter fluorophore at the 5'end (usually one labelled with VIC and the other labelled with 6-FAM), each detecting one of the two possible alleles of a SNP. The probes are also labelled with a quencher dye at the 3' end e.g. TAMRA. When the probe is intact, the proximity of the reporter dye to the quencher results in suppression of the reporter fluorescence by fluorescent resonance energy transfer (FRET) (Clegg 1995). If the specific allele is present in the template DNA, the allele–specific probe will hybridise to the template DNA between the forward and reverse primers. This method then exploits the 5' exonuclease activity of Taq DNA polymerase to cleave the probe in a 5' to 3' direction. As

the Taq DNA polymerase extends from the 3' end of the forward primer, the 5'-3' nucleolytic activity of the Taq DNA polymerase cleaves the probe only if the probe is hybridised to the template DNA (fig.3.2). The probe fragments are displaced and polymerisation of the DNA strand continues. Cleavage of the probe separates the reporter dye from the quencher dye, which results in increased fluorescence of the reporter. The 3' end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every PCR cycle and does not interfere with the exponential accumulation of the amplicon.

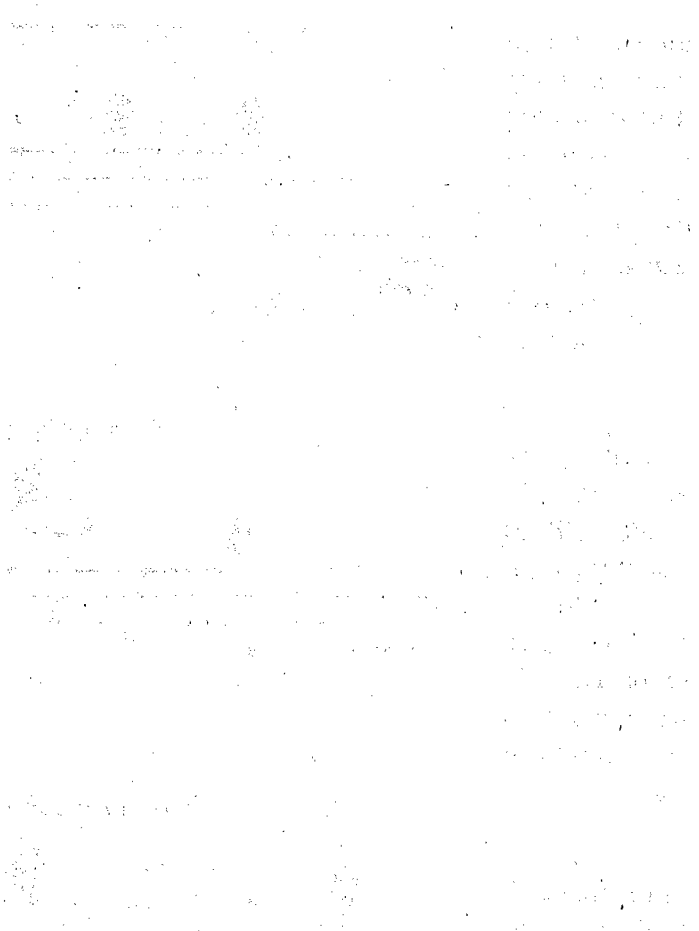
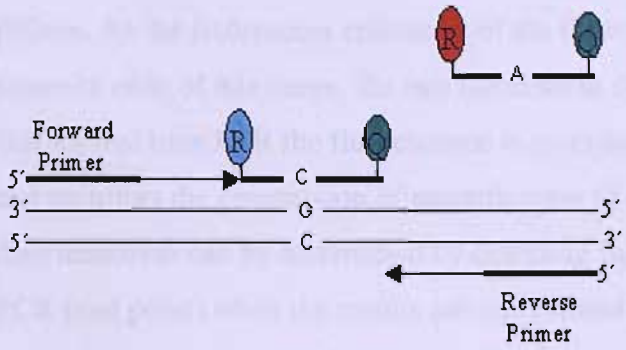


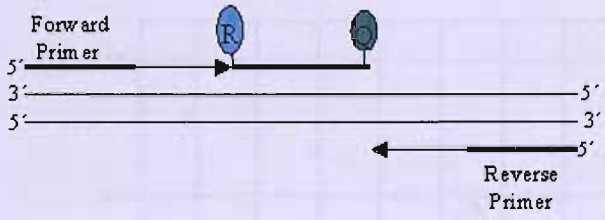
Fig.3.2. Diagram of 5' nuclease allelic discrimination assay.

A. A single tube PCR assay.



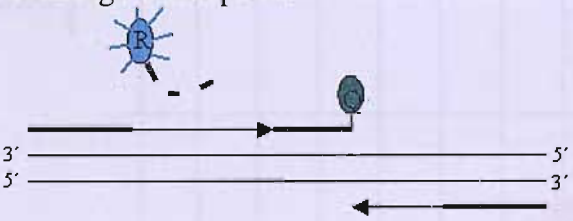
The 5' nuclease method is a single tube PCR assay containing a forward and reverse primer pair to amplify the SNP region and two fluorescent labelled oligonucleotide probes, which detect the two alleles of a SNP. One probe, specific for allele 1, is labelled at the 5' end with the reporter fluorophore 6-FAM ( ). The second probe, specific for allele 2, is labelled with VIC ( ). Both probes are labelled at the 3' end with a quencher dye e.g. TAMRA ( ).

B. Probes and primers bind.



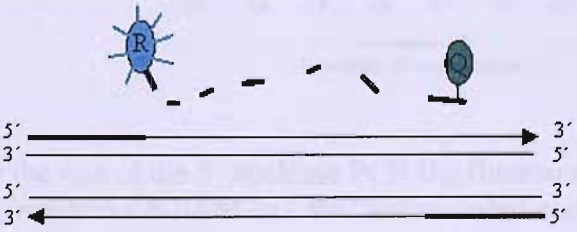
When the probe is intact the reporter fluorescence is quenched due to the proximity of the quencher and FRET. If the target sequence is present on the DNA template, the probe hybridises between the forward and reverse primers.

C. Cleavage of the probe.



The probe is cleaved by the 5'-3' exonuclease activity of the Taq DNA polymerase as it polymerises from the 3' end of the forward primer. The 3' end of the probe is blocked, so it is not extended during the PCR reaction.

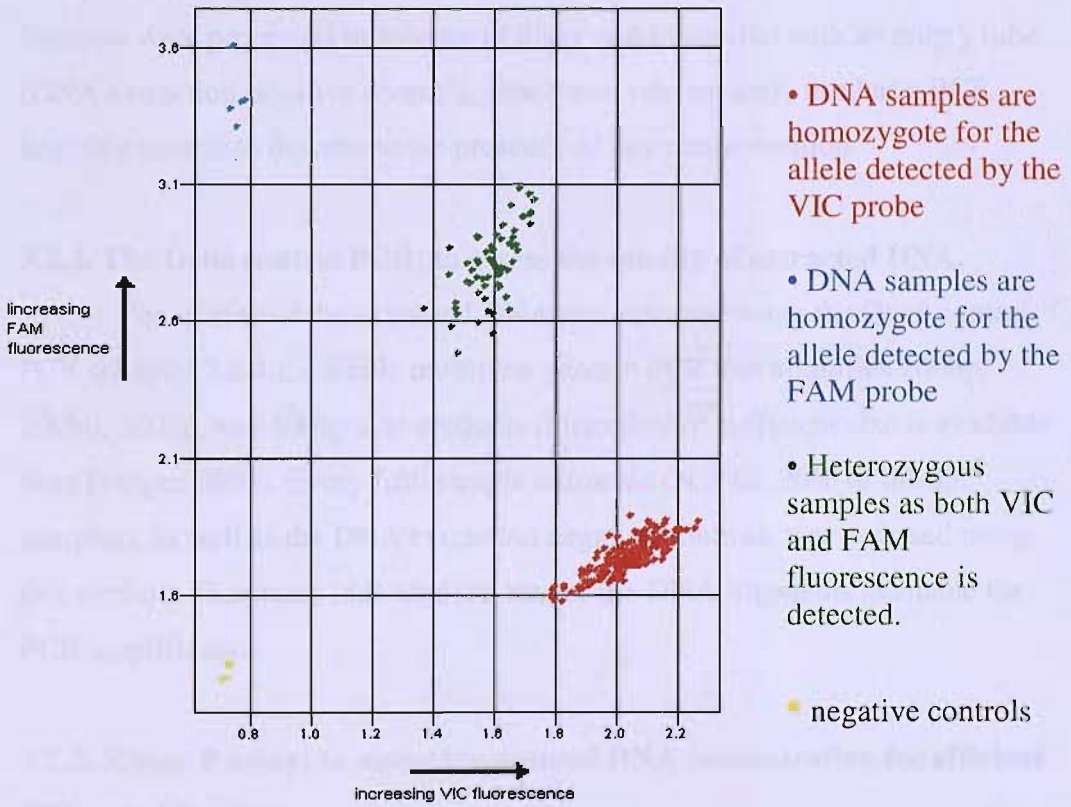
D. Fluorescence is emitted.



Cleavage of the probe separates the 5' fluorophore (6-FAM) from the quencher and the fluorescence is emitted.

The level of fluorescence can be detected by excitation with an argon ion laser and recorded by a CCD camera. This method is used for the ABI 7900HT SDS instrument that detects fluorescent emissions between 500nm and 660nm. As the fluorescent emissions of the fluorophores VIC and 6-FAM are at opposite ends of this range, the two fluorescent dyes can be distinguished. During real time PCR the fluorescence is recorded at every cycle of the PCR, and monitors the progression of amplification of the target DNA. Allelic discrimination can be determined by detecting fluorescence at the end of the PCR (end point) when the results are represented as a scatterplot. The x-axis of the scatterplot is a scale of increasing fluorescence from probe 1 (detecting allele X) and the y-axis is a scale of increasing fluorescence from probe 2 (detecting allele Y) (fig. 3.3).

Fig. 3.3. Allelic discrimination scatterplot.



At the end of the 5' nuclease PCR the fluorescence emitted from the two fluorophores 6-FAM and VIC are recorded and the results presented as a scatterplot. Each dot represents an individual PCR reaction containing a DNA sample, the forward and reverse primers and the two fluorescent-labelled probes.

Alleles that differ in sequence by a single nucleotide can be detected using this method. An advantage of this method is that probes and primers are designed to work under the same PCR conditions therefore no extensive optimisation is required for successful genotyping. In addition, no post-PCR manipulation is required and the system is designed for high throughput genotyping. However, the initial set-up costs are high as specialist equipment and reagents are required, such as an ABI 7900HT SDS instrument.

3.2. Materials and methods.

3.2.1. DNA samples for method comparison.

Ethical approval for this project was obtained from Southampton and SW Hants Local Research Ethics Committee. A sequential series of 335 cases diagnosed as *H. pylori* positive gastritis between 1997 and 2001 at Southampton General Hospital were selected. In all cases, DNA was extracted from an archival, paraffin-embedded, formalin-fixed gastric biopsy (chapter 2.2.3.). Samples were processed in batches of thirty and in parallel with an empty tube (DNA extraction negative control), which was subsequently used as a PCR negative control to determine the presence of any contamination

3.2.2. The Gene control PCR; to assess the quality of extracted DNA.

The quality of the extracted DNA was assessed using the Gene control PCR (chapter 2.2.4.), a single multiplex generic PCR that amplifies 100bp, 200bp, 300bp, and 400bp size products if template of sufficient size is available (van Dongen 2003). Every fifth sample extracted (N = 68, 20% of the 335 samples), as well as the DNA extraction negative controls, was assessed using this method. This assay indicated the size of the DNA fragments available for PCR amplification.

3.2.3. RNase P assay; to assess the optimal DNA concentration for efficient PCR amplification.

The DNA extracted from archival paraffin-embedded, formalin-fixed gastric biopsies was assessed using the RNase P assay (chapter 2.2.5) to determine the optimal DNA concentration for efficient PCR amplification. DNA extracted from this source of material is often degraded and contaminated by the presence of PCR inhibitors. To evaluate the effect of DNA concentration

on PCR amplification when using samples derived from this source, 51 of the DNA samples extracted were tested undiluted, and at a ratio of 1:2, 1:5 and 1:10 dilution with TE buffer and assessed using the RNase P assay. Comparison of the real-time amplification plots and the standard curve generated from a human genomic DNA sample of known concentration determined the optimal dilution for efficient PCR amplification of these samples.

3.2.4. Selection of SNPs for method comparison.

For this comparison the following four SNPs located in cytokine gene promoter regions were selected, IL1B-511 (C/T), IL4-590 (C/T), IL6-174 (G/C), and TNF-308 (G/A). The SNP number indicates the position of the SNP relative to the transcription start site of the gene.

3.2.5. SNP genotyping using the 5' nuclease method.

For each of the four SNPs, 5' nuclease assays were developed using DNA derived from paraffin-embedded, formalin-fixed, tissue biopsies (chapter 2.2.6.). For each assay all 335 DNA samples as well as positive and negative controls were genotyped within 384-well typing plates. Three positive controls were tested for each possible genotype i.e. three controls homozygous for allele 1, three heterozygous controls and three controls homozygous for allele 2, for each SNP. Positive controls were DNA samples kindly provided by Dr.WM Howell. The DNA positive controls had previously been extracted from a peripheral blood sample from bone marrow donors and genotyped using ARMS-PCR (Howell 2003). Samples were genotyped using 2-10ng amplifiable DNA in a 10µl PCR volume (Chapter 2.2.6.2.).

3.2.6. SNP genotyping using ARMS-PCR method.

Primers for the ARMS-PCR assays for these four SNPs had previously been designed and optimised for allelic discrimination of DNA derived from peripheral blood and archival biopsies (Howell 2003) (Chapter 2.2.7.). Of the 335 DNA samples, 87 were tested using the TNF-308 ARMS-PCR assay, 50 were tested in the IL1B-511 ARMS-PCR assay and 30 were tested using the IL6-174 ARMS-PCR assay. Only ten samples were tested using the IL4-590 ARMS-PCR assay, as this assay required further optimisation for successful genotyping of DNA derived from this source. The same positive and negative

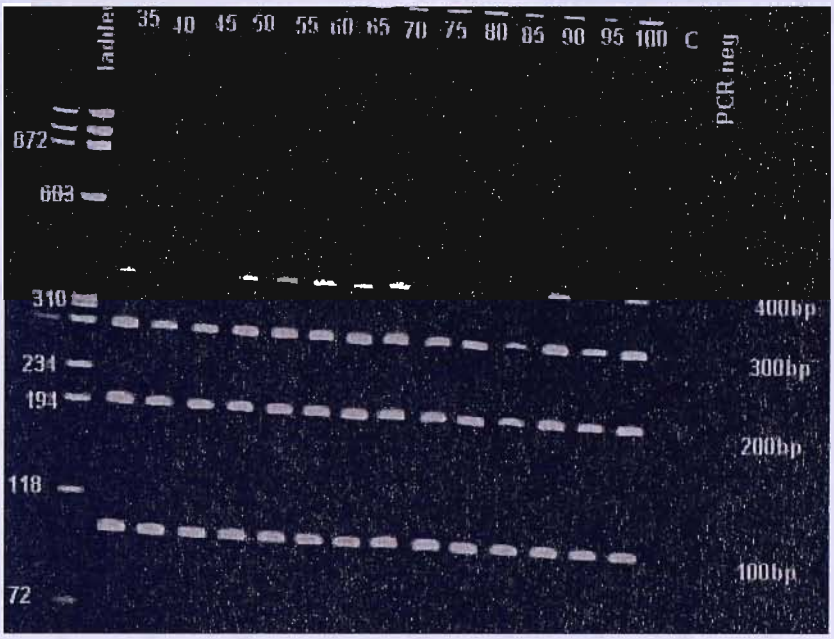
controls used for the 5' nuclease assays (section 3.2.5.) were used to confirm the sensitivity and specificity of the ARMS-PCR assays.

3.3. Results.

3.3.1. Assessment of the quality of DNA.

100bp, 200bp and 300bp size products were amplified from all DNA samples tested (N = 68) (fig.3.4). 400bp amplicons were clearly detected in only 54% (37/68) of the DNA samples. This confirmed that sufficient intact 300bp lengths of DNA were available for PCR amplification in all the samples tested.

Fig.3.4. Gene control PCR to determine the size and quality of extracted DNA.



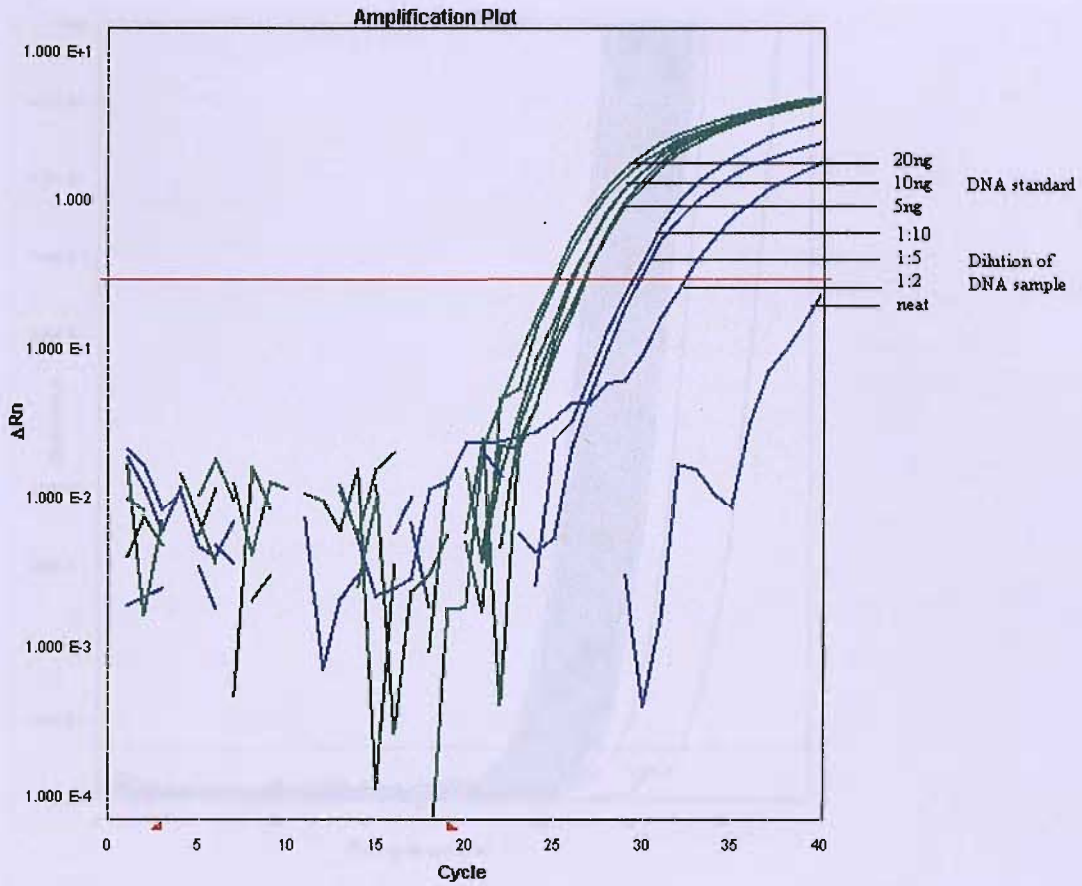
Amplicons of 100bp, 200bp, 300bp and 400bp size were clearly detected in samples 35, 50 to 70, 90 and 100 using the gene control PCR. For the remaining samples 300bp products were clearly detected with weaker detection of the 400bp amplicon. C = DNA extraction negative control; PCR neg = PCR negative control (H_2O);

3.3.2. Assessment of DNA concentration using the RNase P assay.

Initially, 51 DNA samples were tested undiluted and at a ratio of 1:2, 1:5 and 1:10 using the RNase P assay. The effect of diluting the DNA samples derived from an archival biopsy is shown in figure 3.5. Increasing dilution of these DNA samples improved the efficiency of the PCR in spite of the reduction in DNA concentration. This was probably due to the presence of PCR inhibitors

in the DNA samples extracted from archival biopsies. Testing the DNA samples undiluted and at a dilution of 1:2 (final dilution in the 5µl PCR volume was 1:5 and 1:10 respectively) resulted in inhibition of the PCR. However, this inhibition was adequately removed using DNA at a dilution of 1:5 and 1:10 (final dilution in the 5µl PCR volume was 1:25 and 1:50 respectively), which amplified almost identically (fig. 3.5).

Fig.3.5. Effect of diluting a DNA sample derived from an archival biopsy.

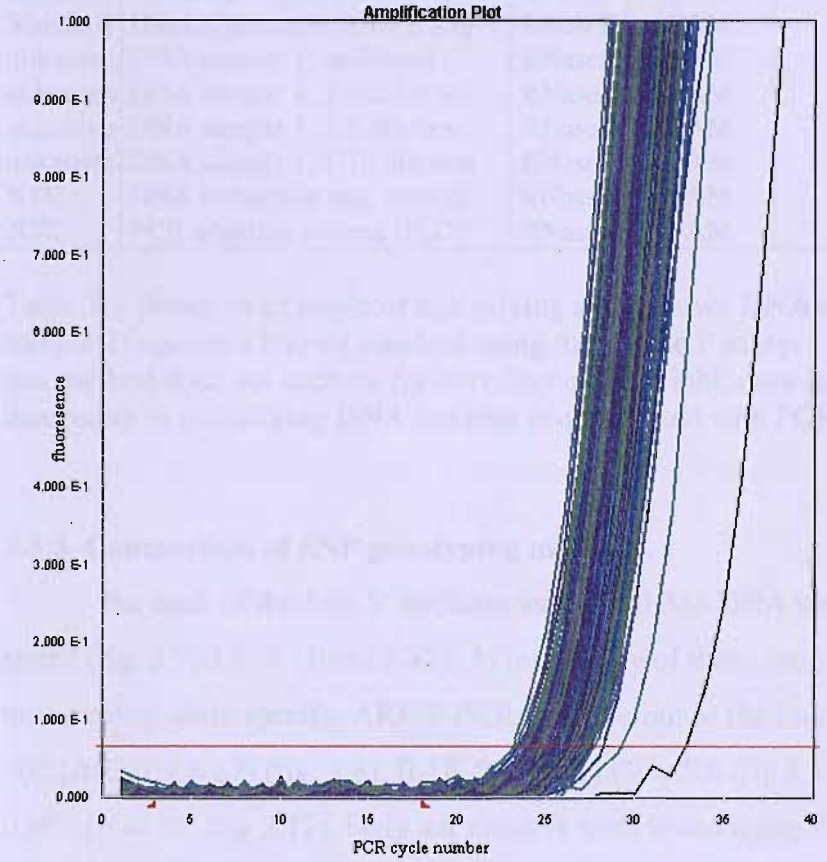


Comparison of the real time PCR amplification of the DNA standard (green) and a DNA sample extracted from an archival biopsy (blue) using the RNase P assay. Increasing the dilution of the DNA sample improves the efficiency of the PCR and correlates with a lower PCR cycle number (x-axis). In contrast, increasing the concentration of the DNA standard correlates with a lower PCR cycle number. The red line on the graph represents the threshold and is the point of detection.

Following the initial assessment of the first 51 samples, all 335 DNA samples were diluted at a ratio of 1:5 and assessed using the RNase P assay. This dilution of DNA resulted in the majority of DNA samples to amplify exponentially from approximately PCR cycle number 22 to 27 (fig. 3.6), whilst

minimising the effect of PCR inhibition. Samples that fell outside of this range were diluted further to standardise all DNA samples. This dilution equated to a concentration of between 2-10ng of amplifiable DNA when compared to the human genomic DNA standard. This optimal concentration/dilution of DNA was then subsequently used for both the ARMS-PCR and 5' nuclease assays.

Fig. 3.6. Standardisation of DNA samples derived from archival biopsies.



Using the RNase P assay the concentration of amplifiable DNA derived from archival biopsies can be standardised for PCR assays. Here all but two of the samples tested are standardised to amplify exponentially from PCR cycle numbers 22-27.

The RNase P assay was used to determine the optimum dilution of DNA for efficient PCR amplification. However, from the data (fig. 3.5) it is evident that this assay is an inappropriate method to quantify the concentration of DNA extracted from archival biopsies. Diluting these samples consequently determined a higher DNA concentration, even though a smaller amount of DNA

was present (table 3.1). This effect is likely to result from the dilution reducing the concentration of PCR inhibitors present in the paraffin-extracted samples.

Table 3.1. Assessment of the DNA concentration by comparison with the human genomic DNA standard in the RNase P assay.

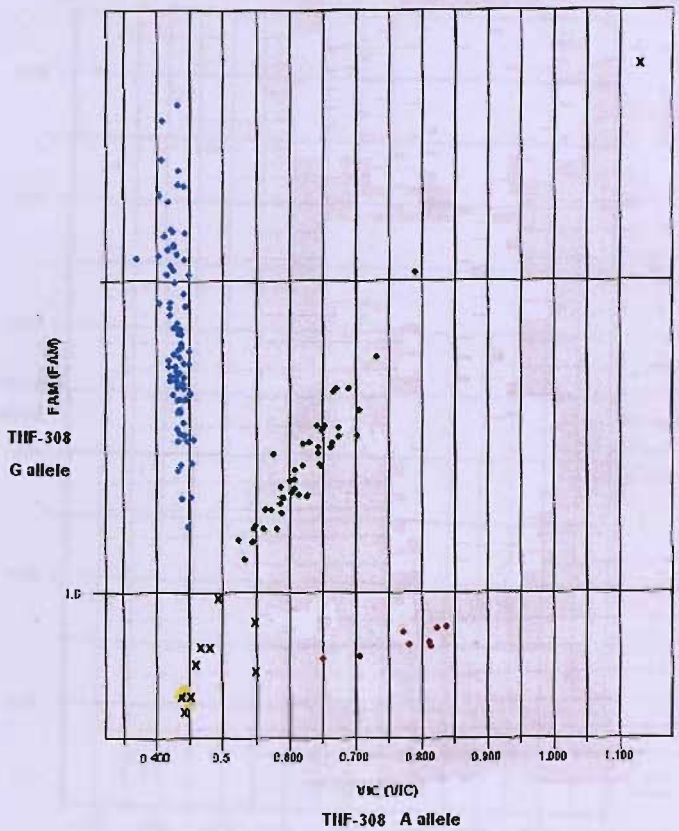
Sample type	Sample Name	Assay	Reporter dye	Ct number	Quantity (ng)
Standard	Human genomic DNA 20ng	RNaseP	FAM	22.88	20
Standard	Human genomic DNA 10ng	RNaseP	FAM	24.29	10
Standard	Human genomic DNA 5ng	RNaseP	FAM	25.85	5
Standard	Human genomic DNA 2.5ng	RNaseP	FAM	27.25	2.5
unknown	DNA sample 1, undiluted	RNaseP	FAM	40	0
unknown	DNA sample 1, 1:2 dilution	RNaseP	FAM	34.48	0.06
unknown	DNA sample 1, 1:5 dilution	RNaseP	FAM	25.67	5.18
unknown	DNA sample 1, 1:10 dilution	RNaseP	FAM	26.07	4.21
NTC	DNA extraction neg. control	RNaseP	FAM	40	0
NTC	PCR negative control (H_2O)	RNaseP	FAM	40	0

Table 3.1 shows an example of quantifying an unknown DNA sample (DNA sample 1) against a known standard using the RNase P assay. Unfortunately, this method does not account for the effect of PCR inhibitors and is therefore, inaccurate in quantifying DNA samples contaminated with PCR inhibitors.

3.3.3. Comparison of SNP genotyping methods.

For each of the four 5' nuclease assays, all 335 DNA samples were tested (fig. 3.7, 3.9, 3.11 and 3.13). At least thirty of these samples were also tested using allele specific ARMS-PCR for three out of the four assays, TNF–308 (A/G) (N = 87) (fig. 3.8), IL1B-511 (C/T) (N = 50) (fig.3.10) and IL6-174 (G/C) (N = 30) (fig 3.12). Only ten samples were tested using the IL4-590 ARMS-PCR method, of which none were adequately genotyped (fig. 3.14). This method required a generic PCR amplification of the IL4 promoter region (253bp amplicon) followed by ARMS-PCR to detect the SNP (131bp amplicon). This assay required extensive optimisation before it would be applicable for these DNA samples from archival tissue and was not pursued any further. The difference in the number of samples tested was dependent on the time required to genotype samples, the 5' nuclease method is designed as a high throughput system, whereas ARMS-PCR is a low to medium throughput system.

Fig. 3.7. The 5' nuclease assay for genotyping the TNF-308 SNP.

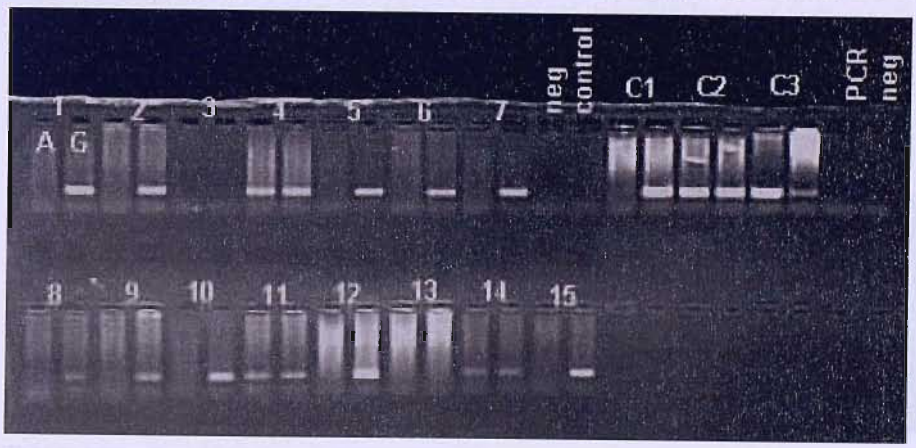


•AA homozygote
• AG heterozygote
• GG homozygote
• negative controls: includes DNA extraction negative controls and PCR negative controls (H_2O).

X undetermined

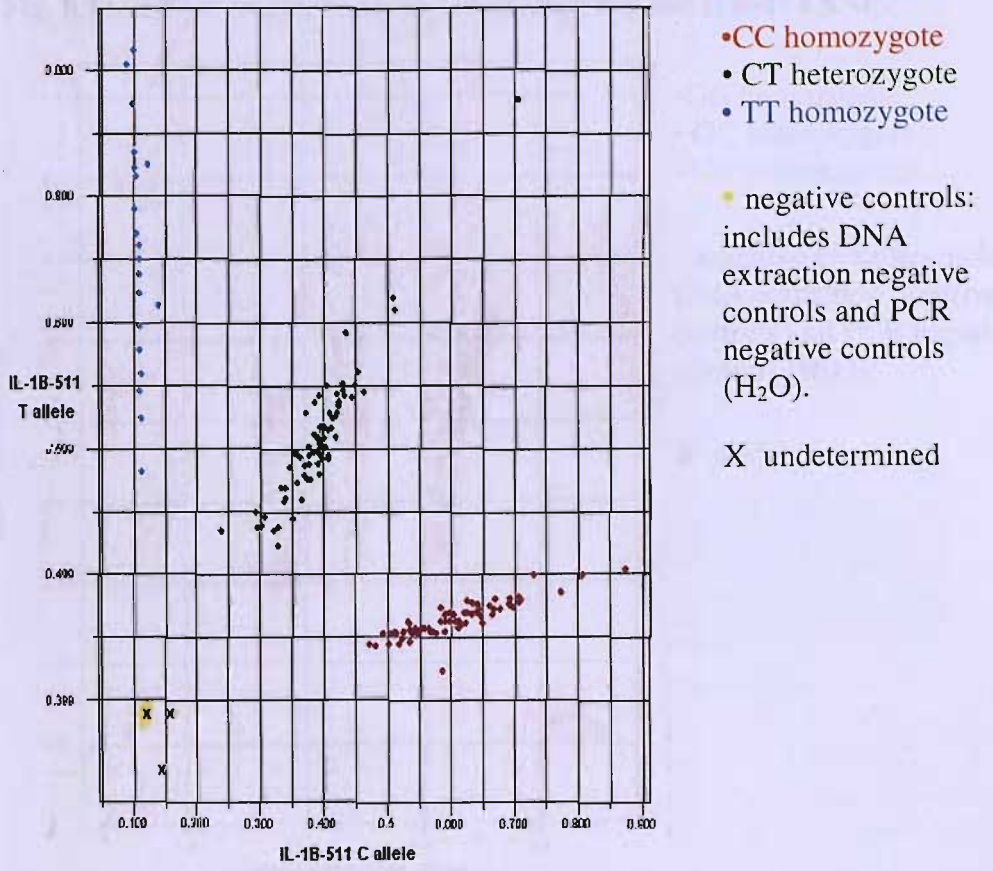
Each dot represents an individual DNA sample, typed by a single 10µl PCR reaction.

Fig. 3.8. Allele specific ARMS-PCR assay for genotyping the TNF-308 SNP.



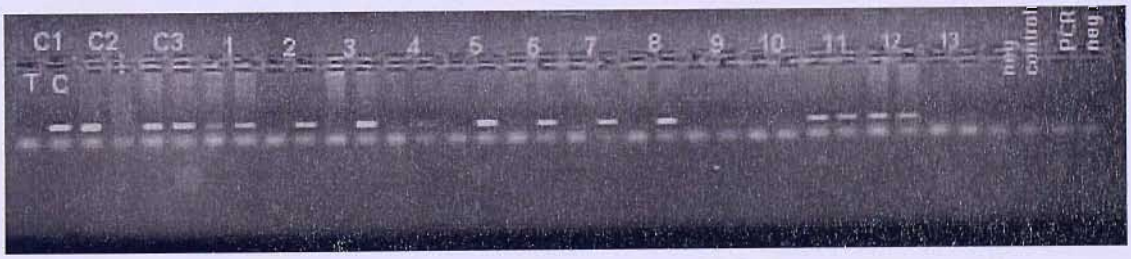
DNA samples 1-15 were extracted from archival gastritis biopsies and tested using the ARMS-PCR assay designed to genotype for the TNF-308 SNP. Two 10µl PCR reactions with primers specific for either the A or G allele are required to type each sample. C1, C2, and C3 are the positive DNA control samples, which genotyped as GG, AG and AA respectively. Samples 1, 2, 5 to 10, 12 and 15 are homozygous for the G allele. Samples 4, 11 and 14 are AG heterozygotes. The rare AA homozygote is not present amongst any of these samples tested. DNA samples 3 and 13 have failed to amplify any specific products. Neg control = DNA extraction negative control; PCR neg = PCR negative control (H_2O).

Fig. 3.9. The 5' nuclease assay for genotyping the IL1B –511 SNP.



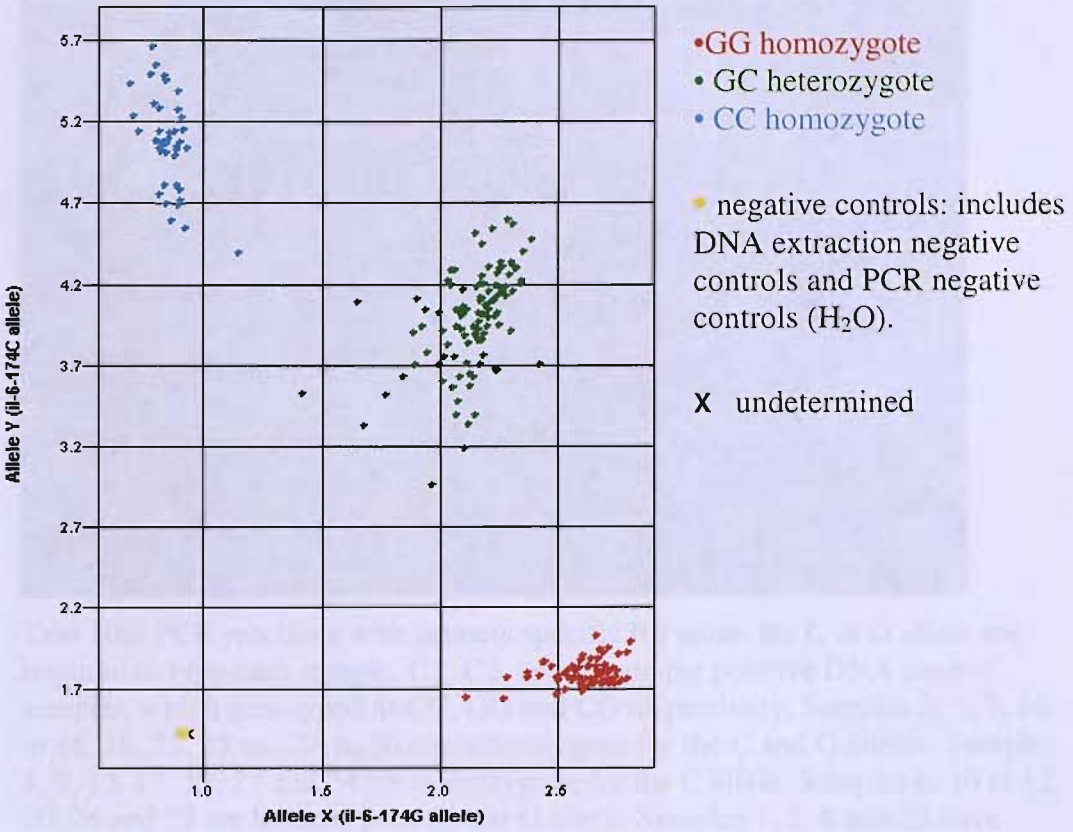
Each dot represents an individual DNA sample, typed by a single 10µl PCR reaction.

Fig. 3.10. Allele specific ARMS-PCR assay for genotyping the IL1B –511 SNP.



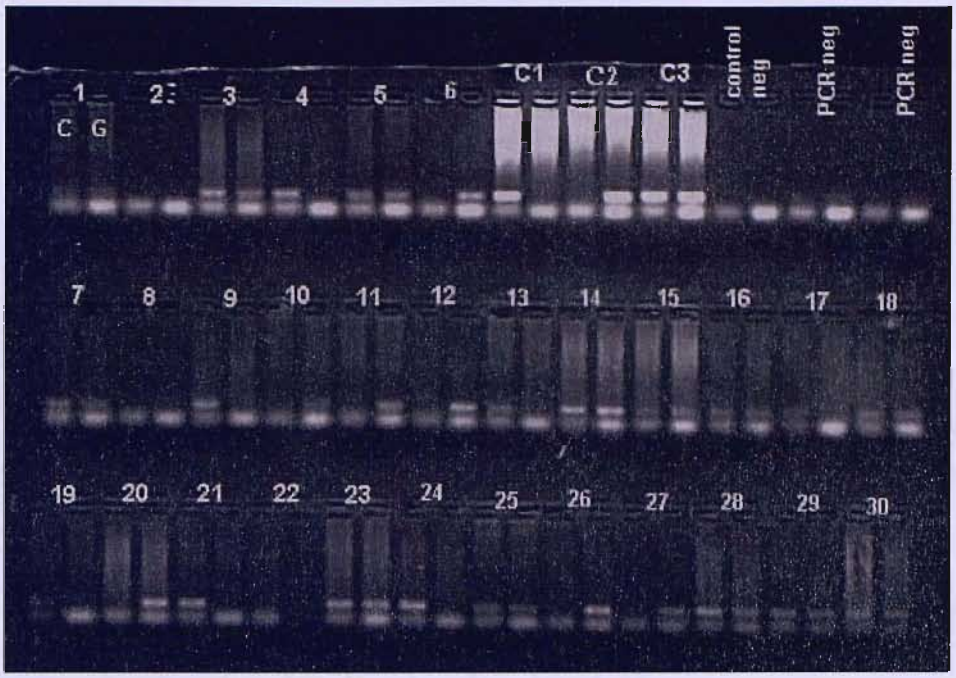
DNA samples 1-13 were extracted from archival gastritis biopsies and tested using the ARMS-PCR assay designed to genotype for the IL1B –511 SNP. Two 10µl PCR reactions with primers specific for either C or T allele are required to type each sample. C1, C2, and C3 are the positive DNA control samples, which genotyped as CC, TT and TC respectively. Samples 1, 11 and 12 are heterozygous for C and T alleles. Samples 2 to 8 are homozygous for the C allele. Samples 9, 10 and 13 have failed, although there are very weak products present for the C allele in samples 9 and 13. Neg control = DNA extraction negative control; PCR neg = PCR negative control (H_2O).

Fig. 3.11. The 5' nuclease assay for genotyping the IL6-174 SNP.



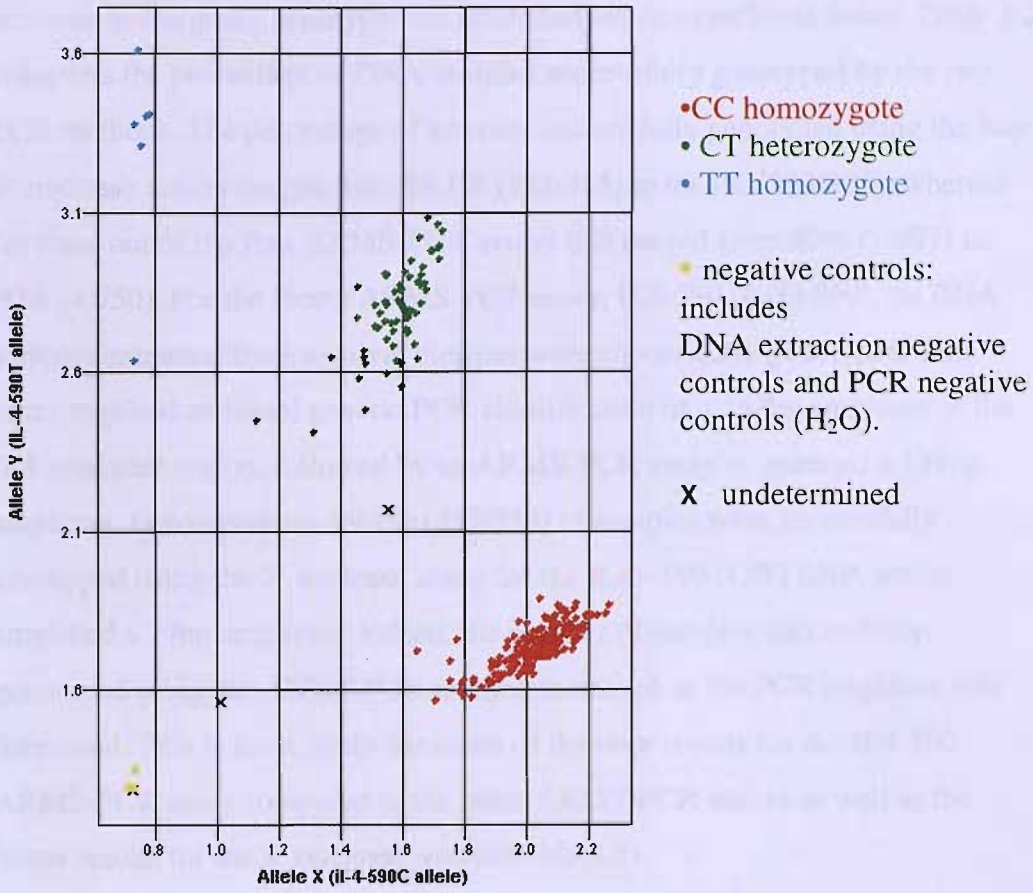
Each dot represents an individual sample, typed by a single 10µl PCR reaction.

Fig.3.12 ARMS-PCR assay for genotyping the IL6-174 SNP.



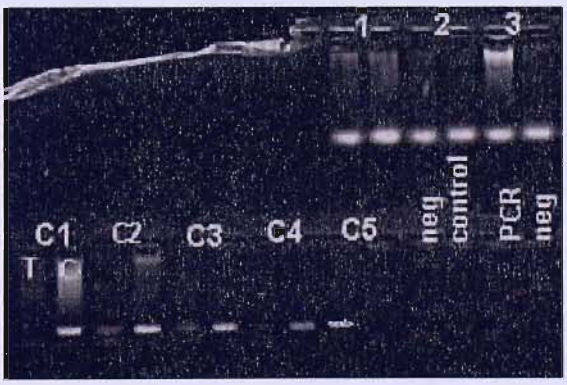
Two 10µl PCR reactions with primers specific for either the C or G allele are required to type each sample. C1, C2, and C3 are the positive DNA control samples, which genotyped as CC, GG and CG respectively. Samples 3, 5, 7, 14 to 16, 18, 23, 25 and 28 to 30 are heterozygous for the C and G alleles. Samples 4, 9, 13, 17, 19, 21 and 24 are homozygous for the C allele. Samples 6, 10 to 12, 20, 26 and 27 are homozygous for the G allele. Samples 1, 2, 8 and 22 have failed out of the 30 samples tested. Neg control = DNA extraction negative control; PCR neg = PCR negative control (H_2O).

Fig. 3.13. The 5' nuclease assay for genotyping the IL4-590 SNP.



Each dot represents an individual sample, typed by a single 10µl PCR reaction.

Fig. 3.14. ARMS-PCR for genotyping the IL4-590 SNP.



Positive DNA control samples were extracted from peripheral blood samples and genotyped as CC for C1, TC for C2 to C4 and TT for C5. DNA samples 1 to 3, which were extracted from archival biopsies, have failed to amplify any PCR products. Neg control = DNA extraction negative control; PCR neg = PCR negative control (H_2O).

There were no typing discrepancies between the two methods for all samples successfully genotyped. This indicated that both assays were equally accurate in assigning genotypes for DNA derived from archival tissue. Table 3.2 compares the percentage of DNA samples successfully genotyped by the two PCR methods. The percentage of samples successfully genotyped using the four 5' nuclease assays ranged from 96.1% (322/335) to 99.4% (333/335), whereas for three out of the four ARMS-PCR assays this ranged from 80% (70/87) to 90% (45/50). For the fourth ARMS-PCR assay, IL4-590 (C/T) SNP, no DNA samples extracted from archival biopsies were successfully genotyped. This assay required an initial generic PCR amplification of a 253bp amplicon of the IL4 promoter region, followed by an ARMS-PCR assay to generate a 131bp amplicon. In comparison, 99.1% (332/335) of samples were successfully genotyped using the 5' nuclease assay for the IL4 –590 (C/T) SNP, which amplified a 79bp amplicon. Indeed, the number of samples successfully genotyped using the ARMS-PCR method increased as the PCR amplicon size decreased. This is most likely the cause of the poor results for the IL4-590 ARMS-PCR assay compared to the other ARMS-PCR assays as well as the better results for the 5' nuclease assays (table 3.2).

Table 3.2. Comparison of the percentage of samples successfully genotyped using ARMS-PCR and 5' nuclease assays.

SNP	5' nuclease assay		ARMS-PCR	
	G/N (%)	amplicon (bp)	G/N (%)	amplicon (bp)
TNF-308 (G/A)	322/335 (96.1)	60	70/87 (80)	184
IL1B-511 (C/T)	332/335 (99.1)	66	45/50 (90)	103
IL6 –174 (C/G)	333/335 (99.4)	80	26/30 (87)	108
IL4 –590 (C/T)	332/335 (99.1)	79	0/10 (0)[a]	253/131[b]

The percentage of samples successfully genotyped using the two PCR methods are shown as well as the size of the PCR amplicons. G = number of samples successfully genotyped; N = total number of samples tested; (a) = none of the ten samples tested were successfully genotyped. This PCR required further optimisation before applicable to these DNA samples; (b) = 1st round generic PCR generates a 253bp amplicon of the IL4 promoter region, the 2nd round ARMS-PCR generates a 131bp amplicon;

3.4. Discussion.

Although 300bp lengths of DNA were amplified in all samples tested using the generic gene control PCR, this did not necessarily result in successful amplification using allele specific PCR, as reflected by the variable but significant failure rate in genotyping. This is likely to be due to the stringent PCR conditions required to prevent mis-priming that are necessary for a successful allelic discrimination PCR as opposed to a generic PCR.

The RNase P assay was found to be an inaccurate method to determine the concentration of amplifiable DNA extracted from archival biopsies when compared with the DNA standard of known concentration. The effect of diluting a DNA sample extracted from archival tissue resulted in increasing the efficiency of the PCR, even though a lower concentration of template DNA had to be present. This was probably due to the presence of PCR inhibitors in these DNA samples extracted from formalin-fixed, paraffin-embedded biopsies (Kosel 2001). Dilution of the DNA sample most likely reduced the effect of the PCR inhibitors, and at a dilution of 1:25 or 1:50 in the final PCR volume, sufficient quantity of template DNA was present for successful PCR amplification, whilst minimising the effect of the PCR inhibitors. PCR inhibitors are usually factors that inhibit Taq DNA polymerase and prevent efficient amplification of the template DNA. Therefore, the RNase P assay was suitable for selecting the optimal dilution of a DNA sample extracted from archival tissue for PCR, but not for quantifying the DNA concentration against the known DNA standard. Consequently, all DNA samples were initially diluted 1:5 with TE buffer and assessed in the RNase P assay and if necessary, diluted further to standardise all samples. This ensured all samples subsequently performed with equal efficiency during PCR. Standardisation of the DNA samples improves genotype calling when using PCR assays.

The ARMS-PCR and 5' nuclease assays are equally accurate in genotyping DNA derived from archival biopsies, as there were no genotyping discrepancies. In this study, the 5' nuclease method was more suitable for genotyping the fragmented DNA samples derived from archival biopsies when compared to the ARMS-PCR method. A much higher percentage of samples were successfully genotyped using the 5' nuclease method, which ranged from 96.1% - 99.4% for the four SNP assays, compared to the ARMS-PCR method, which ranged from 0% - 90% for the four SNP assays. The success of an assay

for genotyping these DNA samples was most likely to be dependent on the size of the PCR amplicon, which for the 5' nuclease assays ranged from 60bp to 80bp, compared to the ARMS-PCR assays, which ranged from 103bp to 253bp. The 5' nuclease assay is designed to amplify very short PCR products as the recommended overall amplicon length should be as short as possible but between 50-150bp (chapter 2.2.6.1.1). However, if the ARMS-PCR assays had been designed to amplify shorter products then the percentage of samples successfully genotyped would possibly have increased, as indicated from the results presented in table 3.2. Indeed, the percentage of samples successfully genotyped using the ARMS-PCR method increased as the amplicon length decreased. This is most likely the main reason for the poor results for the ARMS-PCR IL4-590 assay when using this source of DNA, which required an initial amplification of a 253bp product.

Although genotyping using the 5' nuclease method was very successful, a direct comparison with the ARMS-PCR method does require caution. The overall amount of DNA used for genotyping by the two methods was the same and both used 10µl PCR volumes, however the DNA concentration, primer concentration, PCR reagents and PCR cycling conditions were different. The 5' nuclease method used 1µl of 1:5 dilution of DNA in a single 10µl PCR reaction (final DNA dilution 1:50), whereas the ARMS-PCR assay required two separate reactions, each using 0.5µl of 1:5 dilution of DNA in a 10µl PCR volume (final DNA dilution 1:100). However, both methods were used according to published guidelines and from the results in this study more samples were successfully genotyped using the 5' nuclease method compared to ARMS-PCR when using DNA derived from archival biopsy tissue.

The ARMS-PCR method, as currently established for genotyping DNA from archival tissues, has no internal PCR control. Therefore, there is a chance that if one of the two reactions fails a sample may be mistyped as homozygous rather than heterozygous, although results from this study did not reveal any genotyping discrepancies. Incorporation of appropriate internal PCR controls may be problematic when dealing with variably degraded DNA. PCR optimisation to determine the appropriate concentrations of the allele specific primers and the internal control primers would be extensive. Internal control primers are not necessary for the 5' nuclease assay.

The ARMS-PCR method requires two separate reactions and post-PCR experimental manipulation. The 5' nuclease method is a single reaction with no post-PCR manipulation, offering advantages for medium to high throughput genotyping, and this was reflected in the ease of genotyping 335 samples by the 5' nuclease method compared to genotyping between 30-87 samples using ARMS-PCR. From the single scatterplot generated using the 5' nuclease method, genotypes can be simultaneously interpreted for 384 samples. This is ideal for batch calling and randomisation of cases and control samples on a single plate for association studies.

The 5' nuclease method was faster to establish and apply from primer design through to genotyping samples, than the ARMS-PCR method. However, the 5' nuclease method was more expensive (5μl PCR costs 30p /DNA sample/SNP) compared to the ARMS-PCR method (< 10p /DNA sample/SNP). In addition, the initial cost of specialist equipment for 5' nuclease genotyping is much higher compared to establishing the ARMS-PCR method.

3.5. Conclusions.

A dilution factor of 1:50 of the DNA samples extracted from paraffin-embedded, formalin-fixed archival gastric biopsies in the final PCR volume ensured sufficient template DNA was available for successful PCR whilst minimising the effect of PCR inhibitors.

The 5' nuclease method was faster from primer design through to genotyping of DNA samples compared to the ARMS-PCR method and required no optimisation. From this study, although both methods were equally accurate in genotyping DNA derived from archival tissue, a much higher percentage of samples were successfully genotyped using the 5' nuclease method when compared to the ARMS-PCR method, although set up and running costs were three fold greater. Accordingly, the 5' nuclease allelic discrimination method was selected for genotyping cytokine SNPs using DNA derived from archival tissues for the case-control association study in chapter 4.

Chapter 4: Do polymorphisms in cytokine genes influence the development of primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma following *H. pylori* infection?

4.1. Introduction.

H. pylori infection causes a wide spectrum of gastric pathologies from asymptomatic gastritis and peptic ulcer disease to gastric malignancy. As discussed in chapter one, in addition to bacterial virulence factors, the host immune response plays a pivotal role in the clinical outcome of chronic *H. pylori* infection. There are high inter-individual differences in the extent and pattern of gastric inflammation among *H. pylori* infected patients and clinical consequences develop in only a small subgroup.

4.1.1. Cytokines, polymorphisms and association with disease.

Cytokines play a critical role in the regulation of the immune response and a considerable number of cytokine gene polymorphisms have been identified. The majority of known polymorphisms are found in the upstream promoter sequences, in introns and in 3' untranslated regions which in many cases results in differential *in vitro* expression of the respective pro- or anti-inflammatory cytokine e.g. TNFα (Wilson 1997), IL-1β (El-Omar 2000; Rad 2004) and IL-10 (Turner 1997; Eskdale 1998; Rad 2004). However *in vivo*, the situation is certainly more complex and may be influenced by both the stimulus and the cell type (Kroeger 2000; Allen 1999; Gibson 2001). Polymorphisms in the human IL10, IL1B, TNF, IFNG and IL1RN genes have all been reported to influence cytokine expression (table 4.1). Some of these polymorphisms, considered to alter the rate of gene transcription, are thought to influence inflammatory processes in response to infectious diseases and in support of this, many of these polymorphisms show associations with susceptibility to a number of immune-mediated diseases (reviewed in: Bidwell 1999 and 2001; Haukim 2002). Currently, it is unknown if the polymorphisms associated with differential cytokine expression and the overall cytokine genetic profile of an individual results in qualitative or quantitative programming of the inflammatory response. However, there are plenty of examples in the literature that support this hypothesis. For example, associations between polymorphisms

in the promoter of the TNF gene and rheumatoid arthritis (Brinkman 1997), cerebral malaria (McGuire 1994; McGuire 1999; Knight 1999) and IL10 promoter polymorphisms with systemic lupus erythematosus (Lazarus 1997; Gibson 2001) and pathogenesis to AIDS (Shin 2000). Recent reports have linked cytokine gene polymorphisms with influencing gastric inflammation following *H. pylori* infection (Rad 2004) and subsequent development of gastric adenocarcinoma (El-Omar 2000). In addition, a number of studies have reported associations between TNFα and/or lymphotoxin-α (LTα) and certain cancers, including chronic lymphocytic leukaemia (Demeter 1997), non-Hodgkin's lymphoma (Warzocha 1998; Juszczynski 2002) and breast cancer (Chouchane 1997). In cutaneous malignant melanoma, IL10 genotypes associated with low IL-10 expression *in vitro* are associated with disease susceptibility (Howell 2001).

H. pylori infection is characterised by a strong neutrophil and lymphocyte infiltration. The T helper response towards *H. pylori* is generally considered to be Th1 phenotype, leading to a cell-mediated immune response. This Th1 response may contribute to cancer development as down regulation of the Th1 response in mice with concurrent helminth infection was shown to protect against atrophy, intestinal metaplasia and invasive gastric carcinoma (Fox 2000). IL-12 is the key cytokine in directing the immune response towards a Th1 phenotype, which acts synergistically with IL-18, whereas IL-4 plays a key role in the differentiation of T cells into Th2 cells that promote a humoral response. Important cytokines characterising Th1 mediated immune responses are IFNγ, TNFα, IL-1β and IL-8, all being up-regulated during chronic *H. pylori* infection. IL-10 and TGFβ, which are highly expressed in the *H. pylori* infected stomach (Karttunen 1997), are important regulatory cytokines influencing the balance of the cell- mediated immune response.

Table 4.1. Selection of Cytokine SNPs.

Cytokine Gene	Up-regulated in *H. pylori* infection	Function	Poly-morphism	Effect of polymorphism	Associated with autoimmune/inflammatory disease
IL1A	Yes (1)	Binds same receptor as IL-1β. Significantly more IL1A mRNA in cagA+ compared to cagA- *H. pylori* infected patients (1)	-889	T allele at –889 creates a consensus sequence for the transcription factor skn-1. –889TT associated with increased levels of IL1A mRNA and protein levels compared to –889CC genotype (2, 3).	Juvenile rheumatoid arthritis (4). Alzheimer's disease (2).
IL1B	Yes (1)	Key pro-inflammatory cytokine. Inhibits gastric acid secretion. Significantly more IL-1β in cagA+ compared to cagA- *H. pylori* infected patients (1,41).	+3953	Affects transcription (5). T allele resulted in 2-4 fold increase in IL-1β from monocytes when stimulated with LPS compared to monocytes with two copies of C allele (6).	
			-31	The –31T polymorphism creates a TATA sequence. Using electrophoretic mobility shift analysis of LPS stimulation of PMNs indicated that one or more proteins (presumably transcription factors) interacted with IL1B-31T allele but not the –31C allele (7).	–31C and IL-1RN*2 haplotype associated with increased risk of chronic hypochlorydria and gastric cancer (7). This haplotype is also associated with an increased risk of severe inflammation and intestinal metaplasia (8).
			-511	-511T allele in strong LD with –31C (>90%LD), assoc with slight but not significant increase in IL-1β (9, 10). Effect of –511 polymorphism may be due to complete LD with –31 polymorphism.	Increased risk of severe inflammation and intestinal metaplasia (8).
IL1RN	?	The IL-1 receptor antagonist inhibits binding of IL-1α and IL-1β to their common receptor.	86bp VNTR (intron 2)	Allele 2 associated with increased IL-1ra in plasma and enhanced IL-1β secretion (9, 10, 12, 13).	Increased risk of severe inflammation and intestinal metaplasia (8), hypochlorhydria and gastric adenocarcinoma (7).
		In near complete LD with the ILRN 86bp VNTR (15).	+2018 (exon 2)	IL-1RN+2018 allele2 (C allele) in near complete LD (>99%) with IL-1RN 86bp VNTR allele 2 (15) and is associated with decreased levels of IL-1ra protein (6).	
IL4	No (16,17,18)	Crucial for the differentiation of naïve T cells into Th2 cells that promote humoral immunity. ↑SOM and ↓gastrin (19).	-590	Creates an NFAT binding site. T allele 3-fold increase in transcription compared to C allele (45, 55).	Malignant B-cells from MALT lymphomas proliferate in response to CD40 signalling and IL-4 (20).
IL6	Yes (21,22)	Expression induced in response to IL-1, TNF, LPS. B-cell differentiation.	-174	-180 to –123 crucial for induction of expression in response to IL-1/TNF. -174G→C creates a potential NF-1 transcription binding site. GG genotype is associated with ↑ expression *in vitro* (23).	Systemic onset juvenile chronic arthritis (24).

IL8	Yes (1)	This chemokine attracts neutrophils to the site of inflammation. Significantly more IL-8 in cagA+ compared to cagA- *H. pylori* infected patients (1,14,40,48).	-251	A allele associated with higher IL-8 levels (53).	Associated with severe bronchiolitis following RSV infection (54).
IL10	Yes (25)	Potent anti-inflammatory cytokine. B cell maturation. IL-10 deficient mice develop spontaneous chronic enterocolitis. IL-10 deficient mice have 100 fold decrease in *H. pylori* colonisation (26).	-592	GCC (-1082, -819C, -592C) associated with high IL-10 expression and ATA (-1082A, -819T, -592A) with low expression. -819 in 100% LD with –592 (10,11).	Malignant B-cells from MALT lymphomas proliferate in response to CD40 signalling and IL-10 (20).
IL12	Yes (17,27)	Secreted by macrophages and dendritic cells in response to infections. Promotes Th1 response. Induces IFNγ production by T and NK cells.	IL12B+1188 (Encodes IL-12p40 subunit)	C allele associated with increased IL-12 secretion (28).	Type 1 diabetes (29) Cerebral malaria (30)
IL18	Yes (32)	Mainly produced by activated macrophages. Acts synergistically with IL-12 to promote Th1 response.	-137 and -607	-137C and –607A associated with low expression (35).	Role in chronic inflammatory diseases (33). Up-regulated in Crohn's disease (34).
TNF	Yes (17,21)	Key pro-inflammatory cytokine. Important in development and organisation of lymphoid tissue. Inhibits gastric acid secretion. Transcription controlled by LPS and IFNγ.	–308	A allele in some studies has been associated with heightened TNFα secretion (36). G creates a binding site for AP-2 (activator protein-2).	Anti-TNF is a treatment for Crohn's disease and rheumatoid arthritis. TNF –308A allele associated with greater risk of death from cerebral malaria and associated with higher levels of circulating TNF (50, 51).
			-376	A allele creates an Oct-1 binding site (37,38).	-376A allele associated with cerebral malaria (37).
LTA	?	Involved in development of secondary lymphoid organs. Soluble LTα binds same receptors as TNF.	+252	Lies within a phorbol ester responsive DNA element. G allele is associated with ↑ LTα production (39).	TNF-308A, LTA+252G haplotype associated with increased risk of Diffuse large B-cell lymphoma (40)
IFNG	Yes (16,17)	IFNγ enhances antibacterial immunity. Key activator of macrophages and induces microbicidal state. ↑gastrin, ↓SOM (19).	+874	T allele in LD with allele 2 of a penta-allelic CA microsatellite polymorphism, which has been associated with high IFNγ production (42, 43).	T infiltrating cells in two cases of MALT lymphoma predominantly produce IFNγ (44).

IFNGR1	?	Receptor for IFNγ.	-56	C→ T SNP affects expression levels (31).	Genome wide linkage analysis with *H. pylori* infection (52).
TGFB1	No (46)	Anti-inflammatory. Knock out mice develop severe multi-organ inflammation.	-509	SNP influences expression (47). T allele associated with increased levels TGFβ1.	TGFβ1 detected in tumours of gastric cancer patients (49).

SOM = somatastatin; LPS = lipopolysaccharide; PMNs = polymorphonuclear cells; LD = linkage disequilibrium; ? = unknown.

1 Peek 1995; 2 Dominici 2002; 3 Kornman 1998; 4 McDowell 1995; 5 Pociot 1992; 6 Andus 1997; 7 El-Omar 2000; 8 Rad 2003; 9 Hurme 1998; 10 Rad 2004; 11 Turner 1997; 12 Santtila 1998; 13 Hwang 2002; 14 Crabtree 1993; 15 Smith 2004; 16 Karttunen 1995; 17 D'Elios 1997; 18 Shimizu 2004; 19 Zavros 2003; 20 Greiner 1997; 21 Crabtree 1991a; 22 Lindholm 2001; 23 Fishman 1998; 24 Terry 2000; 25 Karttunen 1997; 26 Chen 2001; 27 Bauditz 1999; 28 Seegers 2002; 29 Morahan 2001; 30 Morahan 2002; 31 Rosenzweig 2004; 32 Tomita 2001; 33 Akira 2000; 34 Monteleone 1999; 35 Giedraitis 2001; 36 Wilson 1997; 37 Knight 1999; 38 Brinkman 1997; 39 Messer 1991; 40 Rothman 2006; 41 Yamaoka 1997; 42 Pravica 1999; 43 Pravica 2000; 44 Hauer 1997; 45 Wray 2003; 46 Monteleone 2004; 47 Grainger 1999; 48 Noach 1994; 49 Ebert 2000; 50 Wilson 1992; 51 McGuire 1994; 52 Thye 2003; 53 Hull 2000; 54 Hull 2001; 55 Rockman 2003.

For this study, SNPs associated with differential expression of candidate pro- and anti-inflammatory cytokines, particularly cytokines with altered expression as a result of the immune response to *H. pylori*, were investigated in primary gastric extra- nodal marginal zone B-cell (MALT) lymphoma patients (abbreviated to GML) and a large *H. pylori* infected patient control group with uncomplicated chronic gastritis from three European populations. The aim of the study was to determine the influence, if any, of polymorphisms in cytokine genes on the development of GML subsequent to *H. pylori* infection. These results will be of interest in comparison with the IL-1β/IL-1 receptor antagonist results associated with gastric adenocarcinoma reported by El-Omar *et al* (2000; 2003; Furuta 2002).

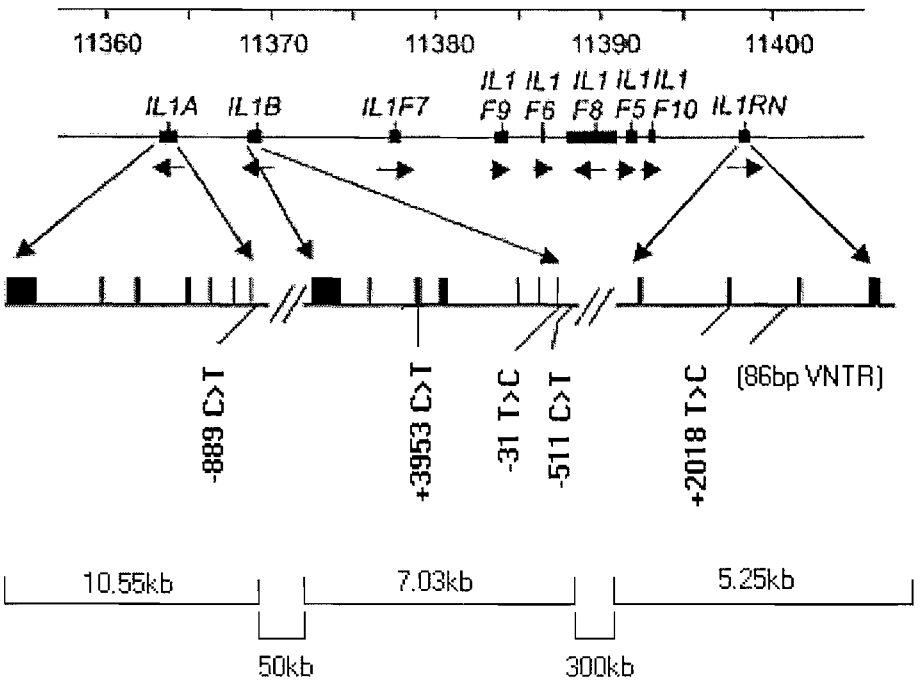
4.2. Materials and methods.

4.2.1. Selection of cytokine gene polymorphisms.

Eighteen SNPs were selected from 14 cytokine genes and their promoter regions. The selection of the SNPs was based on one or more of the following criteria (table 4.1): the cytokine has an altered expression level in response to *H. pylori* infection; there is evidence of the SNP affecting expression levels *in vivo* and/or *in vitro*; there is evidence that the SNP is associated with an inflammatory disease; and finally both alleles are common, with an allele frequency equal to or greater than 20%, in caucasoids. All alleles except the rare TNF-376A allele were of sufficient frequency for the study to have 80% power to detect significant differences in genotype frequencies between cases and control groups conferring an odds ratio of greater than 2 with 95% confidence. Power calculations were performed using Epi Info Revision 2 (2003) statistical package and based upon the number of cases and controls examined using the frequency of the rarest genotype for a SNP among the control subjects.

Based on these criteria, five SNPs within the IL1 gene cluster (chromosome 2q12-14) were selected (fig. 4.1); IL1A-889 (C/T), IL1B-31 (T/C), IL1B-511(C/T), IL1B+3953 (C/T) and IL1RN+2018 (T/C). Three SNPs within the TNF region (chromosome 6p21.3) were selected; TNF-308 (G/A), TNF-376 (G/A), and LTA+252(A/G). Finally, a further ten SNPs from cytokines or their receptors that modulate T helper lymphocyte responses were selected; IL4-590 (C/T), IL6-174 (G/C), IL8-251 (T/A), IL10-592 (C/A), IL12B+1188 (A/C), IL18-137 (G/C), IL18-607 (C/A), IFNG+874 (A/T), IFNGR1-56 (T/C), and TGFB1-509 (C/T).

Fig. 4.1. IL1 gene cluster on chromosome 2q12-14.



Organisation of the IL1 gene cluster on chromosome 2q showing the approximate base position ($x10^4$). Direction of transcription is shown beneath each gene by a horizontal arrow. Exons are shown by vertical lines or boxes. Polymorphic nucleotides examined in the IL1A, IL1B and IL1RN genes are shown. The position of the IL1RN 86bp VNTR in relation to +2018 SNP is also shown. Adapted from Smith 2004.

4.2.2. Selection of GML patients and *H. pylori* gastritis controls.

Ethical approval for this project was obtained from Southampton and SW Hants Local Research Ethics Committee. This project has received German and Dutch ethical approval for the use of archival human material for genetic studies.

4.2.2.1. German patients and controls.

Professor Andreas Neubauer and colleagues from the Phillips University, Marburg, Germany, kindly provided DNA extracted from peripheral blood from 79 confirmed primary gastric extra-nodal marginal zone B-cell lymphoma cases (GML). All cases were diagnosed as low-grade, stage EI, GML with *H. pylori* infection. In addition, archival gastric biopsies from 154 German patients diagnosed with uncomplicated *H. pylori* gastritis were

provided as a German control group. The ratio of male to female for the GML cases was 1 : 1.3 and 1.3 : 1 for the control group. The age range was 35-90 years with a mean of 69.5 years for the GML group compared to an age range of 14-91 years with a mean of 55.8 years for the control group (table 4.2).

4.2.2.2. Netherlands patients and controls.

Professor Daphne de Jong from the Netherlands Cancer Institute, Amsterdam, kindly provided 72 cases diagnosed between 1994 and 2002, and confirmed as low-grade primary gastric extra-nodal marginal zone B-cell lymphoma. The majority (90%, 65/72) were confirmed as *H. pylori* positive, for the remaining seven cases infection status with *H. pylori* was unknown. A consecutive series of 224 uncomplicated *H. pylori* gastritis cases diagnosed between 1999 and 2002 were provided as a control group. Tissue sections from an archival gastric biopsy of uninvolved, non-malignant mucosa were provided for all cases and controls. The ratio of males to females was 1.3 : 1 for the GML group compared to 1 : 1.1 for the control group. The age range was 27-86 years with a mean of 59.2 years for the GML group compared to the control group, which had an age range of 19-88 years with a mean of 60.1 years.

4.2.2.3. UK patients and controls.

Dr Andrew Wotherspoon from the Royal Marsden Hospital, Sutton, UK kindly provided twenty-three cases confirmed as *H. pylori* positive, primary gastric low-grade extra-nodal marginal zone B-cell lymphoma. A further 32 cases diagnosed between 1990 and 2001 at Southampton General Hospital, UK were selected for this study. Only 11 of the 32 Southampton cases (34%) were confirmed as *H. pylori* positive. For the remaining Southampton cases infection with *H. pylori* was unknown. The lack of detection of *H. pylori* in the Southampton cases may be due to the presence of malignant tissue in these biopsies. *H. pylori* is often absent at the site of the lymphoma where destruction of the normal tissue architecture appears to be incompatible with the ability of the bacteria to reside in this micro-niche. The UK control group was a sequential series of 190 cases diagnosed between 1997 and 2001 at Southampton General Hospital as *H. pylori* positive gastritis with acute and chronic inflammation. The gastritis controls had no history of gastro-intestinal

dysplasia or malignancy, Coeliac or Crohn's Disease. Diagnosis of all cases and controls was based on histological examination from gastric biopsies. The pathology reports and biopsy sections were reviewed again by Dr Adrian Bateman prior to inclusion in this study. Tissue sections from an archival gastric biopsy were provided for all cases and controls. The ratio of male to female in the GML group was 2.4 : 1 with an age range of 26-94 years and the mean age was 65.5 years. The ratio of male to female in the control group was 1.1 : 1 with an age range of 21-97 years and the mean age was 60.5 years.

Table 4.2. Cases and controls of the association study:

	number of cases	age range (yrs)	mean age (yrs)	ratio of male : female	diagnosis	known infection with *H. pylori* (%)
Netherlands:						
GML cases	72	27-86	59.2	1.3 : 1.0	low-grade GML	90%
gastritis controls	224	19-88	60.1	1.0 : 1.1	*H. pylori* gastritis	100%
German:						
GML cases	79	35-90	69.5	1.0 : 1.3	low-grade GML	100%
gastritis controls	154	14-91	55.8	1.3 : 1.0	*H. pylori* gastritis	100%
UK:						
Royal Marsden GML cases	23	26-94	65.5	2.4 : 1.0	low-grade GML	100%
Southampton GML cases	32				low-grade GML	34%
gastritis controls	190	21-97	60.5	1.1 : 1.0	*H. pylori* gastritis	100%
GML= primary gastric extra-nodal marginal zone B-cell lymphoma.						

4.2.2.4. Risk of population stratification in the patient and control groups.

Population stratification due to inappropriate matching of cases and controls is a confounding factor in association studies. In this study, the male to female ratio in the Netherlands and German GML cases and gastritis control groups and the UK gastritis control group are not significantly different and are comparable to the ratio seen in GML described by Isaacson *et al* (2001). Isaacson state that most GML cases occur in adults with a median age of 61yrs with a slight female preponderance and a male: female ratio of 1.0 : 1.2. However, caution must be applied to any interpretation of the UK GML cases, as there are nearly 2.5 times more males to females in this study group. In addition, careful consideration must be given to the age differences that are present between each GML and gastritis control group (table 4.2), as this has the potential to result in spurious associations, due to differences in population

stratification between cases and controls. In-order to reduce this risk, cases and controls should ideally be age-, sex- and ethnicity matched. However, this will reduce the power of this study as the numbers of cases and controls would be reduced. Therefore, the risk of these factors would be considered post-analysis of the genotype frequencies.

4.2.3. DNA extraction.

For all cases and controls DNA was extracted from an archival, paraffin wax embedded, formalin-fixed, archival gastric biopsy except for the 79 German GML cases from which peripheral blood derived DNA was provided. In addition, as previously mentioned, DNA was extracted from non-malignant tissue except for the 32 Southampton GML cases, which had variable amounts of malignant tissue. For all archival biopsies five 10μM sections were cut as previously described in chapter 2.2.3. During the DNA extraction process no more than thirty samples were processed at any one time in order to minimise the risk of contamination. In addition, batches were processed in parallel with an empty 1.5ml microcentrifuge tube, which was subsequently used as a negative control. This DNA extraction negative control was tested in all subsequent PCR assays to detect the presence of any contamination.

4.2.4. Assessment of the quality and concentration of DNA.

The quality of extracted DNA was assessed for a proportion of cases using the multiplex generic gene control PCR as previously described (chapter 2.2.4.). For the Netherlands group, 51/72 (71%) of the GML cases and 102/224 (46%) of the *H. pylori* gastritis control group were assessed (table 4.2); For the UK group, 35/55 (64%) of the GML cases and 31/190 (16%) of the *H. pylori* gastritis control group were assessed; For the German *H. pylori* gastritis control group 31/154 (20%) were assessed. As DNA was extracted from peripheral blood for the German GML cases it was assumed good quality DNA of a high molecular weight was obtained and therefore these samples were not assessed using the gene control PCR. However, all DNA samples were assessed using the RNase P assay. To determine the optimal DNA dilution for subsequent genotyping, the DNA from all cases and controls were initially diluted 1:5 with

TE buffer and assessed using the RNase P assay and real time quantitative PCR as described in chapter 2.2.5. This determined the optimal dilution of DNA for efficient PCR amplification and standardised the samples for the SNP genotyping assays.

4.2.5. Preparation of DNA for SNP genotyping for the case-control association study.

The methodology for the preparation of genotyping trays is presented here to emphasize the generation of good quality 384-well typing plates that contained a mix of GML patient and gastritis control group DNA samples from the three European populations, as well as positive and negative DNA controls and at least 4% of replicate samples. Using the following method the risk of bias in genotype calling was reduced.

The Quadra robotic arm (Tomtec) is an automated instrument that will accurately dispense reagents in to a 384-well plate format. This instrument was used to dispense 2µl DNA (1-5ng/µl) into 384-well plates for subsequent genotyping using the 5' nuclease method. To achieve this, four x 96-well microtitre plates (AB gene), each well containing 60µl of DNA (approximately 1-5ng/µl) were used as mastertrays from which 2µl of DNA was dispensed into 384-well TaqMan® optical microplates (Applied Biosystems) using the Quadra robotic arm. This resulted in each 384-well plate comprised of an automated mix of cases and control DNA samples, negative controls consisting of no DNA template controls (10/384 wells) and DNA extraction negative controls (12/384 wells), positive controls of known genotype if available, and between 4% and 12.5% replicates. The percentages of replicates were as follows; Netherlands GML cases 9/72 (12.5%), Netherlands gastritis controls 18/224 (8%), UK GML cases 6/55 (11%), UK gastritis controls 11/190 (6%) and German gastritis controls 6/154 (4%). The positive controls where peripheral blood derived DNA samples previously genotyped by ARMS-PCR and at least two samples of known genotype for each of the three possible genotypes (homozygous for allele 1, heterozygous and homozygous for allele 2) were tested in each of the following assays; IL1B-31, IL1B-511, IL4-590, IL6-174, IL8-251, IL10-592, IL12B+1188, IFNG+874, TNF-308, LTA+252, and TNF-376 (the rare TNF-376AA genotype was not tested). Positive controls were not available for the

following assays; IL1A-889, IL1B+3953, IL1RN+2018, IL18-137, IL18-607, IFNGR1-56, and TGFB1-509. The 384-well plates were sealed with film (AB gene) and stored at -20°C until required and used within one month of production.

4.2.6. Genotyping using 5' nuclease allelic discrimination assays.

TaqMan® 5' nuclease allelic discrimination assays (5' nuclease assay) were developed for genotyping DNA derived from paraffin-embedded, formalin-fixed biopsies and peripheral blood (chapter 2.5). For these assays, DNA samples derived from archival biopsies were genotyped using 10μl PCR volumes. DNA from the German GML cases were derived from peripheral blood and were tested separately using 5μl PCR volumes.

The specificity of PCR amplification of these 5' nuclease assays was confirmed by visualisation of the size of the amplicon product using 6% polyacrylamide gel electrophoresis and staining with ethidium bromide (chapter 2.2.1-2). This was performed for all 5'nuclease assays using the amplicon from the UK gastritis samples 1, 2 and 3. In addition, to confirm the accuracy of genotype calling for the eighteen SNP assays, three samples, one of each genotype, were subsequently confirmed by sequencing as described in chapter 2.2.9.

4.2.7. Statistical analysis.

All sample information and genotyping results were entered on to the statistical program database, SPSS (version 10.0) and the genotype and allele frequencies determined. The Hardy-Weinberg (H-W) equation was used to determine whether the genotype frequencies (GF) observed were in agreement with expected values according to H-W equilibrium. Genotype frequencies were compared between cases and controls for each population using Fisher's Exact 2x2 contingency tables and a *P* value of less than 0.05 was considered significant.

Dr. N. Maniatis, Division of Human Genetics, University of Southampton, kindly analysed the linkage disequilibium (LD) and the probable haplotype frequencies of the four SNPs within the IL1A and IL1B genes. The disequilibrium coefficient (Rho) was used (cedar.genetics.soton.ac.uk) and is a

statistical measure of the LD between two SNPs. It is dependent on the distance and the rate of recombination between the two SNPs. The probable haplotypes were determined using the program 'SNPHAP' (www-gene.cimr.cam.ac.uk/clayton/software/snphap). This program estimates the haplotype frequencies using data from unrelated individuals by an expectation-maximisation algorithm to calculate maximum likelihood estimates of haplotype frequencies from the genotype frequencies.

4.3. Results.

4.3.1. The quality and concentration of extracted DNA.

The DNA extraction negative controls were tested using the gene control PCR, the RNase P assay and all 5' nuclease genotyping assays and were found to be negative in all assays indicating there was no detectable contamination from DNA extraction through to PCR set up. Table 4.3 shows the percentage of samples tested using the gene control PCR and the percentage of these samples able to amplify 100, 200, 300 or 400bp products. From this, the overall DNA quality of the Netherlands samples (fig. 4.2), the German samples and the UK gastritis control samples was excellent as the majority of samples amplified over 300bp products. The quality of DNA from the UK GML cases was variable and of a poorer quality compared to the DNA extracted from the UK gastritis control samples. DNA from four of the assessed UK GML samples were very degraded and very little 100bp amplicon was detected using the gene control PCR. In addition, of the 35 samples tested, 200bp and 300bp amplicons were only detected in 13 (37%) samples and 400bp products were not amplified from any of the UK GML cases. Therefore, the quality of the UK GML DNA samples was relatively poor. The variation in DNA quality detected between the different groups was likely to be due to the source of material from which the DNA was extracted. The control DNA samples from all three populations were extracted from archival gastric biopsies and, as can be seen from table 4.3, had comparable DNA quality. DNA samples from the German GML cases were derived from peripheral blood and therefore not assessed using the gene control PCR, as it was assumed that the DNA fragment length would not be extensively degraded. DNA from the Netherlands GML cases was extracted from archival gastric biopsies of uninvolved, non-malignant gastric mucosa and although the

DNA quality appeared slightly reduced (300bp amplicons were detected in 39/ 51 (76%) of samples tested), it was still very good. However, for the UK GML cases the DNA was extracted from an archival biopsy containing variable amounts of lymphoma tissue. The presence of lymphoma tissue may have been a factor influencing the overall poorer quality of the DNA from this group. In addition, the formalin-fixation time is a critical factor in influencing the size of extracted DNA. Fixation in un-buffered formalin for >24hours results in the extraction of smaller fragments of DNA (Inoue 1996). The fixation protocols for the archival samples in this study are unknown, but this may have influenced the quality of the DNA from the UK GML cases. However, this assay confirmed that sufficient intact 100bp lengths of DNA were available for PCR amplification in all samples tested except for four of the UK GML case samples. This length of DNA is sufficient for successful genotyping using the designed 5' nuclease assays, which require less than 80bp lengths of DNA for amplification.

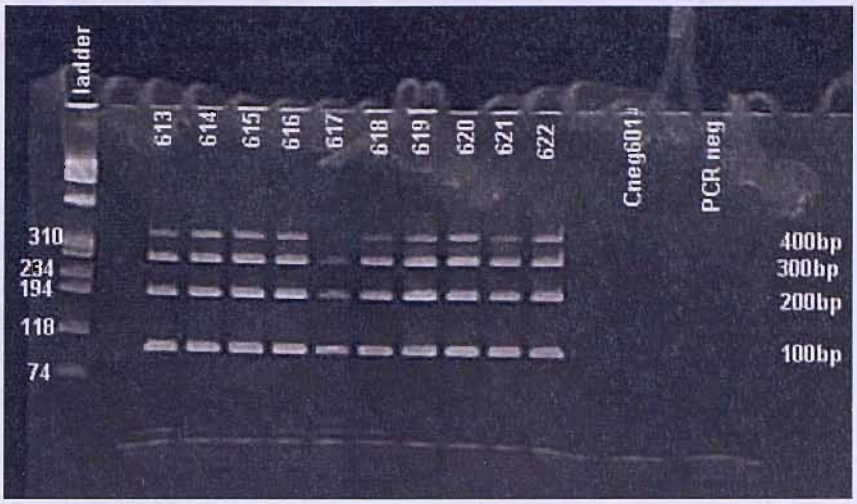
Table 4.3. Assessment of the quality of DNA using the gene control PCR.

Sample type	Percentage tested (N)	Gene control PCR amplicon size: Percentage detection rate in samples tested.			
		100bp	200bp	300bp	400bp
Netherlands gastritis	46% (102/224)	100%	100%	100%	83% (85/102)
Netherlands GML	71% (51/72)	100%	100%	76% (39/51)	16% (8/51)
German gastritis	20% (31/154)	100%	100%	100%	81% (25/31)
German GML	Not tested*	-	-	-	-
UK gastritis	16% (31/190)	100%	100%	100%	84% (26/31)
UK GML	64% (35/55)	89% (31/35)	37% (13/35)	37% (13/35)	0%

* Peripheral blood derived DNA.

Table 4.3 and fig. 4.2 show the results of the gene control PCR. This assay indicated the quality and fragment size of extracted DNA available for PCR amplification.

Fig. 4.2. Example of the quality of DNA extracted from archival biopsies.



Gene control PCR of Netherlands gastritis samples 613-22. All samples except sample 617 amplified up to 400bp products.

4.3.2. Standardisation of DNA samples for subsequent genotyping.

All DNA samples were assessed using the RNase P assay (chapter 2.2.5) to determine the optimal DNA concentration for efficient PCR amplification and standardise the DNA samples for subsequent genotyping. DNA from German GML cases (DNA provided from peripheral blood), diluted at 1:5 had a DNA concentration of between 2ng/µl to 400ng/µl DNA when assessed against the known DNA standard in the RNase P assay (table 4.4). From these results the DNA concentration was adjusted to between 2-10ng/µl to standardise all DNA samples for subsequent genotyping.

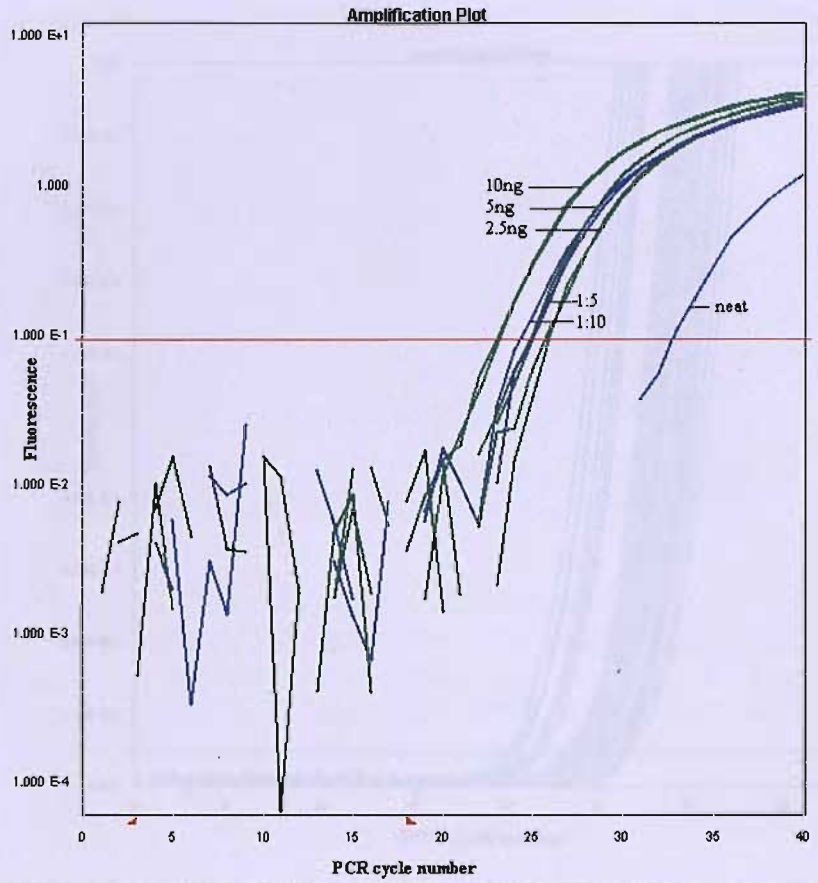
Table 4.4. Quantification of German GML cases against DNA standard in the RNase P assay.

Sample type	Sample Name	Assay	PCR cycle number	Quantity (ng)
Standard	control DNA 10ng	RNaseP	22.11	10
Standard	control DNA 5ng	RNaseP	23.7	5
Standard	control DNA 2.5ng	RNaseP	25.57	2.5
Unknown	German GML DNA 1	RNaseP	25.3	4.16
Unknown	German GML DNA 2	RNaseP	25.53	3.78
Unknown	German GML DNA 3	RNaseP	21.33	22.56
Unknown	German GML DNA 4	RNaseP	22.3	14.93
NTC	no template control	RNaseP	40	0

Standards of known DNA concentration (2.5ng, 5ng, 10ng) were used to generate a standard curve using the RNase P assay. The concentration of DNA in the German GML cases was determined from this standard curve. Table 4.4 shows the concentration of DNA determined for German GML cases 1-4.

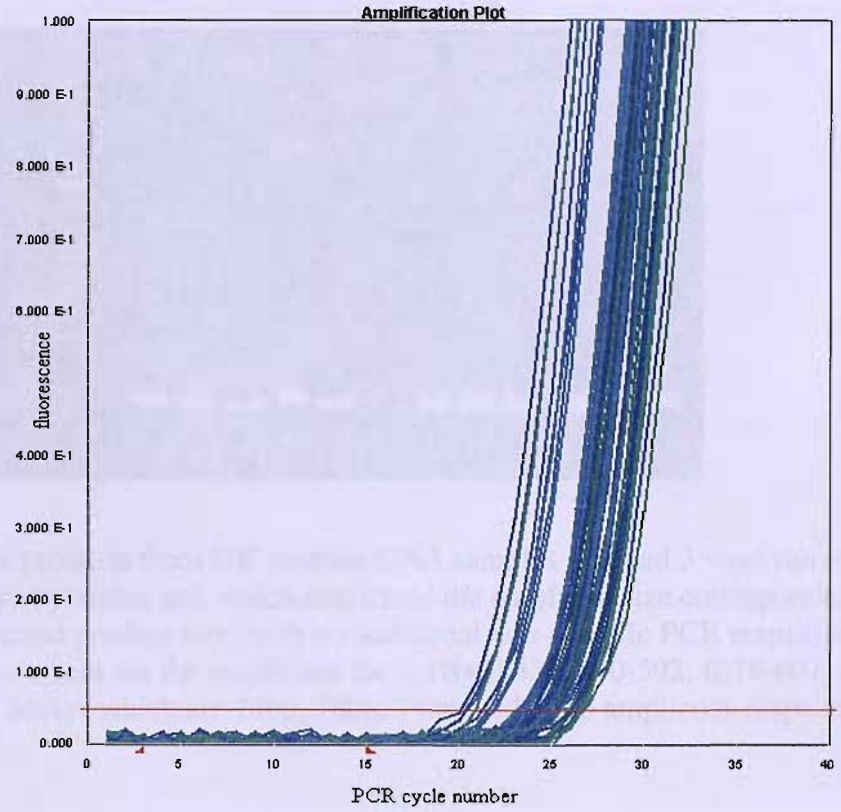
As previously discussed in chapter 3, the RNase P assay is an inappropriate method for determining the concentration of DNA derived from archival biopsies due to the presence of PCR inhibitors. However, this method is suitable to select the optimal dilution of DNA derived from these biopsies for efficient PCR amplification and genotyping. Figure 4.3 shows the Netherlands GML sample 501 tested undiluted and at a dilution of 1:5 and 1:10 with TE buffer using the RNase P assay. From the three real-time amplification curves for this sample, the PCR inhibition detected in the undiluted DNA sample was adequately reduced using DNA at a dilution of 1:5 and 1:10, which amplified almost identically. All of the Netherlands DNA samples (fig. 4.4), and all German and UK gastritis samples amplified efficiently at a dilution of 1:5 using the RNase P assay. This optimal dilution for efficient PCR amplification equated to a concentration of between 2-10ng of amplifiable DNA when compared to the human genomic DNA standard (fig.4.3). This dilution was selected for these samples for subsequent genotyping. The majority (42/55, 76%) of UK GML DNA samples amplified efficiently using a dilution of 1:5, although a different optimal dilution for some samples was selected, and this ranged from 1:2 to 1:10.

Fig. 4.3. The effect of diluting DNA derived from an archival biopsy in the RNase P assay.



Comparison of the real-time PCR amplification of the DNA standard (green) and Netherlands GML DNA sample 501 extracted from an archival biopsy (blue) using the RNase P assay. Increasing DNA concentration of the standard correlates with a lower PCR cycle number, whereas increasing dilution of sample 501 correlates with a lower PCR cycle number.

Fig. 4.4. Standardisation of Netherlands DNA samples using the RNase P assay.



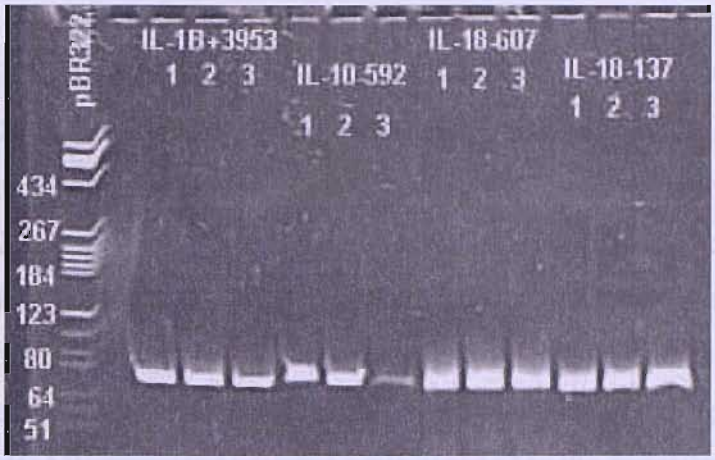
The optimum DNA dilution for samples derived from archival biopsies was determined for efficient PCR amplification. Here a dilution of 1:5 resulted in the majority of Netherlands gastritis DNA samples to amplify exponentially from approximately PCR cycle number 22 to 27 using the RNase P assay.

4.3.3. Cytokine genotyping using 5' nuclease assays.

All cases and control samples were genotyped using the eighteen 5' nuclease SNP assays. In all assays, the negative controls clustered together near the baseline on the allelic discrimination scatterplots, with no PCR amplification detected from the real-time amplification plots. All hidden replicates agreed giving 100% precision. All positive control samples tested typed in agreement with previous genotyping by ARMS-PCR.

To confirm the specificity of amplification for each of the eighteen 5' nuclease assays, the PCR products amplified from three of the DNA samples (UK gastritis samples 1, 2 and 3) were run on a 6% polyacrylamide gel. This confirmed that the amplicon from all assays corresponded to the expected product size, and that there were no additional PCR products (fig. 4.5).

Fig. 4.5. Amplicons generated from 5' nuclease allelic discrimination assays.



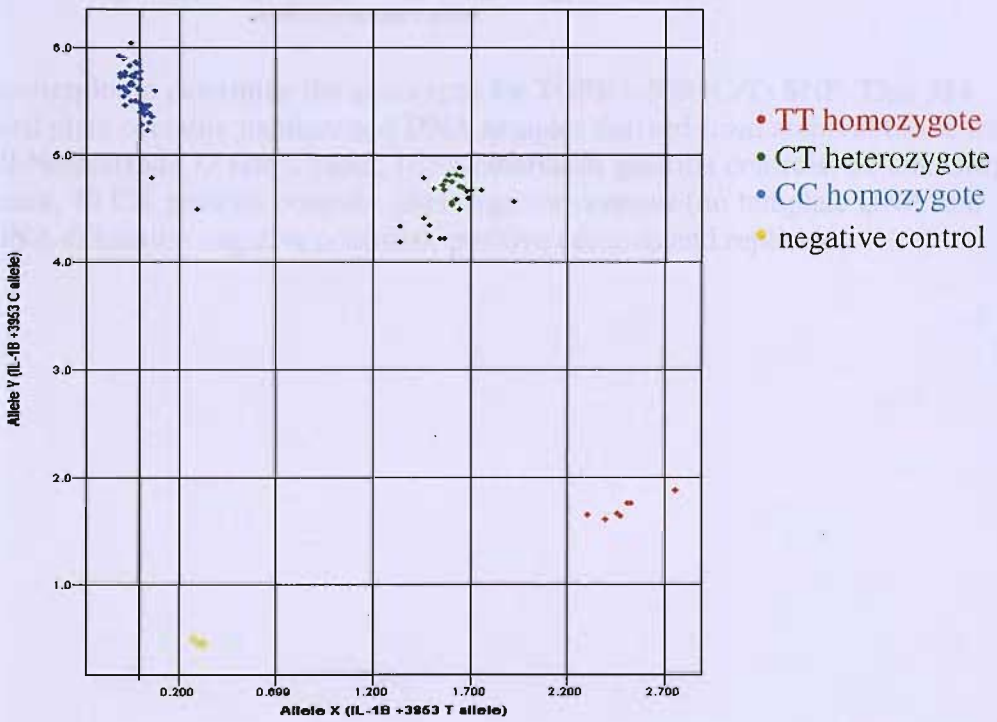
PCR products from UK gastritis DNA samples 1, 2 and 3 were run on a 6% polyacrylamide gel, which confirmed the amplicon size corresponded to the expected product size, with no additional non-specific PCR amplifications. Shown here are the amplicons for IL1B+3953, IL10-592, IL18-607, and IL18-137 assays which are 74bp, 78bp, 74bp, and 73bp amplicons respectively.

Genotypes were assigned for the majority of GML cases and gastritis controls for each population for the eighteen SNPs (tables 4.5, 4.6, and 4.7). Genotype calling was determined by clustering of the data points into three distinct groups on the scatterplot which were clearly distinguishable from the negative controls (fig. 4.6 and appendix A). However, the genotype for a small percentage of samples for some assays was undetermined (fig 4.7). There was variability in the quality of the different SNP assays and the number of undetermined samples reflected this. The greater the increase in fluorescence along the x and y-axis, and if there was no or minimal mis-priming, the greater the distinction between the three genotypic clusters on the scatterplot (fig. 4.6), and consequently genotype calling by grouping each of the three clusters was easy. In assays where the increase in fluorescence was minimal (0.5) and mis-priming occurred (invariably involving the 6-FAM labelled probe), there was reduced distinction between the three genotypic clusters and consequently there were a greater number of samples undetermined (fig. 4.7). For all undetermined samples the real-time PCR amplification plots were used to determine the likely

genotype to detect if there was skewing of the genotype frequencies. There was no significant difference in the genotype frequencies when the likely genotype was included. This indicated that the data did not appear to be distorted.

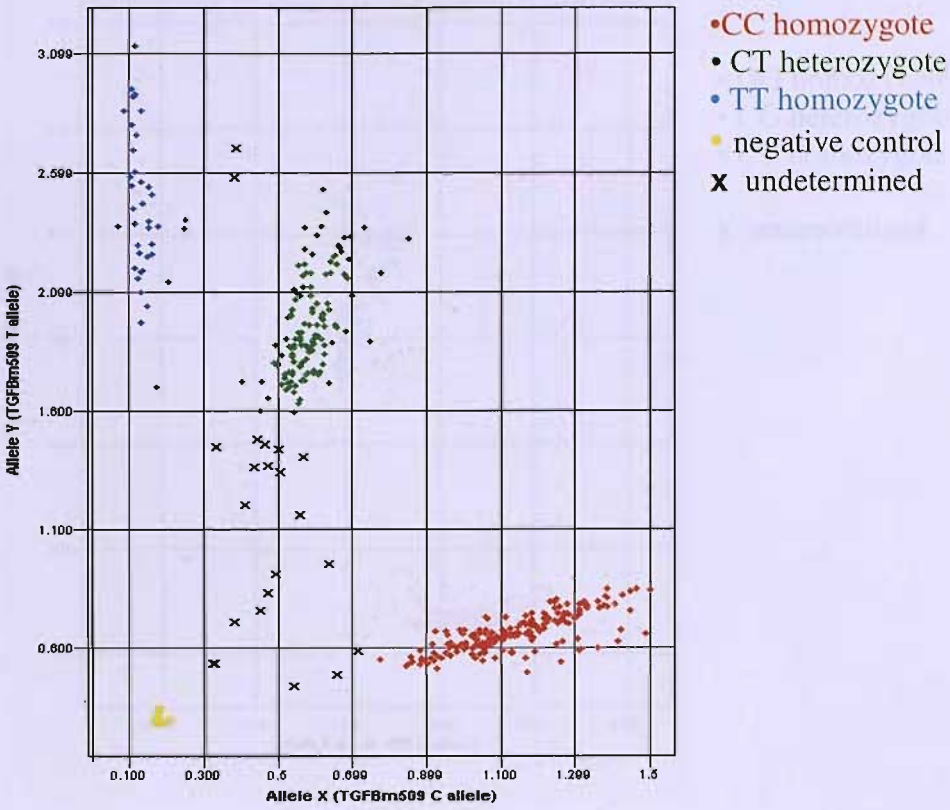
For the 5' nuclease assays where no positive controls were tested (IL1A-889, IL1B+3953, IL1RN+2018, IL18-137, IL18-607, IFNGR1-56, and TGFB1-509) the three genotypic clusters were determined from the scatterplot, i.e. homozygous for allele 1, homozygous for allele 2 and heterozygous for alleles 1 and 2, and genotypes assigned (fig. 4.8). To confirm the accuracy of genotype calling for the eighteen SNP assays, three samples one of each genotype, homozygous for allele 1, heterozygous for alleles 1 and 2 and homozygous for allele 2, were subsequently confirmed by sequencing as described in chapter 2.2.9. All sequenced samples corresponded to the genotype assigned using the 5' nuclease assays (fig 4.9 and 4.10).

Fig. 4.6. Scatterplot of German GML cases for IL1B+3953 SNP.



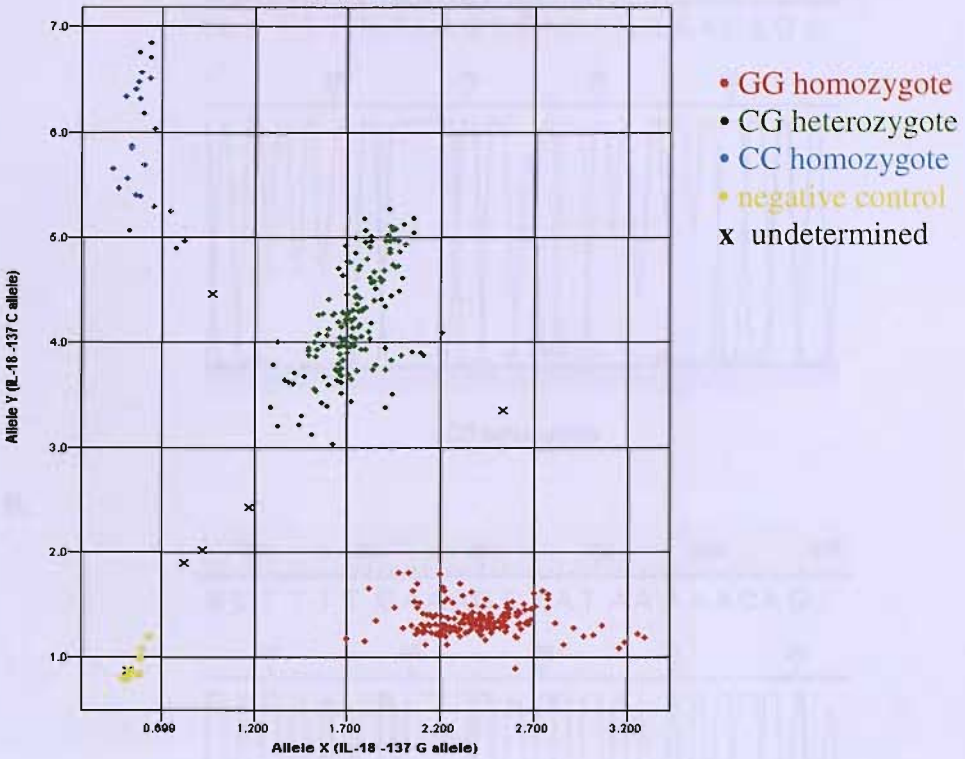
Genotype calling for the German GML DNA samples derived from peripheral blood using a 5µl PCR volume. In this assay there was a substantial increase in fluorescence along the x- and y-axis from the negative controls and minimal mis-priming. Consequently all samples were easily genotyped.

Fig. 4.7. Scatterplot of TGFB1-509 for archival derived DNA samples.



Scatterplot to determine the genotypes for TGFB1-509 (C/T) SNP. This 384 well plate contains standardised DNA samples derived from archival tissue for all Netherlands 72 GML cases, 165 Netherlands gastritis controls, 32 UK GML cases, 49 UK gastritis controls, plus negative controls (no template DNA and DNA extraction negative controls), positive controls and replicates.

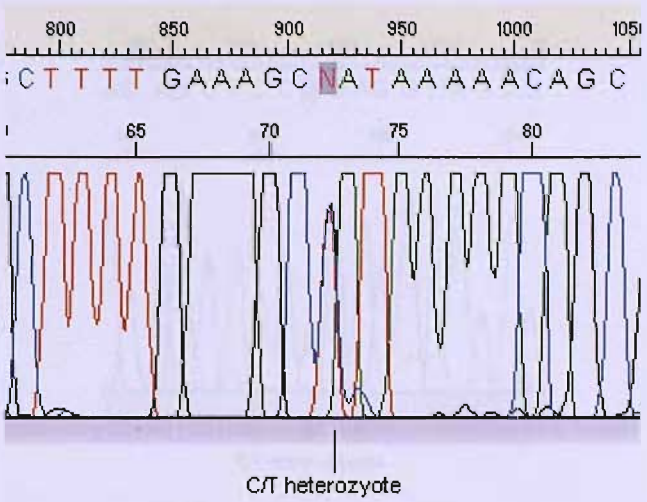
Fig. 4.8. Scatterplot for IL18-137 assay.



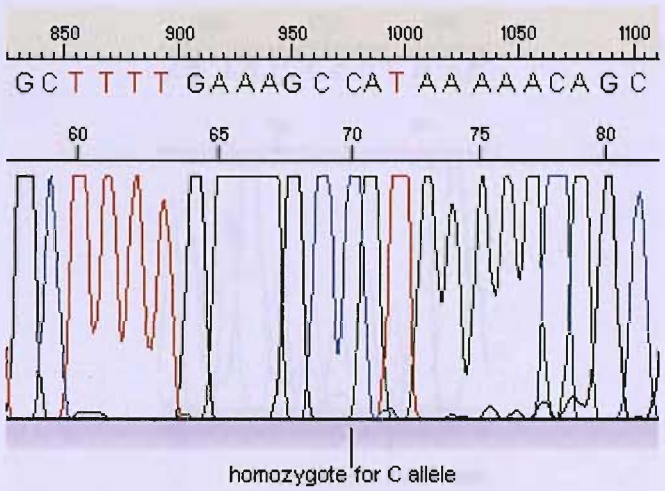
IL18-137 scatterplot for archival derived DNA samples. Genotypes were assigned by grouping of the three clusters. Although all hidden replicates agree, no positive controls were available for testing. Therefore, the accuracy of genotype calling was confirmed by sequencing three samples.

Fig. 4.9. Sequencing of the IL1B-31 SNP.

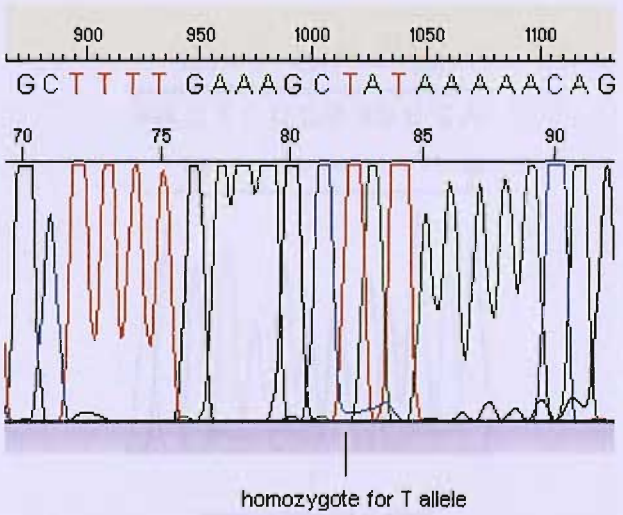
A.



B.



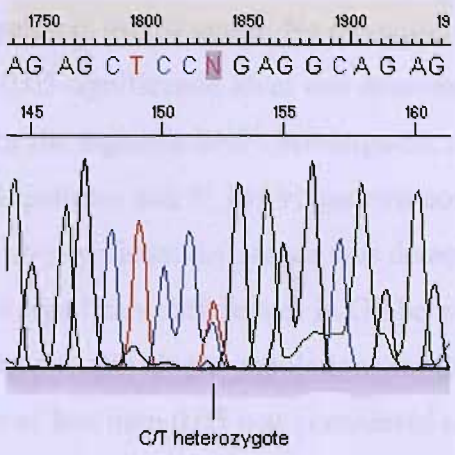
C.



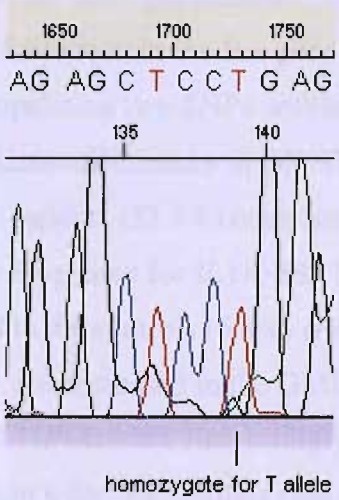
German GML cases 8, 10 and 20 were subsequently sequenced for the IL1B-31 SNP as (**A**) C/T heterozygote, (**B**) C homozygote and (**C**) T homozygote, respectively. This was in agreement with the genotyping results from the 5' nuclease assay for IL1B-31 SNP.

Fig. 4.10. Sequencing for the IL1B-511 SNP.

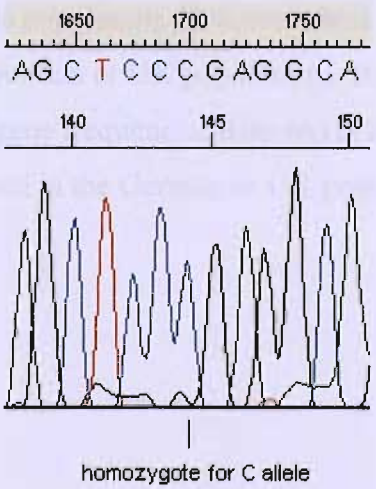
A.



B.



C.



German GML cases 8, 10 and 20 were subsequently sequenced for the IL1B-511 SNP as (**A**) C/T heterozygote, (**B**) T homozygote and (**C**) C homozygote, respectively. This was in agreement with the genotyping results from the 5' nuclease assay for IL1B-511 SNP.

4.3.4. Genotype frequencies (GF).

Tables 4.5, 4.6 and 4.7 show the genotype frequencies (GF) for the eighteen SNPs investigated in the 14 genes. No deviation from Hardy-Weinberg equilibrium at the $P = 0.05$ significance level was demonstrated for the control group for any locus. For the eighteen SNPs investigated, none were significantly different between GML patients and *H. pylori* gastritis controls in all three populations. Although no significant difference was detected in all three populations, there were significant differences in GF between GML patients and *H. pylori* gastritis controls within single populations for three of the SNPs investigated. A P value of less than 0.05 was considered significant (highlighted in red). For the remaining 15 SNPs, the GF did not differ significantly between cases and controls in any of the three populations.

The GF of the five SNPs within the IL1 gene cluster are shown in table 4.5. For the Netherlands population two SNPs within this region, IL1A-889 and IL1B+3953, showed significant differences in GF. The GF for IL1A-889 CC was decreased in the GML patients (32.8%) compared to the gastritis controls (53.9%) (P=0.005) and the frequency for IL1A-889 TT was increased in patients (17.2%) compared to the controls (6.8%) (P=0.02). Also in this population IL1B+3953 TT was increased in the GML patients (12.9%) compared to controls (3.4%) (P=0.006). Interestingly, the +3953T allele shows strong linkage disequilibrium with –889T allele (see section 4.3.6) and both SNPs were significantly increased in GML patients compared to *H. pylori* controls in the Netherlands population. However, these significant differences were not detected in the German or UK populations. In addition, this pattern of significant differences in gene frequencies detected in the Netherlands population was not mirrored in the German or UK populations.

Table 4.5. IL1 region (chromosome 2q12-14) genotype frequencies.

SNP		Netherlands			Germany			UK		
		GML N=72 (%)	gastritis N=224 (%)	*P*	GML N=79 (%)	gastritis N=154 (%)	*P*	GML N=52 (%)	gastritis N=190 (%)	*P*
IL1A-889	CT	29 (50.0)	86 (39.3)	0.18	25 (33.3)	73 (48.0)	0.05	16 (34.8)	63 (36.4)	0.86
	CC	19 (32.8)	118 (53.9)	0.005	41 (54.7)	64 (42.1)	0.09	25 (54.3)	94 (54.3)	1
	TT	10 (17.2)	15 (6.8)	0.02	9 (12.0)	15 (9.9)	0.65	5 (10.9)	16 (9.2)	0.78
TOTALS		58	219		75	152		46	173	
IL1B+3953	CT	27 (38.6)	83 (39.9)	0.89	28 (35.4)	54 (39.1)	0.66	18 (35.3)	60 (34.3)	1
	CC	34 (48.6)	118 (56.7)	0.27	44 (55.7)	71 (51.4)	0.57	32 (62.7)	105 (60.0)	0.75
	TT	9 (12.9)	7 (3.4)	0.006	7 (8.9)	13 (9.4)	1	1 (2.0)	10 (5.7)	0.46
TOTALS		70	208		79	138		51	175	
IL1B-31	CT	34 (49.3)	104 (53.6)	0.56	36 (45.6)	78 (52.3)	0.33	25 (49.0)	72 (44.2)	0.63
	CC	9 (13.0)	25 (12.9)	1	7 (8.9)	8 (5.4)	0.4	9 (17.6)	22 (13.5)	0.5
	TT	26 (37.7))	65 (33.5)	0.56	36 (45.6)	63 (42.3)	0.67	17 (33.3)	69 (42.3)	0.33
TOTALS		69	194		79	149		51	163	
IL1B-511	CT	34 (47.9)	106 (48.2)	1	33 (43.4)	78 (50.6)	0.33	28 (53.8)	85 (45)	0.28
	CC	27 (38.0)	82 (37.3)	1	35 (46.1)	68 (44.2)	0.89	18 (34.6)	81 (42.9)	0.34
	TT	10 (14.1)	32 (14.5)	1	8 (10.5)	8 (5.2)	0.17	6 (11.5)	23 (12.2)	1
TOTALS		71	220		76	154		52	189	
IL1RN+2018	CT	25 (34.7)	89 (40.5)	0.4	27 (36.5)	68 (45)	0.25	18 (36)	70 (37.8)	0.87
	CC	6 (8.3)	12 (5.5)	0.4	6 (8.1)	4 (2.6)	0.08	6 (12)	13 (7)	0.25
	TT	41 (56.9)	119 (54.1)	0.69	41 (55.4)	79 (52.3)	0.67	26 (52)	102 (55.1)	0.75
TOTALS		72	220		74	151		50	185	

Table 4.5 shows the genotype frequencies of the SNP's within the IL1 gene region for cases (GML) and *H. pylori* gastritis controls. *P* values were calculated using Fisher's Exact test. A value was considered significant if <0.05. All SNPs were in accordance with Hardy -Weinberg equilibrium.

Table 4.6 shows the GF of the three SNPs located within the TNF region. In the Netherlands population the frequency of the LTA+252 GG genotype was decreased in the GML patients (2.9%) compared to the controls (13.5%) (*P*=0.01). This SNP also showed a significant difference in the UK group as LTA+252 AA was decreased in patients (23.1%) compared to the gastritis controls (41.5%) (*P*=0.02). However, these differences were not consistent between the three populations. In addition, a decrease in the LTA+252 GG frequency in Netherlands GML patients is inconsistent with a decrease in the LTA+252 AA frequency in UK GML patients. Table 4.7 shows the GF of the remaining 10 SNPs investigated. No significant differences were identified between GML cases and gastritis controls for any of these SNPs in any population.

Table 4.6. TNF region (chromosome 6p21.3) genotype frequencies.

SNP		Netherlands			Germany			UK		
		GML N=72 (%)	gastritis N=224 (%)	*P*	GML N=79 (%)	gastritis N=154 (%)	*P*	GML N=52 (%)	gastritis N=190 (%)	*P*
TNF-308	AG	16 (26.2)	56 (26.3)	1	15 (22.4)	34 (22.2)	1	17 (34.0)	57 (30.5)	0.73
	AA	1 (1.6)	12 (5.6)	0.31	2 (3.0)	0 (0.0)	0.09	3 (6.0)	7 (3.7)	0.44
	GG	44 (72.1)	145 (68.1)	0.64	50 (74.6)	119 (77.8)	0.61	30 (60.0)	123 (65.8)	0.51
TOTALS		61	213		67	153		50	187	
TNF-376	AG	2 (2.8)	4 (1.8)	0.63	1 (1.3)	6 (3.9)	0.43	2 (3.8)	6 (3.2)	0.68
	AA	0 (0.0)	0 (0.0)	1	0 (0.0)	0 (0.0)	1	0 (0.0)	0 (0.0)	1
	GG	69 (97.2)	218 (98.2)	0.63	77 (98.7)	147 (96.1)	0.43	50 (96.2)	184 (96.8)	0.68
TOTALS		71	222		78	153		52	190	
LTA+252	AG	39 (56.5)	103 (46.2)	0.17	39 (50.0)	68 (44.4)	0.49	28 (53.8)	79 (43.2)	0.21
	AA	28 (40.6)	90 (40.4)	1	33 (42.3)	77 (50.3)	0.27	12 (23.1)	76 (41.5)	0.02
	GG	2 (2.9)	30 (13.5)	0.01	6 (7.7)	8 (5.2)	0.56	12 (23.1)	28 (15.3)	0.21
TOTALS		69**	223		78	153		52	183	

Table 4.6 shows the genotype frequencies of the SNP's within the TNF gene region for cases (GML) and *H. pylori* gastritis controls. *P* values were calculated using Fisher's Exact test. A value was considered significant if <0.05. All SNPs were in accordance with Hardy -Weinberg equilibrium except **.

Table 4.7. Genotype frequencies for remaining cytokine SNPs.

SNP		Netherlands			Germany			UK		
		GML N=72 (%)	gastritis N=224 (%)	*P*	GML N=79 (%)	gastritis N=154 (%)	*P*	GML N=52 (%)	gastritis N=190 (%)	*P*
IL4-590	CT	21 (30.9)	61 (28.5)	0.76	20 (27.0)	37 (24.0)	0.63	14 (26.9)	47 (25.0)	0.86
	CC	47 (69.1)	147 (68.7)	1	49 (66.2)	109 (70.8)	0.54	35 (67.3)	137 (72.9)	0.49
	TT	0 (0.0)	6 (2.8)	0.34	5 (6.8)	8 (5.2)	0.76	3 (5.8)	4 (2.1)	0.18
TOTALS		68	214		74	154		52	188	
IL6-174	CG	32 (45.7)	105 (46.9)	0.89	34 (43.0)	68 (45.3)	0.78	24 (47.1)	94 (49.5)	0.87
	CC	16 (22.9)	38 (17.0)	0.29	18 (22.8)	22 (14.7)	0.14	9 (17.6)	32 (16.8)	0.84
	GG	22 (31.4)	81 (36.2)	0.57	27 (34.2)	60 (40.0)	0.47	18 (35.3)	64 (33.7)	0.87
TOTALS		70	224		79	150		51	190	
IL8-251	AT	31 (50.0)	94 (50.3)	1	43 (54.4)	63 (42.9)	0.12	16 (37.2)	69 (46.9)	0.3
	AA	14 (22.6)	40 (21.4)	0.86	15 (19.0)	35 (23.8)	0.5	10 (23.3)	34 (23.1)	1
	TT	17 (27.4)	53 (28.3)	1	21 (26.6)	49 (33.3)	0.37	17 (39.5)	44 (29.9)	0.27
TOTALS		62	187		79	147		43	147	
IL10-592	CA	22 (32.8)	84 (38.0)	0.47	34 (43.6)	49 (31.8)	0.08	18 (40.0)	67 (35.4)	0.61
	CC	42 (62.7)	125 (56.6)	0.4	41 (52.6)	89 (57.8)	0.49	23 (51.1)	107 (56.6)	0.51
	AA	3 (4.5)	12 (5.4)	1	3 (3.8)	16 (10.4)	0.13	4 (8.9)	15 (7.9)	0.77
TOTALS		67	221		78	154		45	189	
IL12B+1188	AC	24 (34.3)	73 (33.6)	1	18 (24.0)	57 (37.3)	0.05	22 (41.5)	65 (34.8)	0.42
	CC	0 (0.0)	5 (2.3)	0.34	4 (5.3)	9 (5.9)	1	0 (0.0)	7 (3.7)	0.35
	AA	46 (65.7)	139 (64.1)	0.89	53 (70.7)	87 (56.9)	0.06	31 (58.5)	115 (61.5)	0.75
TOTALS		70	217		75	153		53	187	
IL18-137	CG	31 (43.7)	86 (38.6)	0.49	34 (44.2)	62 (40.5)	0.67	18 (35.3)	86 (46.2)	0.2
	CC	8 (11.3)	18 (8.1)	0.47	7 (9.1)	11 (7.2)	0.61	2 (3.9)	12 (6.5)	0.74
	GG	32 (45.1)	119 (53.4)	0.28	36 (46.8)	80 (52.3)	0.49	31 (60.8)	88 (47.3)	0.11
TOTALS		71	223		77	153		51	186	
IL18-607	CA	30 (45.5)	84 (45.7)	1	38 (51.4)	70 (47.0)	0.57	19 (45.2)	75 (45.7)	1
	CC	19 (28.8)	62 (33.7)	0.54	23 (31.1)	56 (37.6)	0.37	18 (42.9)	66 (40.2)	0.86
	AA	17 (25.8)	38 (20.7)	0.39	13 (17.6)	23 (15.4)	0.7	5 (11.9)	23 (14.0)	1
TOTALS		66	184		74	149		42	164	
IFNGR1-56	CT	21 (39.6)	101 (45.9)	0.44	39 (50.0)	72 (46.8)	0.68	13 (50.0)	73 (41.2)	0.4
	CC	11 (20.8)	40 (18.2)	0.7	13 (16.7)	25 (16.2)	1	4 (15.4)	29 (16.4)	1
	TT	21 (39.6)	79 (35.9)	0.64	26 (33.3)	57 (37.0)	0.66	9 (34.6)	75 (42.4)	0.53
TOTALS		53	220		78	154		26	177	
IFNG+874	AT	41 (59.4)	102 (47.4)	0.1	38 (48.7)	73 (52.5)	0.67	18 (39.1)	97 (51.9)	0.14
	AA	16 (23.2)	63 (29.3)	0.36	26 (33.3)	42 (30.2)	0.65	14 (30.4)	45 (24.1)	0.45
	TT	12 (17.4)	50 (23.3)	0.4	14 (17.9)	24 (17.3)	1	14 (30.4)	45 (24.1)	0.45
TOTALS		69	215		78	139		46	187	
TGFB1-509	CT	26 (37.1)	85 (38.8)	0.89	36 (46.2)	53 (38.4)	0.31	22 (45.8)	58 (32.2)	0.09
	CC	39 (55.7)	111 (50.7)	0.49	38 (48.7)	71 (51.4)	0.78	23 (47.9)	104 (57.8)	0.25
	TT	5 (7.1)	23 (10.5)	0.49	4 (5.1)	14 (10.1)	0.31	3 (6.3)	18 (10.0)	0.58
TOTALS		70	219		78	138		48	180	

Table 4.7 shows the genotype frequencies of the SNP's for cases (GML) and *H. pylori* gastritis controls. *P* values were calculated using Fisher's Exact test. A value was considered significant if <0.05. All SNPs were in accordance with Hardy -Weinberg equilibrium. No significant differences were identified between GML cases and gastritis controls for any of these SNPs in any population. IL-12B+1188 AC for the German population is highlighted red as the *P* value = 0.05.

4.3.5. Allele frequencies (AF).

The allele frequencies (AF) of the 18 SNPs investigated for the GML cases and gastritis controls for each population are shown in table 4.8. The allele frequencies for the gastritis control groups for all SNPs investigated were not significantly different between the three populations and are in accordance with published data (shown in table 4.8). This indicated that the method of genotyping was accurate and gave further assurance of the quality of data.

The allele frequencies for the majority of SNPs (15/18, 83%) did not differ significantly between cases and controls, as well as between the different populations. This would indicate that the selection of the cases and controls are not at risk of confounding factors and that the significant differences identified in section 4.3.4 for IL1A-889, IL1B+3953 and LTA+252 are not due to population stratification.

Finally, it should be noted that the heterozygous GF and the AF for the UK GML cases compared to the UK gastritis control group did not differ significantly, and indicated that the quality of genotyping was comparable in the two groups. As discussed previously (section 4.3.1), the DNA from the UK GML cases was derived from biopsy tissue containing variable amounts of lymphoma tissue. Neoplastic tissue contains somatic-cell genetic alterations, which tend to accumulate as the tumour progresses to advanced stages. Loss of heterozygosity (LOH) is the most common molecular genetic alteration observed in human cancers (Zheng 2005). LOH is caused by a variety of genetic mechanisms, including deletion, mitotic recombination and gene conversion. LOH is a common form of allelic imbalance and therefore, as the UK GML cases contained malignant tissue, the genotype frequencies of these samples may have been affected by LOH. However, as the heterozygous GF did not differ from the control group, this indicated that the genotype frequencies were not affected by LOH for the SNPs investigated in this study.

Table 4.8. Allele frequencies of the SNPs investigated.

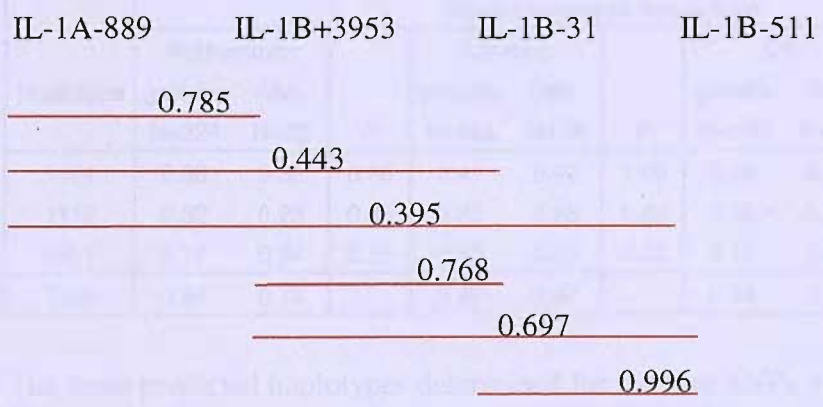
Allele		Allele Frequency						Normal Caucasian Population (Hp status unknown)
		Netherlands		Germany		UK		
		GML	gastritis	GML	gastritis	GML	gastritis	Allele frequency (number)
IL1A-889	C	0.58	0.74	0.71	0.66	0.72	0.73	0.71 (398) [a]
	T	0.42	0.26	0.29	0.34	0.28	0.27	0.29 (398) [a]
IL1B+3953	C	0.68	0.77	0.73	0.71	0.8	0.77	0.79 (245) [j]
	T	0.32	0.23	0.27	0.29	0.2	0.23	0.21 (245) [j]
IL1B-31	C	0.38	0.4	0.32	0.32	0.42	0.36	0.37 (164) [b]
	T	0.62	0.6	0.68	0.68	0.58	0.64	0.63 (164) [b]
IL1B-511	C	0.62	0.61	0.68	0.69	0.62	0.65	0.59 (261) [b]
	T	0.38	0.39	0.32	0.31	0.38	0.35	0.41 (261) [b]
IL1RN+2018	C	0.26	0.26	0.26	0.25	0.3	0.26	0.23 (100) [c, d]
	T	0.74	0.74	0.74	0.75	0.7	0.74	0.77 (100) [c, d]
TNF-308	A	0.15	0.19	0.14	0.11	0.23	0.19	0.19 (220) [e]
	G	0.85	0.81	0.86	0.89	0.77	0.81	0.81 (220) [e]
TNF-376	A	0.01	0.01	0.01	0.02	0.02	0.02	0.01 (220) [e]
	G	0.99	0.99	0.99	0.98	0.98	0.98	0.99 (220) [e]
LTA+252	A	0.69	0.63	0.67	0.73	0.5	0.63	0.61 (220) [e]
	G	0.31	0.37	0.33	0.27	0.5	0.37	0.39 (220) [e]
IL4-590	C	0.85	0.83	0.8	0.83	0.81	0.85	0.89 (208) [b]
	T	0.15	0.17	0.2	0.17	0.19	0.15	0.11 (208) [b]
IL6-174	C	0.46	0.4	0.44	0.37	0.41	0.42	0.42 (224) [b]
	G	0.54	0.6	0.56	0.63	0.59	0.58	0.58 (224) [b]
IL8-251	A	0.48	0.47	0.46	0.45	0.42	0.47	0.45 (235) [b]
	T	0.52	0.53	0.54	0.55	0.58	0.53	0.55 (235) [b]
IL10-592	C	0.79	0.76	0.74	0.74	0.71	0.74	0.77 (660) [h]
	A	0.21	0.24	0.26	0.26	0.29	0.26	0.23 (660) [h]
IL12B+1188	C	0.17	0.19	0.17	0.25	0.21	0.21	0.22 (229) [b]
	A	0.83	0.81	0.83	0.75	0.79	0.79	0.78 (229) [b]
IL18-137	C	0.33	0.27	0.31	0.27	0.22	0.3	0.25 (278) [i]
	G	0.67	0.73	0.69	0.73	0.78	0.7	0.75 (278) [i]
IL18-607	C	0.52	0.57	0.57	0.61	0.65	0.63	0.62 (278) [i]
	A	0.48	0.43	0.43	0.39	0.35	0.37	0.38 (278) [i]
IFNG+874	A	0.53	0.53	0.58	0.56	0.5	0.5	0.55 (222) [b]
	T	0.47	0.47	0.42	0.44	0.5	0.5	0.45 (222) [b]
IFNGR1-56	C	0.41	0.41	0.42	0.4	0.4	0.37	0.38 (128) [f]
	T	0.59	0.59	0.58	0.6	0.6	0.63	0.62 (128) [f]
TGFB1-509	C	0.74	0.7	0.72	0.71	0.71	0.74	0.69 (322) [g]
	T	0.28	0.3	0.28	0.29	0.29	0.26	0.31 (322) [g]

Table 4.8 shows the allele frequencies of the SNP's investigated for the cases (GML) and *H. pylori* gastritis control groups for each population. For comparison, the allele frequencies from normal Caucasian populations taken from the literature are also provided: [a] Cox 1998; [b] Howell 2003; [c] Guasch 1996; [d] Karasneh 2003; [e] Howell 2002; [f] Rosenzweig 2004; [g] Grainger 1999; [h] Reynard 2000; [i] Giedraitis 2001; [j] Kaijzel 2002;

4.3.6. Linkage Disequilibrium between the four SNPs within IL1A and B genes.

As there was a significant difference in the Netherlands IL1 gene frequencies between cases and controls, the IL1 region was investigated further. There was no statistical difference in GF or AF in the three *H. pylori* gastritis control groups and therefore, these groups was amalgamated for linkage disequilibrium and haplotype analyses. The disequilibrium coefficient (Rho) between two SNPs was calculated for all combinations of the four SNPs genotyped within the IL1A and IL1B genes (fig. 4.11). This statistical measure of linkage between two SNPs identified strong LD between IL1A-889 and IL1B+3953 (79% LD) and between IL1B-31 and IL1B-511 (>99% LD). These results are in accordance with published data (Cox 1998). In addition, strong linkage between IL1B+3953 and IL-1B31 was also detected (77% LD). Although linkage between these two SNPs had previously been reported (Cox 1998), it had not been detected in all populations studied (Guasch 1996), which may have been due to the small sample size studied (n<50) compared to this study (n = 568).

Fig. 4.11. Linkage disequilibrium between the four SNPs within the IL1A and IL1B genes.



The linkage disequilibrium coefficient (Rho) was calculated between the four SNPs investigated within the IL1A and IL1B genes using the *H. pylori* gastritis controls from the three populations (n =568).

4.3.7. Frequency of probable haplotypes for the IL1A and ILB gene region.

In collaboration with Dr. Niklas Maniatis, University of Southampton, the predicted haplotypes and their frequencies of the IL1A and IL1B genes were determined for each population using an expectation-maximisation algorithm (section 4.2.7). Haplotypes were inferred from the genotypes frequencies determined for the three *H. pylori* gastritis groups (n=568), as well as for the individual gastritis and GML groups for each population. Three common haplotypes were identified that represented over 78% of samples. The remaining possible haplotypes had very low frequencies (<0.04) and therefore were not considered in any further analysis. Table 4.9 shows the three predicted haplotypes and their frequency for each population. There were no significant differences in haplotype frequencies between the three gastritis control groups, which were consistent with haplotype analysis previously published by Kornman *et al* (1999). In addition, there was no significant difference in haplotype frequencies between GML cases and gastritis control groups for all three populations. Therefore, the significant differences identified for the Netherlands population for the individual IL1A-889 and IL1B+3953 SNPs was not identified when the IL1 haplotypes were analysed.

Table 4.9. The predicted haplotypes and their frequencies for the IL1 gene region.

	Inferred haplotype frequencies									
	Netherlands			Germany			UK			Kornman*
Haplotype	gastritis N=224	GML N=72	*P*	gastritis N=154	GML N=79	*P*	gastritis N=190	GML N=52	*P*	N=1343
1121	0.36	0.32	0.88	0.41	0.42	1.00	0.39	0.38	0.86	0.38
1112	0.32	0.23	0.35	0.22	0.25	0.62	0.29	0.28	0.86	0.27
2221	0.17	0.24	0.15	0.23	0.20	0.73	0.16	0.12	0.65	0.17
Total	0.85	0.79		0.86	0.87		0.84	0.78		0.82

The three predicted haplotypes determined for the four SNPs investigated in the IL1A and IL1B genes, which represented over 78% of the samples. Position 1 of the haplotype = IL1A-889 gene (allele 1 = C allele); position 2 = IL1B+3953 gene (allele 1 = C allele); position 3 = IL1B-31 gene (allele 1 = C allele); position 4 = IL1B-511 gene (allele 1 = C allele). Kornman* – data is from the reference Kornman *et al*, 1999.

4.4. Discussion.

Taking these results as a whole would tend to suggest that the cytokine polymorphisms investigated in this study are unlikely to have a major influence in the development of primary GML following *H. pylori* infection, as no significant difference between GML cases and *H. pylori* controls was detected in all three populations. In addition, no trend towards significance was detected in all three populations for any of the SNP's investigated. This may have indicated a potential influence of a cytokine SNP in the development of GML subsequent to *H. pylori* infection. Therefore, it is unlikely that the SNPs investigated in this study for IL1A, IL1B, IL1RN, IL4, -6, -8, 10, -12, -18, IFNG, IFNGR1, TGFB1, LTA and TNF cytokine genes play a major role in the development of GML following *H. pylori* infection. However, this does not exclude a role of the cytokine in the development of this disease. Investigation of SNPs that span the entire gene and its promoter is required to fully evaluate the influence of a particular cytokine in the development of this disease. For example, there are numerous SNPs within the TNF gene (reviewed in: Hajeer 2001; Spink 2006) and, as discussed in chapter 1, until the full extent of the polymorphisms within a cytokine gene and their influence on expression and functional activity are determined, then the SNPs required to be investigated to determine a cytokine's role in the development of disease will remain controversial.

A significant difference was detected for the lymphotoxin alpha cytokine gene polymorphism at position +252, located in the first intron of the gene. Presence of the G allele has been shown to result in raised LTα concentrations in stimulated mononuclear cells (Messer 1991; Knight 2003). This SNP showed a significant difference in GF between GML cases and controls for the Netherlands and the UK populations but not for the German population. However, the significant differences were not consistent for the Netherlands and UK populations, since a decrease in the LTA+252 GG frequency in Netherlands GML patients is inconsistent with a decrease in the LTA+252 AA frequency in UK GML patients compared to their control groups. However, these results do require exploration in a larger study group to determine if this is due to chance and to clarify this result. Interestingly, systemic release of TNFα and LTα has

been found to be associated with the severity of non-Hodgkin lymphoma (NHL) (Warzocha 1998) and TNF/LTA haplotypes associated with development of NHL (Spink 2006). However in contrast to this study, cases were mainly follicular and diffuse large B-cell lymphoma patients. Warzocha *et al* showed patients with high circulating levels of TNFα and LTα, and high TNFα plasma levels were associated with poorer disease outcome. The TNF-308A allele was significantly associated with higher plasma levels of TNFα at diagnosis. Presence of two or more TNF/LTA 'high producing' alleles (TNF-308A and LTA+252G) was significantly associated with higher rate of relapse and progression of disease. In addition, Spink *et al* identified that TNF haplotypes were associated with disease risk.

Comparison of the IL1 results shows that this study found no significant differences between cases and controls in any population for the IL1B-31 CC genotype or the IL1RN+2018 CC genotype. IL1RN+2018C allele, located in exon 2, is in near complete linkage disequilibrium with allele 2 of the 86bp VNTR in the second intron of the gene encoding the interleukin-1 receptor antagonist (IL1RN*2 allele) (Smith 2004). This contrasts with a significant association of the haplotype IL1B-31C/ IL1RN*2 that has been detected in several European populations to be associated with a significant increase in the risk of developing chronic hypochlorhydria and gastric adenocarcinoma following *H. pylori* infection (El-Omar 2000; Machado 2001; Furuta 2002; Zambon 2002). This might indicate that the aetiology of *H. pylori* associated gastric adenocarcinoma and GML are different. However, our results for IL1B-31 polymorphism are in agreement with the published findings from Rollinson *et al* (2003), which were published during the course of this study. This group looked at IL1 gene polymorphisms in relation to 66 GML cases and 163 healthy controls for a UK (Sheffield) based study and found there was no significant difference for the IL1B-31 CC genotype between cases and controls. However, in the Sheffield study the IL1RN*2/2 genotype (homozygous for allele two of the VNTR within intron 2 of the IL1 receptor antagonist) was significantly increased in GML patients (33.9%) compared to healthy controls (8%) (OR 5.51, CI 2.61-14.07). An aim of this study was then subsequently to seek independent confirmation of the IL1RN finding by Rollinson *et al.* In this study, we were unable to genotype for the IL1RN VNTR as the size of the alleles were

too large to be detected using the fragmented DNA extracted from archival biopsies (allele 1 consists of 4 repeats, allele 2 consists of two repeats, allele 3 consists of 5 repeats, allele 4 consists of 3 repeats and allele 5 consists of 6 repeats of the 86bp VNTR). However, this VNTR in intron 2 of the IL1 receptor antagonist is in strong linkage disequilibrium (>90%) with a SNP in exon 2, IL1+2018 (Smith 2004). Therefore, this SNP was used to genotype all GML cases and gastritis controls. However, we did not identify a significant difference in the genotype frequency between cases and controls in any of the three European populations and therefore, did not replicate the finding by the Rollinson study. This may be due to the use of a more appropriate control group, namely *H. pylori* infected controls rather than a control group of healthy individuals as used in the Rollinson study. In addition, the results presented here are concordant with the findings published in 2004 (Hellmig 2004), which used 344 *H. pylori* positive patients without gastric lymphoma as a control group and compared with 153 GML cases. This study, as does ours, found no significant association for IL1-31 or IL1RN 86bp VNTR with the development of GML subsequent to *H. pylori* infection. In addition, the allele frequencies for these two SNPs in the cases and control groups were in agreement with our frequencies.

Interestingly, there were significant differences within the IL1 gene cluster between Netherlands GML cases and gastritis controls, but this trend was not detected in the German or UK populations. Differences in confounding factors, such as ethnicity, age and sex, between the Netherlands GML cases and gastritis controls could result in the significant differences identified. However, as the majority of SNPs investigated (15/18, 83%) did not differ significantly between the Netherlands cases and controls, this would indicate that the selection of the cases and controls are not at risk of confounding factors and the significant differences identified for IL1A-889 and IL1B+3953 is unlikely to be due to population stratification.

Sampling variation could account for the differences detected and therefore to eliminate this risk these significant differences need to be confirmed in a second study group. Differences in environmental factors may account for the differences in significance detected but the cultural and geographical locations of the three populations at first sight would lead the observer to

believe they are not dramatically different. The quality of genotyping could affect the significance but as cases and controls were a randomised mix and suitably controlled this risk has been reduced and subsequently confirmed by sequencing. Finally, a difference in the selection of the cases in the three populations could explain these findings. As previously discussed in chapter 1 certain translocations, in particular t(11;18)(q21;q21), have been identified in a proportion of GML cases and this may be a difference between the GML cases in the three populations studied. Therefore, if the presence of this translocation is significantly different between the GML groups and if the frequencies of the IL1 polymorphisms differ between translocation positive and translocation negative GML patients, then this may explain the differential IL1 associations identified between the different populations. In chapter five this translocation was determined for the GML cases from each population to evaluate the hypothesis 'are IL1 genotypes associated with the presence or absence of the t(11;18)(q21;q21) translocation?' Interestingly the two SNPs, IL1A-889 and IL1B+3953, that show a significant difference between the GML cases and gastritis controls within the Netherlands population are closest together compared to the other SNPs studied within the IL1 gene cluster (fig. 4.1). These SNPs may potentially reflect an association with the development of GML in this Netherlands group of patients or are in LD with a polymorphism centromeric to this region.

Chapter 5: Detection of the t(11;18)(q21;q21) translocation in primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma cases and its relation to polymorphisms in the IL1 gene region.

5.1. Introduction

Mucosa-associated lymphoid tissue (MALT) lymphomas can arise in a variety of extra-nodal sites such as the gastrointestinal tract, lung, eye and thyroid. At least three different, apparently site-related, chromosomal translocations have been implicated in the development and progression of MALT lymphoma, t(11;18)(q21;q21), t(1;14)(p22;q32) and t(14;18)(q32;q21). These three translocations appear to affect the same signalling pathway, the nuclear factor-κB pathway (NF- κB) resulting in activation of NF-κB (Lucas 2001). NF- κB has a central role in immunity, inflammation and apoptosis (reviewed in: Bours 2000) and is a transcription factor for several genes including cytokines, growth factors, cell adhesion molecules and apoptosis inhibitors.

The most common translocation t(11;18)(q21;q21) can be present in 15-40% of cases. The frequency of this translocation is site – related and is common in MALT lymphomas arising in the gastrointestinal tract and lung, but rare in lymphomas of the conjunctiva and orbit and almost absent in lymphomas of the salivary glands, thyroid, liver and skin (Ye 2003). The t(1;14)(p22;q32) and t(14;18)(q32;q21) translocations have infrequently been identified in MALT lymphomas arising in the stomach (table 5.1). In addition, the t(11;18)(q21;q21) translocation has been identified specifically in MALT lymphoma and has not been detected in any other lymphoma subtypes including the closely related nodal and splenic marginal zone lymphoma (Isaacson 2005).

Table 5.1. The three common translocations identified in MALT lymphomas.

Translocation	**t(11;18)(q21;q21)**	**t(1;14)(p22;q32)**	**t(14;18)(q32;q21)**
Effect	API2 – MALT1 chimera	↑ BCL10	↑ MALT1
% of cases	15-40%	1-2%	20%
Location of lymphoma	GI tract, lung	GI tract, lung	Rarely identified in lymphomas arising in the GI tract.
MALT1 expression	Cytoplasmic weak	Cytoplasmic weak	Cytoplasmic strong
BCL10 expression	Nuclear strong	Nuclear strong	Cytoplasmic strong
NF-κB activation	Yes	Yes	Yes
Additional genomic abnormalities	No	Yes	Yes

API2 – cellular inhibitor of apoptosis protein 2.; MALT1 – a paracaspase; BCL10 – adaptor protein; ↑ - over-expression; GI – gastrointestinal. Adapted from Bertoni 2006.

Thus, presence of the t(11;18)(q21;q21) translocation is identified specifically in a proportion of patients with MALT lymphoma, particularly lymphomas arising in the gastrointestinal tract and lungs. This translocation results in the fusion of the amino terminal of the API2 gene (apoptosis inhibitor gene) on chromosome 11q21 with the carboxyl terminal of the MALT1 gene on chromosome 18q21 and generates a functional chimera product. The API2 gene encodes a protein that belongs to the inhibitor of apoptosis protein family. This molecule has 3 N-terminal BIR (baculoviral IAP repeat) domains, a middle caspase recruitment domain (CARD), and a C-terminal zinc binding RING finger domain (figure 5.1). MALT1, a paracaspase, comprises an N-terminal death domain (DD), two immunoglobulin-like C2 domains and a caspase-like domain (figure 5.1). All the breakpoints in the API2 gene of the t(11;18)(q21;q21) translocation occur downstream of the third BIR domain but upstream of the C-terminal RING domain, with over 90% of all breakpoints occurring just before the CARD domain (figure 5.1). Conversely, the breakpoints in the MALT1 gene are variable but always upstream of the caspase-like domain. Thus, the resulting fusion product always comprises the N-terminal region of API2, with three intact BIR domains and the C-terminal MALT1 region containing an intact caspase-like domain (Ye 2003). The presence of these domains in the functional fusion product generated from this

translocation strongly suggests they have an important role in oncogenesis. The t(11;18)(q21;q21) fusion protein can activate NF-κB, which is functionally increased when compared to wild-type MALT1 (Lucas 2001; Zhou 2005). NF-κB activation is one of the downstream effects following stimulation of the T and B cell-surface receptors. In unstimulated cells, NF-κB molecules are sequestered in the cytoplasm due to binding with inhibitory κB proteins. Upon degradation of the inhibitory κB binding proteins, NF-κB is released and migrates to the nucleus where it can act as a transcription factor for various genes.

Detection of the t(11;18)(q21;q21) translocation is a valuable aid in the diagnosis of MALT lymphoma and in determining prognosis of primary gastric GML, as tumours with this translocation do not respond to *H. pylori* eradication (Liu 2001).

As identified in chapter 4, two of the SNPs investigated within the IL1 gene region, IL1A-889 and IL1B+3953, showed significant differences in genotype frequency between the Netherlands GML cases and the *H. pylori* infected control group. However, these differences were not detected in the German or UK populations, indicating that these IL1 genotypes do not have a major influence in the development of primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma subsequent to *H. pylori* infection. However, it is unknown if the presence of the t(11;18)(q21;q21) translocation is associated with these IL1 genotypes. Therefore, in this chapter, the presence of the t(11;18)(q21;q21) translocation was determined for the GML cases from each population, Netherlands, Germany and UK. The presence of this translocation was identified to evaluate the hypothesis 'are the IL1 genotypes associated with the presence or absence of the t(11;18)(q21;q21) translocation in GML patients subsequent to *H. pylori* infection?'. The presence of the translocation can be identified from an archival paraffin-embedded, formalin-fixed tissue biopsy of the malignancy by detecting the chimeric fusion product using cDNA generated from RNA extracted from the biopsy.

5.2. Materials and methods.

Detection of the t(11;18)(q21;q21) translocation in the UK GML cases was performed at Cambridge University, Department of Histopathology, Addenbrooke's Hospital under the guidance of Professor M-Q Du and Dr. H Liu. The presence of the translocation was identified from an archival paraffin-embedded, formalin-fixed tissue biopsy of the malignancy by detecting the chimeric fusion product using cDNA generated from RNA extracted from the biopsy. The presence of this translocation had previously been determined for the German and Netherlands GML cases using this method and the results kindly provided by Professors Andreas Nuebauer and Daphne De Jong.

5.2.1. RNA extraction and transcription into cDNA.

Four 10μM sections of malignant tissue were cut from each archival paraffin-embedded biopsy as previously described in chapter 2.2.8.1. To reduce the risk of contamination no more than four biopsy samples were processed together. To detect the presence of any contamination, 4x10μM sections were cut from an empty paraffin wax block following the cutting of the four biopsies. This negative (no tissue) control was subsequently used to detect the presence of any contamination from the cutting of biopsies, through to extraction of RNA, reverse- transcription into cDNA and finally detection of the translocation by PCR.

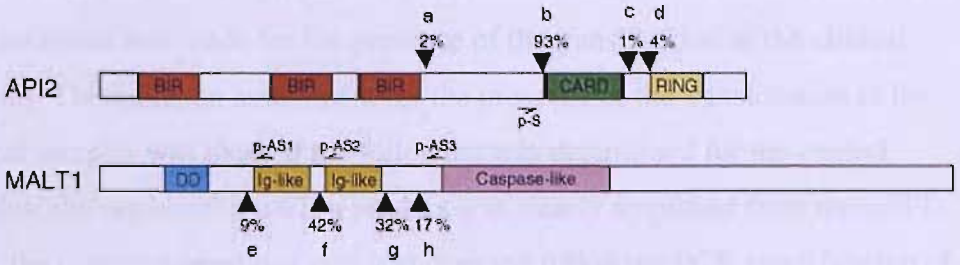
RNA was extracted from the tissue sections and transcribed into cDNA as described in chapter's 2.2.8.1. and 2.2.8.3. cDNA was generated using four gene specific primers, which had previously been designed by Liu *et al* (2002a). These gene specific primers used to generate the cDNA comprised of three MALT1 specific reverse primers, p-AS1, p-AS2, p-AS3 (figure 5.1), and a reverse primer specific for the glucose-6-phosphate dehydrogenase gene (G6PD). The combination of the three MALT1 specific primers ensured that all MALT1 gene regions would be transcribed irrespective of the translocation breakpoints. Generation of cDNA for the G6PD gene region was required to validate RNA quality and reverse-transcription PCR efficiency.

5.2.2. Detection of the t(11;18)(q21;q21) translocation.

The presence of the t(11;18)(q21;q21) translocation was detected by amplifying a short segment of the fusion junction of the API2-MALT1 chimera transcript. Three separate primer mixes were used to detect 93% of all known breakpoints. The three primer mixes consisted of a common API2 forward primer, p-s, that detected 93% of the known API2 breakpoints in combination with one of the following MALT1 reverse primers, p-AS1, p-AS2 or p-AS3 (figure 5.1). These three reverse MALT1 primers had previously been used to generate the cDNA from RNA, as discussed in section 5.2.1, and detected all four known variable breakpoints of the MALT1 gene. In parallel, a fourth set of primers that amplified 67bp and 151bp products of the G6PD gene was included as a control to validate RNA quality and reverse transcription-PCR efficiency. The G6PD primers were designed to span an exon-exon junction to avoid amplification from genomic DNA. Therefore, for each cDNA sample four separate PCR assays were performed with each primer pair (chapter 2.2.8.4.), three detected 93% of all known breakpoints and the G6PD assay validated the quality of the cDNA sample. For each primer pair, a known t(11;18)(q21;q21) positive control cDNA sample (kindly provided by H. Liu which and previously been sequenced to confirm the location of the fusion junction), the paraffin extraction negative control (no template) and a PCR negative control (H_2O) were also tested in parallel with samples. Following PCR amplification, products were analysed by electrophoresis using 10% polyacrylamide gels. Figure 5.2 shows the expected size of the amplicon products for each primer pair.

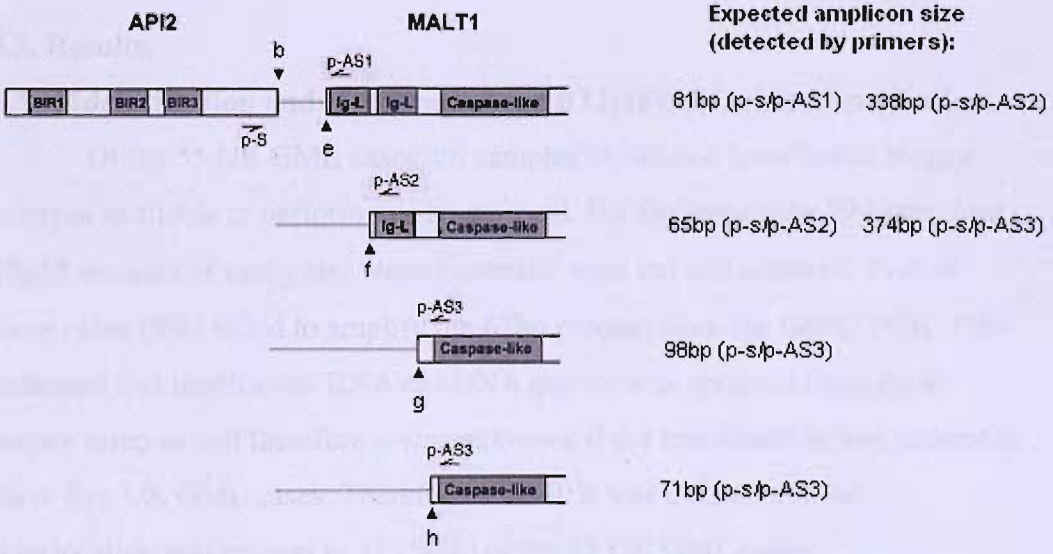
All primers were designed by Liu *et al* (2002a) for amplification from archival biopsies. Amplicon products were small (67-98bp) and therefore suitable for cDNA generated from the fragmented RNA obtained from archival biopsies. The method had previously been validated for the detection of the t(11;18)(q21;q21) translocation using archival, paraffin-embedded tissue biopsies of primary gastric MALT lymphomas (Liu 2002a). The assay was determined to be very sensitive as the translocation was detected from as few as 100 micro-dissected cells (Liu 2002b).

Fig. 5.1 Schematic diagram of the API2 and MALT1 genes.



Arrowheads indicate position of the breakpoints, a-h, and their frequency. The position of the primers, p-S, p-AS1, p-AS2 and p-AS3, used to detect the t(11;18)(q21;q21) translocation product by PCR is shown. BIR = baculovirs IAP repeat; CARD = caspase recruitment domain; DD = death domain. Adapted from Isaacson 2005.

Fig. 5.2. Schematic diagram showing examples of the API2-MALT1 fusion transcripts.



The API2-MALT1 fusion transcripts detected by the three primer mixes and the expected amplicon size. If sufficient length of cDNA is present the fusion transcript generated from joining API2 breakpoint b with MALT1 breakpoint e can be detected in mixes p-s/p-AS1 (81bp amplicon) and p-s/p-AS2 (338bp). This is also true for the fusion of API2 breakpoint b with MALT1 breakpoint f, which can be detected in mixes p-s/p-AS2 (65bp) and p-s/p-AS3 (374bp). In addition splice variants of the API2-MALT1 transcripts have been seen. Adapted from Liu 2002a.

5.2.3. Interpretation of the PCR amplicons.

PCR products amplified from the control samples were examined before an assessment was made for the presence of the translocation in the clinical samples. Therefore, an assessment for the presence of the translocation in the clinical samples was made if the following was determined for the control samples; the presence of a 67bp product was clearly amplified from the G6PD PCR; the expected amplicon size was detected following PCR amplification of the positive control (known t(11;18)(q21;q21) cDNA sample); the paraffin extraction negative control (no template) and the PCR negative control (H_2O) were both clearly negative following amplification. Detection of a prominent amplicon of expected size in any of the three PCR assays specific for the API2-MALT1 fusion products indicated the presence of the translocation. If the controls did not yield the expected results then the assay was deemed invalid and an interpretation of the results from the clinical sample could not be made. In this instance, it was unknown if the biopsy sample contained the translocation.

5.3. Results.

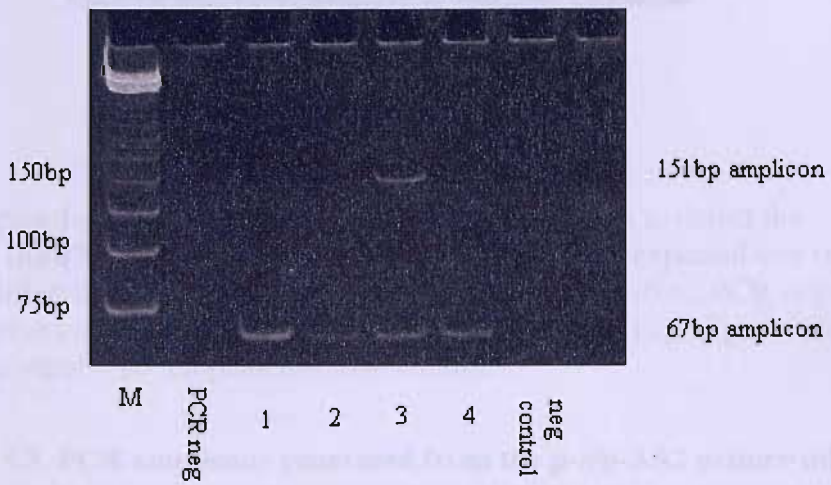
5.3.1. Identification and frequency of the t(11;18)(q21;q21) translocation.

Of the 55 UK GML cases, 26 samples (47%) had insufficient biopsy material available to perform this assessment. For the remaining 29 cases, four 10μM sections of malignant biopsy material were cut and assessed. Five of these cases (9%) failed to amplify the 67bp product from the G6PD PCR. This indicated that insufficient RNA or cDNA quality was obtained from these biopsy samples and therefore it was unknown if the translocation was present in these five UK GML cases. Therefore overall, it was unknown if the translocation was present in 31 (56%) of the 55 UK GML cases.

Of the remaining 24 samples, six (11%) were positive for the t(11;18)(q21;q21) translocation. Three cases amplified the 98bp amplicon or a splice variant using the p-s/p-AS3 primer pair, a further two cases amplified the 65bp amplicon using the p-s/p-AS2 primer pair and one case amplified the 81bp amplicon using the p-s/p-AS1 primer pair. Figures 5.3 to 5.6 show the results for the UK GML cases 1 to 4.

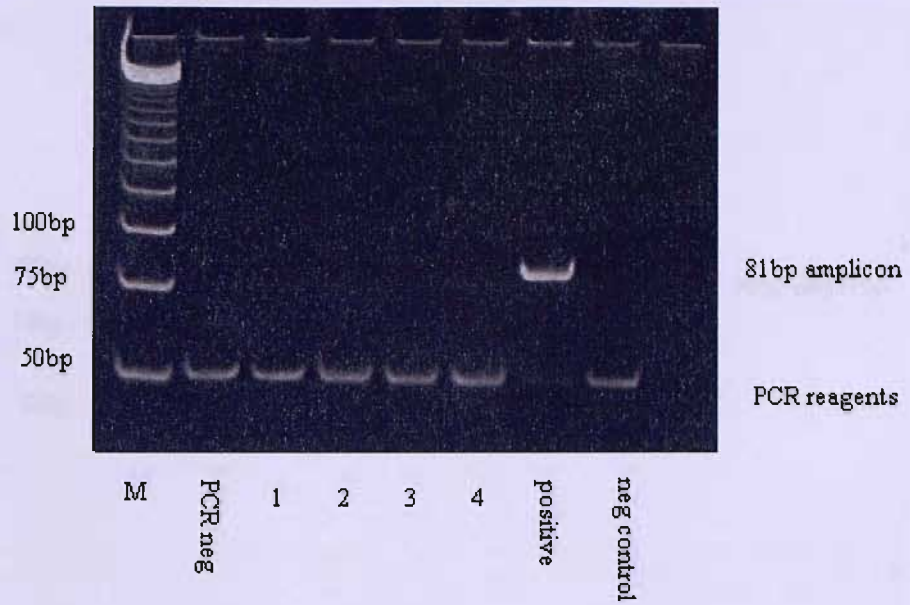
For the remaining 18 samples (33%), all controls amplified as expected and at least a 67bp amplicon was detected using the G6PD assay. As no t(11;18)(q21;q21) specific amplicons were detected using cDNA from these 18 cases it was assumed that the t(11;18)(q21;q21) translocation was not present in these samples. However, the method used only detects 93% of all known breakpoints.

Fig. 5.3 The G6PD assay to determine cDNA quality.



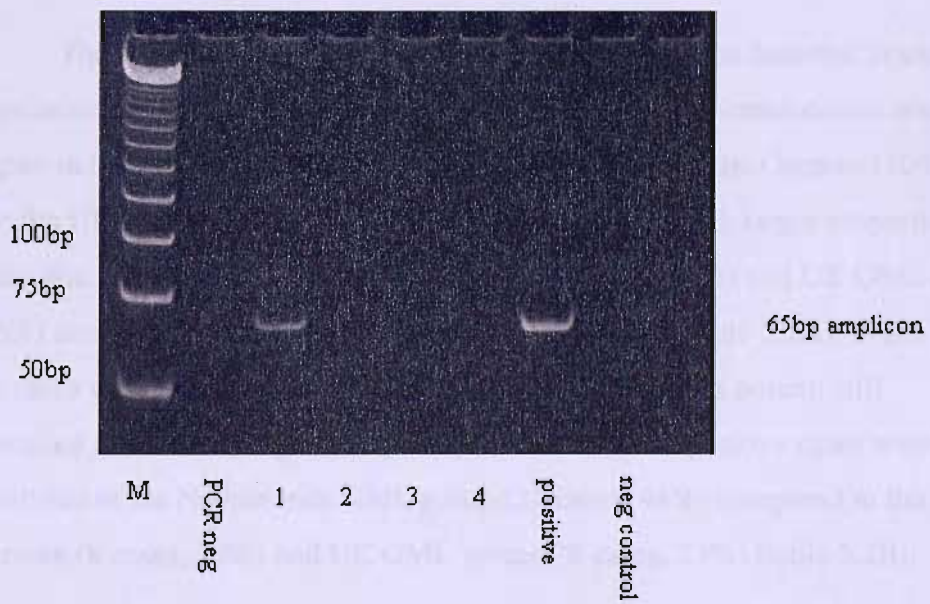
Samples 1-4 assessed using the G6PD PCR assay. The 67bp amplicon was amplified from all four cDNA samples. In addition, the 151bp amplicon was amplified from sample 3 and weakly from sample 4. This indicated that sufficient quality and quantity of cDNA was available for samples 1-4. M = molecular marker. PCR neg = PCR negative control (H_2O). neg control = no template negative control.

Fig. 5.4. PCR amplicons generated from the p-s/p-AS1 primer mix.



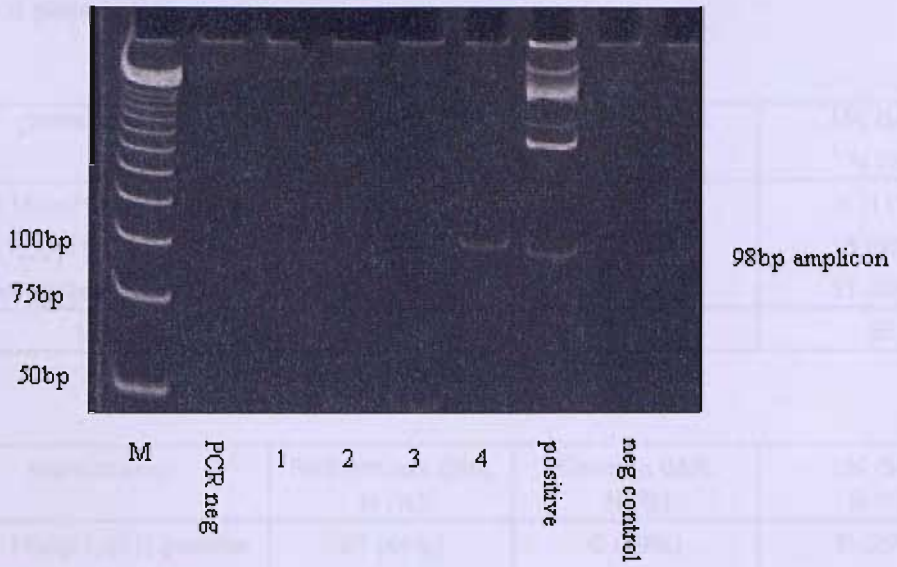
Samples 1-4 assessed using the p-s/p-AS1 primer mix to detect the t(11;18)(q21;q21) translocation. No amplicons of the expected size were amplified from any of samples 1-4. M = molecular marker. PCR neg = PCR negative control (H_2O). positive = known t(11;18)(q21;q21) positive control. neg control = no template negative control.

Fig. 5.5. PCR amplicons generated from the p-s/p-AS2 primer mix.



Samples 1-4 assessed using the p-s/p-AS2 primer mix to detect the t(11;18)(q21;q21) translocation. The 65bp amplicon was amplified from sample 1. This indicated that sample 1 was positive for the t(11;18)(q21;q21) translocation. No amplicons of the expected size were amplified from samples 2-4. M = molecular marker. PCR neg = PCR negative control (H_2O). positive = known t(11;18)(q21;q21) positive control. neg control = no template negative control.

Fig. 5.6. PCR amplicons generated from the p-s/p-AS3 primer mix.



Samples 1-4 assessed using the p-s/p-AS3 primer mix to detect the t(11;18)(q21;q21) translocation. A splice variant of the 98bp amplicon was detected in sample 4. This indicated that sample 4 was positive for the t(11;18)(q21;q21) translocation. No amplicons of the expected size were amplified from samples 1-3. M = molecular marker. PCR neg = PCR negative control (H_2O). positive = known t(11;18)(q21;q21) positive control. neg control = no template negative control.

The frequency of the t(11;18)(q21;q21) translocation detected in each population is presented in table 5.2. The frequency of this translocation was higher in the Netherlands GML cases (32%) compared to the German (10%) and the UK GML cases (11%). However, there was a much larger proportion of cases that were not tested or unknown in the German (46%) and UK GML cases (56%) compared to the Netherlands GML cases (28%) (table 5.2A). When only the cases where a result was obtained were considered, this pattern still prevailed and a much higher frequency of translocation positive cases were identified in the Netherlands GML group (23 cases, 44%) compared to the German (8 cases, 19%) and UK GML groups (6 cases, 25%) (table 5.2B).

Table 5.2. Frequency of the t(11;18)(q21;q21) translocation detected in each population.

A

translocation	Netherlands GML N (%)	German GML N (%)	UK GML N (%)
t(11;18)(q21;q21) positive	23 (32%)	8 (10%)	6 (11%)
t(11;18)(q21;q21) negative	29 (40%)	35 (44%)	18 (33%)
not tested/unknown	20 (28%)	36 (46%)	31 (56%)
total	72	79	55

B

translocation	Netherlands GML N (%)	German GML N (%)	UK GML N (%)
t(11;18)(q21;q21) positive	23 (44%)	8 (19%)	6 (25%)
t(11;18)(q21;q21) negative	29 (56%)	35 (81%)	18 (75%)
total	52	43	24

The frequency of the t(11;18)(q21;q21) translocation for all GML cases included in this study is shown in table A. Table B shows the frequency of the GML cases that were either positive or negative for the translocation.

5.3.2. The IL1 region genotypes and the presence of the t(11;18)(q21;q21) translocation.

Table 5.3 shows the genotype frequencies of the SNPs within the IL1 region in relation to the translocation positive and negative GML cases for each population. Although a much higher frequency of the t(11;18)(q21;q21) translocation was detected in the Netherlands GML cases compared to the German and UK GML cases (table 5.2), this did not correlate with a significant difference in genotype frequencies for any of the SNPs within the IL1 region (table 5.3).

Table 5.3. Genotype frequencies of the SNPs within the IL1 region in relation to the presence of the t(11;18)(q21;q21) translocation.

SNP		Netherlands GML t(11;18) translocation pos	neg	*P*	German GML t(11;18) translocation pos	neg	*P*	UK GML t(11;18) translocation pos	neg	*P*
IL1A-889	CT	13	11	0.39	2	8	1.00	2	6	1.00
	CC	7	9	1.00	5	19	1.00	4	9	0.65
	TT	3	7	0.31	1	5	1.00	0	3	0.55
	Total	23	27		8	32		6	18	
IL1B+3953	CT	8	12	0.57	3	12	1.00	2	6	1.00
	CC	11	11	0.78	5	18	0.70	4	11	1.00
	TT	4	4	1.00	0	5	0.56	0	1	1.00
	Total	23	27		8	35		6	18	
ILB-31	CT	13	14	0.78	6	15	0.13	2	13	0.15
	CC	3	2	0.65	1	2	0.47	1	0	0.25
	TT	7	11	0.56	1	18	0.06	3	5	0.36
	Total	23	27		8	35		6	18	
IL1B-511	CT	13	14	0.78	5	14	0.22	2	13	0.15
	CC	7	11	0.57	1	17	0.11	3	5	0.36
	TT	3	3	1.00	1	3	0.54	1	0	0.25
	Total	23	28		7	34		6	18	
IL1RN+2018	CT	6	10	0.56	4	13	0.69	2	6	1.00
	CC	2	3	1.00	0	4	0.57	0	2	1.00
	TT	15	16	0.57	4	17	1.00	4	10	1.00
	Total	23	29		8	34		6	18	

P values were calculated using Fisher's Exact test. There was no significant difference in the genotype frequencies for any of the IL1 region SNPs and the presence of the t(11;18)(q21;q21) translocation for any of the populations.

In addition, the genotype frequencies for all 18 SNPs from the 14 cytokine genes investigated did not differ significantly between translocation positive and translocation negative GML cases in any of the populations (data not shown).

5.4. Discussion.

There was a much higher frequency of t(11;18)(q21;q21) translocation positive cases within the Netherlands GML group compared to the German and UK GML groups. Therefore, there was clinical heterogeneity between the Netherlands GML group and the German and UK GML groups. Thus, the selection of the Netherlands GML cases may have been biased towards patients with a poor response to *H. pylori* eradication as a form of treatment compared to the German and UK GML cases. This difference in the GML cases may have influenced the significant differences detected in the IL1 genotype frequencies identified in the Netherlands

population but not in the German or UK populations. However, a direct association between the presence of the t(11;18)(q21;q21) translocation and the SNPs within the IL1 region was not identified.

Chapter 6: Final conclusions.

6.1. Infection and susceptibility to disease.

Susceptibility to infection and the severity of ensuing disease is dependent on several factors including environmental and microorganism based variables. However, studying only the environmental and virulence factors of the microorganism cannot explain differences in the course of an infection between individuals. The role of host genetics, particularly genes encoding immune mediators, provides insight into infection and the development and severity of disease (Coussens 2002). Evidence is accumulating that both the infectious agent and the host immunogenetic background contribute to the disease outcome following infection.

Infection with *Helicobacter pylori* has been associated with several upper gastrointestinal diseases, including distal gastric adenocarcinoma, gastric and duodenal ulcer disease and primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma (GML). Several *H. pylori* virulence genes, such as cagA, have been identified and associated with an increased risk of developing severe gastritis, atrophic gastritis, both gastric and duodenal ulcers (Nomura 2002a) and distal gastric adenocarcinoma (Nomura 2002b). However, most individuals infected with *H. pylori* do not develop severe disease, strongly indicating that host factors are also involved in disease development.

There is growing evidence that regulatory polymorphisms outside the exonic regions are important modulators of gene expression (Wray 2003). Regulatory polymorphisms can be classified as either *cis*-acting (present in or near the locus of the gene it regulates such as a transcription-factor binding site) or *trans*-acting (a polymorphism in one gene affecting the expression of another gene). Several groups have performed genome-wide analysis of the variability in gene expression in humans (Cheung 2003; Morley 2004). By examining the variation in gene expression these groups have identified genes whose transcript levels differed greatly among unrelated and related individuals. Furthermore, they found evidence for familial aggregation of the expression phenotype using monozygotic twins, siblings and family members. This variation in expression was associated with both *cis* and *trans*-acting markers at the population and familial level (Morley 2004). In addition, SNPs contributed to the variation in

human gene expression. Genetic polymorphisms can be assessed using reporter gene assays in which cells are transiently transfected with allele or SNP – specific promoter constructs. A review of these studies confirmed that functionally significant polymorphisms were located in the proximal regulatory promoter regions of genes and 3' untranslated regions (Rockman 2002). These functional polymorphisms included sites that affected the binding of transcription factors. Notably, the transcription factors, Sp1, Oct-1, NF-κB, GATA and USF, which all showed variation of binding using gel shift assays (reviewed in Knight 2005). Rockman and Wray (2002) estimate that humans are heterozygous at more functional *cis*-acting sites than at amino acids positions, with 10,700 functional bi-allelic *cis*-regulating polymorphisms in an individual. A recent study found that 34% of promoter polymorphisms significantly modulated reporter gene expression more than 1.5 fold (Hoogendoorn 2003).

The effects of functional polymorphisms in genes, particularly genes encoding cytokines and chemokines, on the outcome of infection are providing information concerning the susceptibility to and severity of infection and disease. Indeed, El-Omar (2003) has shown in several populations that individuals with cis-acting regulatory polymorphisms within promoter regions of cytokine genes that are believed to enhance a pro-inflammatory immune response following *H. pylori* infection have a greater risk of developing distal gastric adenocarcinoma. Furthermore, possession of multiple pro-inflammatory polymorphisms in TNF, IL1B, IL1RN, and IL10 genes conferred the greatest risk (OR of 2.8 for one, 5.4 for two, 27.3 for three or four high risk genotypes), indicating that a genetic pro-inflammatory cytokine profile increases the risk of distal gastric adenocarcinoma in response to *H. pylori* infection.

In contrast to gastric adenocarcinoma, a malignancy of gastric epithelial cells, GML, a MALT lymphoma, develops from the acquired extra-nodal mucosa-associated lymphoid tissue that develops following chronic infection with *H. pylori.* MALT lymphomas develop in extra-nodal sites, which in the absence of infection are normally devoid of organised lymphoid tissue. Evidence indicates that MALT lymphomas are associated with chronic antigenic stimulation by microbial pathogens and/or autoantigens. A scenario involving chronic and sustained stimulation of the immune system leading to lymphoid

transformation has emerged. It defines a distinct category of infection-associated lymphoid malignancies, in which the infectious agent does not directly infect and transform lymphoid cells, such as the oncogenic Epstein-Barr virus, but rather indirectly increases the probability of lymphoid transformation by chronically stimulating the immune system to maintain a protracted proliferative state. Sustained activation of the lymphoid system and lymphocyte proliferation, which can be observed during chronic infection, increases the risk of transformation and constitutes a risk factor for lymphomas. Infections may contribute to lymphomagenesis by promoting favourable conditions for lymphocyte transformation, such as increased proliferation, decreased apoptosis of lymphoid cells and a highly toxic environment that can cause DNA damage due to the presence of ROI and RNI during chronic inflammation. These conditions favour the occurrence of oncogenic DNA damage in proliferating lymphocytes (Coussens 2002). The intrinsic genetic instability of B-cells during isotype class-switching and somatic hypermutation also increases the risk of transformation (Goossens 1998; Greeve 2003).

Gastric inflammation and development of acquired MALT are a prerequisite for the development of GML. In addition, the topography of inflammation is associated with clinical outcome and GML is associated with pangastritis. Cytokines play a crucial role in regulating inflammation, are essential for the development of acquired MALT and modulate gastric physiological responses following infection. Therefore, cytokines play a key role in all of these pre-clinical sequelae of GML. In addition, there is individual variation in the level of expression of cytokines and some of this variation is due to *cis*-acting polymorphisms in the promoter regions of cytokine genes. Inter-individual differences in the level of cytokine expression may lead to differences in the immune response to an infection. In this case-control association study, polymorphisms in cytokine genes that are thought to influence the inflammatory process in response to infection were investigated to determine if host cytokine polymorphisms influenced the development of GML following *H. pylori* infection and if these polymorphisms were associated with the presence or absence of the t(11;18)(q21;q21) translocation.

6.2. Significance in case-control association studies.

Complex diseases do not exhibit classical Mendelian patterns of inheritance and it is harder to identify causal genes due to several factors. Disease development is likely to be influenced by multiple genetic as well as environmental factors. There may be incomplete penetrance where subjects inheriting a disease gene do not develop the disease. In addition, phenocopy may affect the ability to identify causal genes as more than one gene may cause the disease trait. All of these problems will reduce the power to identify causal genes. Genetic linkage and positional cloning are appropriate methods for the identification of relatively high-risk genes, but this approach has not been successful for the identification of genes in complex forms of polygenic diseases, such as infectious-disease susceptibility and severity. However, allelic association studies do provide the most powerful method for locating genes of small effect contributing to complex diseases (Daniels J, 1998). Candidate gene studies can detect small to moderate relative risks in the context of aetiological and genetic heterogeneity by studying the relevance of functional single nucleotide polymorphisms (SNPs) in genes.

The power of a study is the probability that a study will detect an effect of a particular size. Overall, the study presented in this thesis, comparing 203 cases with 568 controls, had 80% power to detect a risk of disease of at least 1.5 to carriers of an allele compared to non-carriers, for all SNPs with a lowest allele frequency of 20% or higher. Overall, the detectable risk varies from 1.2 for IL8-251 SNP to 4 for TNF-376 SNP. However, for the individual populations, German, Netherlands or UK, the detectable risk varies from 1.4 for IL8-251 to 7 for TNF-376. In addition, the study was designed to identify trends across all three populations that may indicate a locus is associated with the development of GML, rather than a statistical significant result. However, none of the cytokine polymorphisms investigated were significantly associated with the development of primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma subsequent to *H. pylori* infection in all three European populations. In addition, there was no trend towards significance in all three populations to indicate any of the SNPs investigated were associated with development of disease. Therefore, the SNPs investigated do not have a major influence in the development of GML subsequent to *H. pylori* infection. This is in accordance

with the study by S. Hellmig *et al* (2004), and confirms the conclusion that the pro-inflammatory cytokine polymorphisms that favour the development of gastric adenocarcinoma do not appear to have a major influence in the development of GML subsequent to *H. pylori* infection. However, this does not exclude a role of the cytokine in the development of this disease. Investigation of SNPs that span the entire gene and its promoter is required to fully evaluate the influence of a particular cytokine in the development of this disease. Until the full extent of the polymorphisms within a cytokine gene and their influence on expression and functional activity are determined, then the SNPs required to be investigated to determine a cytokine's role in the development of disease will remain controversial.

Selection of the phenotype of cases and controls is critical to the success of identifying causal genes. If the cases include a heterogeneous collection of aetiologies for a complex disease, then the power to detect significant association is reduced. Similarly, if the control group are affected but undiagnosed or pre-clinical, again power is reduced. In this study, cases were selected as low-grade primary gastric extra-nodal marginal zone B-cell (MALT) lymphoma from the Netherlands, Germany and the UK. The controls were confirmed *H. pylori* gastritis cases with no indication of atrophy, gastric adenocarcinoma or MALT lymphoma. However, in chapter 5 clinical heterogeneity in the GML cases was identified, as a much higher proportion of Netherlands cases (44%) were t(11;18)(q21;q21) translocation-positive compared to the German and UK GML cases (19% and 25%, respectively). Studies by H. Liu *et al* (2002a) identified that the majority of primary GML patients who are negative for this translocation respond to *H. pylori* eradication resulting in regression of the lymphoma. In translocation-positive GML patients the lymphoma does not regress in response to *H. pylori* eradication. In addition, in translocation-positive patients the lymphoma is more likely to have spread to local lymph nodes and distal sites, whereas in translocation-negative cases the lymphoma tends to remain localised in the stomach. Thus, the t(11;18)(q21;q21) translocation is associated with adverse clinical features. This difference between the Netherlands GML cases and the German and UK GML cases may have influenced the significant differences identified in the genotype frequencies for the IL1-889 and IL1+3953 SNPs (P= 0.005 and 0.006,

respectively) detected for the Netherlands population but not the German or UK populations. However, a direct association between the IL1 SNPs and the presence of the translocation was not detected when analysed in chapter 5. The levels of the significant differences identified for the IL1-889 and IL1+3953 SNPs (P= 0.005 and 0.006, respectively) in the Netherlands population are unlikely to be due to spurious associations. Case-control association studies can be susceptible to spurious associations, which are due to differences in population stratification between cases and controls, such as age, sex, ethnicity and exposure to environmental factors and that the selection of the cases are more related to each other than the control group. Therefore, the risk of confounding factors must be removed and controls and clinical cases selected from the same population. Significant differences between the Netherlands cases and controls seem unlikely as the allele frequencies for the control group where not significantly different from the German or UK control groups, or significantly different from the Netherlands cases for the majority of SNPs investigated. As in any statistical test, false positives or false negatives may result by chance. However, the level of significance identified for the IL1A-889 and IL1B+3953 SNPs in the Netherlands population is unlikely to have occurred by chance. Although ideally, due to multiple testing (18 tests) on a single data set, any statistical significance should be adjusted for multiple comparisons. Bonferroni correction can be applied to correct for multiple comparisons, and under these circumstances the differences in the genotype frequencies for the IL1A-889 and IL1B+3953 SNPs are not statistically significant (P= 0.09 and 0.1, respectively). However, Bonferroni correction is very strict and may result in loss of a weak association. In addition, the appropriate correction for multiple statistical comparisons remains unclear for case – control association studies (reviewed in Silverman 2000). Therefore, in the design of this study we considered looking for trends in the three populations to indicate a SNP was associated with development of disease, rather than a true significant result. However, the difference in the frequency of the t(11;18)(q21;q21) translocation between the three GML populations may have reduced the power of this study. Therefore, to clarify this result the SNPs within the IL1 gene region require further investigation in a second study of known t(11;18)(q21;q21) translocation-positive and translocation-negative

GML cases. Ideally, to ensure the risk of confounding factors are minimised, cases and controls should be age-, sex- and ethnicity-matched.

It is unknown if the significant differences identified for the Netherlands and UK populations within the TNF and LTA region are due to chance or reflect an influence of this gene region in the development of GML. LTA+252 GG genotype was decreased in the Netherlands GML patients (2.9%) compared to their control group (13.5%) (P=0.01). A significant difference in genotype frequencies was also identified in the UK group, as LTA+252 AA was decreased in patients (23.1%) compared to the gastritis control group (41.5%) (P=0.02). However, these differences were not consistent between the three populations. A decrease in the LTA+252 GG frequency in the Netherlands GML patients is inconsistent with a decrease in the LTA+252 AA frequency in the UK GML patients. In addition, no significant differences were identified in the German group for the LTA+252 SNP. Furthermore, no significant differences in the TNF-308 or -376 genotype frequencies were identified for any population. This contrasts with other studies where systemic release of TNFα and LTα has been found to be associated with the development of (Spink 2006; Rothman 2006) and severity of non-Hodgkin's lymphoma (NHL) (Warzocha 1998; Juszczynski 2002). However in contrast to this study, cases were mainly follicular and diffuse large B-cell lymphoma patients (DLBCL). Warzocha *et al* (1998) showed patients with high circulating levels of TNFα and LTα, and high TNFα plasma levels were associated with poorer disease outcome. The TNF-308A allele was significantly associated with higher plasma levels of TNFα at diagnosis. Recently a study by the International Lymphoma Epidemiology Consortium on 3586 cases of NHL and 4018 controls of European descent, identified TNF-308A was associated with increased risk for DLBCL (P<0.0001, OR=1.65, CI=1.16-2.34) the main histological subtype studied, but not for follicular lymphoma (Rothman 2006). In addition, Spink *et al* (2006) identified that TNF haplotypes were associated with disease risk. The TNF/LTA gene region shows genetic variation and is associated with susceptibility to a number of infectious and autoimmune conditions, as well as NHL. Stable 'high' and 'low' TNFα producers can be identified in populations and is dependent on a genetic component. However, this region shows a complex pattern of

haplotypes and the exact functional polymorphisms remain extremely controversial and appear highly context specific, depending on the cell type and stimulus. It is important to understand the full haplotype structure if fundamental effects of regulatory SNPs are to be resolved and disease associations finely mapped (reviewed in Knight 2005). Defining specific functional regulatory polymorphisms remains problematic and no doubt has contributed to the differences in results of association studies. There is difficulty in fine mapping the functional polymorphism due to linkage disequilibrium and the complex pattern of haplotypes of some loci. In addition, the experimental tools available to resolve the effects of non-coding sequence variation on gene expression are often highly influenced by the cell type, stimulus and environmental conditions (Rockman 2002). This results in difficulty in defining the specific functional polymorphism(s) at the experimental level. For example, the presence of the Duffy blood group antigen on the surface of erythrocytes is essential for invasion of the malarial parasite *Plasmodium vivax*. Individuals who lack the Duffy protein are protected from malaria caused by this parasite (Horuk 1993). The molecular basis for this protection was found to be due to a SNP at nucleotide position –46, located in the promoter region of the FY gene. In the presence of the T to C SNP, the binding site for the transcription factor GATA-1 was disrupted (Tournamille 1995). Individuals that posses this allele do not express the Duffy protein on erythrocytes but do express it on all other cell types. This arises because GATA-1 is a transcription factor specific for erythrocytes and therefore, this SNP only causes a functional change in this specific cell. Thus, the functional effect of the SNP is only detected in erythrocytes.

Therefore, the inconsistent results in our study for the LTA+252 SNP requires exploration in a larger study group of known translocation status patients. Ideally, examination of the TNF/LTA extended haplotype should be performed to clarify the role of this region in the development of GML and to determine if the results of this study are due to chance.

6.3. Development of GML.

Therefore, what factors favour the development of GML subsequent to *H. pylori* infection? This study has shown that the polymorphisms investigated are unlikely to have a major influence in the development of GML. However, the role of the IL1 gene region and the presence of the t(11;18)(q21;q21) translocation remains to be evaluated. This is in contrast to the development of distal gastric adenocarcinoma (El Omar 2000 and 2003). This indicates that although both malignancies require the same pre-clinical sequaele of chronic inflammation, acquired MALT and pangastritis, the events leading to transformation are potentially different. Epidemiological evidence strongly supports a causal role for *H. pylori* in gastric carcinogenesis. The infection was recognized as a Class I human carcinogen by the International Agency for Research on Cancer in 1994. However, the bacterium does not induce carcinogenesis by itself. The present scientific consensus, for both GML and distal gastric adenocarcinoma, is that the bacterial oncogenic role is mediated by the chronic active inflammation it elicits in the gastric mucosa (Correa 2006; Suarez 2006). Although the ultimate basic mechanism of carcinogenesis is unknown, strongly suggestive evidence points to oxidative stress as having a pivotal role in the process of gastric adenocarcinoma (Correa 2006). Correa has proposed the 'atrophy-metaplasia-dysplasia-carcinoma' model leading to development of distal gastric adenocarcinoma, which can be described as a series of sequential phases. Initially, chronic active inflammation occurs in response to *H. pylori* infection. Infiltration of the gastric mucosa by polymorphonuclear neutrophils, the development of mucosa-associated lymphoid tissue and damage to the epithelial cells, characterize this phase. The second phase is dominated by alterations of the epithelial cell cycle, especially increased rates of apoptosis and cell proliferation. During the third phase nuclear and architectural abnormalities develop, which represent progressive mutational events as expected in classical molecular models of carcinogenesis. Thus, in the presence of a continuing antigen drive, chronic pro-inflammatory immune response and a lower acid environment, greater bacterial colonisation of the gastric epithelium results in pangastritis and a high epithelium turnover. These conditions favour the development of distal gastric adenocarcinoma. In addition, bacterial virulence factors (cagPAI, VacA, BabA), which are

associated with enhanced inflammation, are risk factors for the development of distal gastric adenocarcinoma.

Following chronic infection and development of acquired MALT, survival and selection of the B-cells is determined by the B-cell antigen receptor (BCR) specificity, ligation of co-receptors and cytokine-receptor signal transduction. In the presence of persisting antigens a polyclonal B-cell response is elicited. The mature B-cells are heterogeneous with respect to their micro-anatomic location in the lymphoid organs and functional properties (reviewed in Shapiro-Shelef 2005). Follicular B-cells constitute the major subset of B-cells and participate in T-cell dependent immune responses in the germinal centre (GC), where they receive help from antigen-specific T-cells through specific receptors such as CD40-CD40L engagement. The GC reaction leads to isotype class-switched B-cells with somatic hypermutations (SHM) in their immunoglobulin gene segments. These B-cells differentiate into either plasma cells secreting high-affinity antibody or memory B-cells. In contrast, the marginal zone (MZ) which surrounds B-cell follicles in the spleen and in extra-nodal lymphoid tissue, contains a distinct subset of B-cells, the marginal zone B-cells (IgM^{high} IgD^{low} $CD21^{high}$ $CD23^{low/-}$). MZ B-cells are generally believed to participate in T-cell independent immune responses to microbial pathogens. The specificity of their BCR is skewed towards recognition of T-cell independent antigens, although they can participate in T-cell dependent immune responses (Weller 2005). They contribute significantly to the immunity of invasive infections with polysaccharide-encapsulated bacteria (Guo 1997; Benedict 1999; Guinamard 2000). Unlike follicular B-cells, they respond rapidly to lipopolysaccharide, CD40 ligation or cross-linking of their BCR (Snapper 1993; Oliver 1999). This property, together with their 'activated' phenotype of $CD21^{high}$ $CD35^{high}$ and $CD23^{low}$ (Gray 1984; Snapper 1993; Oliver 1999; Morse 2001) suggest MZ B-cells are equipped to react immediately to pathogens. Upon recognition of antigen, they rapidly proliferate and differentiate into primarily IgM-secreting plasma cells, producing the bulk of the primary antibody response. There is evidence that their BCR maybe poly-reactive (Dammers 2005). However, in contrast to rodent MZ B-cells, over 90% of adult human MZ B-cells are somatically mutated. This has resulted in the development pathway of MZ B-cells to be strongly debated (Weller 2005;

Willenbrock 2005). However, there is evidence that recruitment and selection of MZ B-cells can occur in the absence of exogenous antigen and may occur early in B-cell development by low affinity self-antigens (Dammers 2005; Wen 2005).

The neoplastic cells from MALT lymphomas exhibit features of MZ B-cells from which they are thought to have derived. In MALT lymphomas, the MZ is expanded and surrounds residual GCs. Strikingly, neoplastic B-cells from GML are not specific for *H pylori* antigens but rather for auto-antigens found in the gastric mucosa. These auto-reactive B-cells are thought to receive cognate help from *H pylori*–specific T-cells displaying cross-reactivity with gastric auto-antigens. Interestingly, the clonal B-cells were present in the gastritis years before the clinical emergence of the lymphoma (Zucca 1998). Therefore, what triggered them to become malignant? Proliferation of cells alone does not cause cancer. However, sustained cell proliferation in an environment rich in inflammatory cells, growth factors and DNA-damage promoting agents all increase neoplastic risk. These cells continue to proliferate in a microenvironment that supports their growth and survival. Suarez *et al* (2006) have described a model for the development of MALT lymphomas, which manifest initially as indolent low-grade proliferations. Co-stimulation is provided by cytokines and members of the tumour necrosis factor superfamily such as CD40-CD40L in T-cell–dependent responses and B-cell activating factor (BAFF) primarily produced by dendritic cells in T-cell–independent responses. In the case of *H pylori,* T-cells specific for *H pylori* epitopes provide help to B-cells that recognize cross-reactive auto-antigens present in the gastric mucosa such as fucosylated sialyl–Lewis x through CD40-CD40L co-stimulation. Pathogens have selected countless mechanisms allowing them to persist in the host and colonize their specific niches. Molecular mimicry, a situation in which microbial pathogens express antigenic motifs shared with the host, is a mechanism that favours microbial persistence in the tolerogenic immune system towards auto-antigens. Several *H pylori* antigens resemble auto-antigens, notably the fucosylated Lewis antigens expressed on the surface of the gastric mucosa.

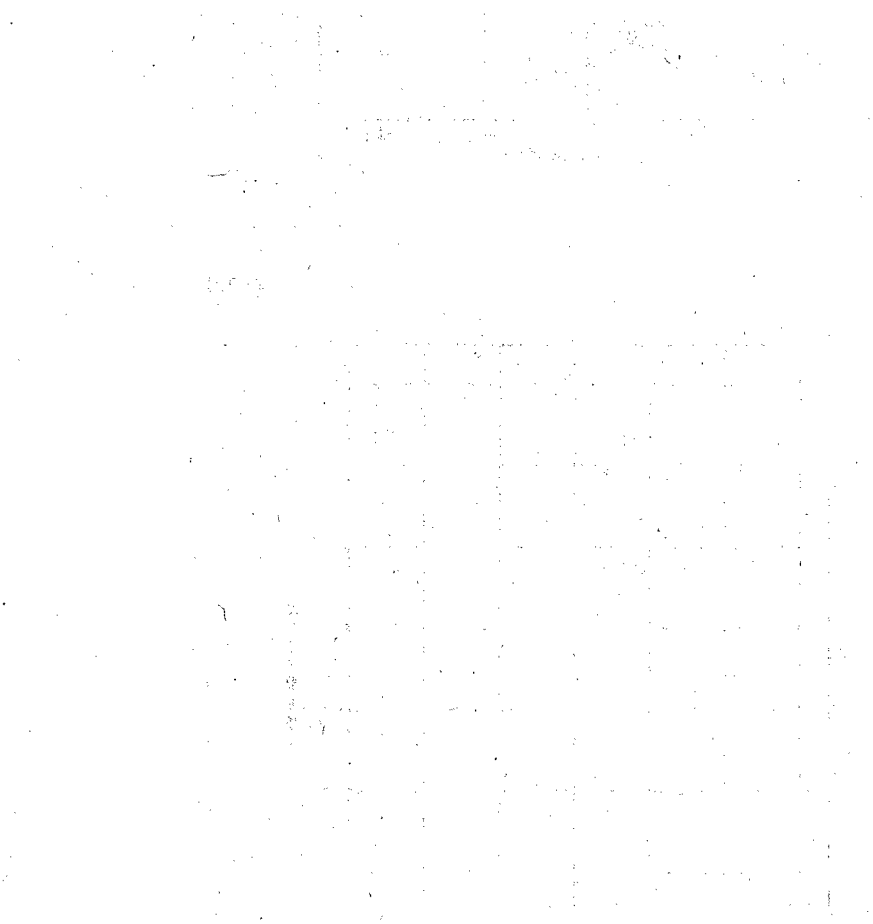
Occurrence of genetic events provides a selective advantage leading to the outgrowth of an antigen-responsive clone. Antigen dependence reflects the

requirement for BCR signals and activation of the NF-κB pathway for cell survival (reviewed in Thome 2004). Development of antigen-independent MALT lymphoma is associated with the presence of the t(11;18)(q21;q21) translocation. This results in the ability of neoplastic B-cells to proliferate in an antigen-independent fashion. The t(11;18)(q21;q21), t(1;14)(p22;q32) and t(14;18)(q32;q21) translocations appear to affect the NF-κB signalling pathway, resulting in activation of NF-κB (Lucas 2001). Furthermore, Zhou *et al* (2006) have compared the chromosomal abnormalities in translocation negative and translocation-positive GML cases using comparative genomic hybridisation. In general, both chromosomal gains and losses were far more frequent in t(11;18)-negative (median = 3.4 imbalances) than t(11;18)-positive cases (median = 1.6 imbalances), with gains being more frequent than losses. Recurrent chromosomal gains involving whole or major parts of a chromosome were seen for chromosomes 3, 12, 18 and 22 (23%, 19%, 19% and 27% respectively) and discrete recurrent chromosomal gains were found at 9q34 (11/26 = 42%). In addition, bioinformatic analysis of genes mapping to the 9q34 region revealed potential targets. Among them, TRAF2 and CARD9 are known interaction partners of BCL10, playing a role in NF-κB activation. Interphase fluorescent in-situ hybridisation confirmed genomic gain of the TRAF2, CARD9 and MALT1 loci in 5/6 and 2/2 cases showing chromosomal gains at 9q34 and 18q21 respectively. These results suggest that genomic gain of genes that modulate NF-κB activation, such as MALT1, TRAF2 and CARD9, may play a role in the pathogenesis of translocation-negative GML (Zhou 2006).

Therefore, these studies suggest a role of NF-κB activation in not only the progression but also the development of GML. In addition, mice deficient or mutated for CARMA1, BCL10 and MALT1, components of NF-κB signalling pathway, have apparently normal development of immature B-cells but the differentiation into MZ B-cells is markedly affected (Thome 2004). This indicates that signalling from the BCR via these molecules is required for the development of MZ B-cells. In addition, specific NF-κB family members have crucial roles in the development of MZ B-cells, as mice deficient in the p50 subunit of NF-κB have markedly reduced numbers of MZ B-cells. The molecular function of the NF-κB family members with respect to generation and survival of MZ B-cells is not understood (reviewed in Thome 2004) but

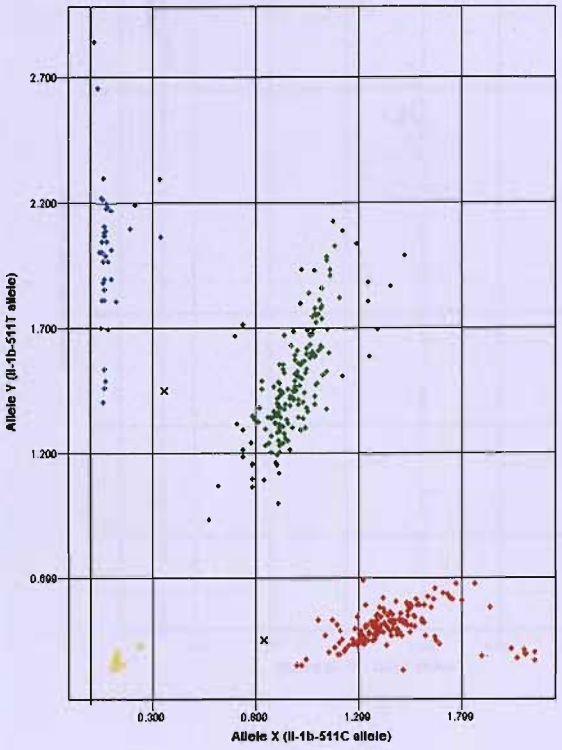
possibly involve the activation of transcriptional targets following ligation of the BCR and/or survival regulating TNF family members such as BAFF. Therefore, a reduced threshold for triggering proliferation may pre-dispose MZ B-cells to neoplasia and mutations in genes regulating apoptosis, such as the anti-apoptotic role of the NF-κB pathway, may lead to malignancy (Morse 2001).

In conclusion, a role for human genetic variation in the pathogenesis of GML remains elusive.

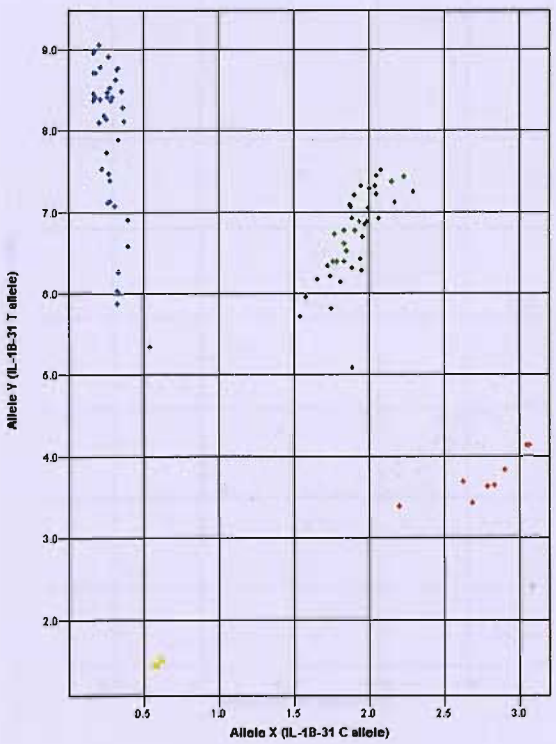


Appendix A: Examples of the eighteen 5' nuclease assays.

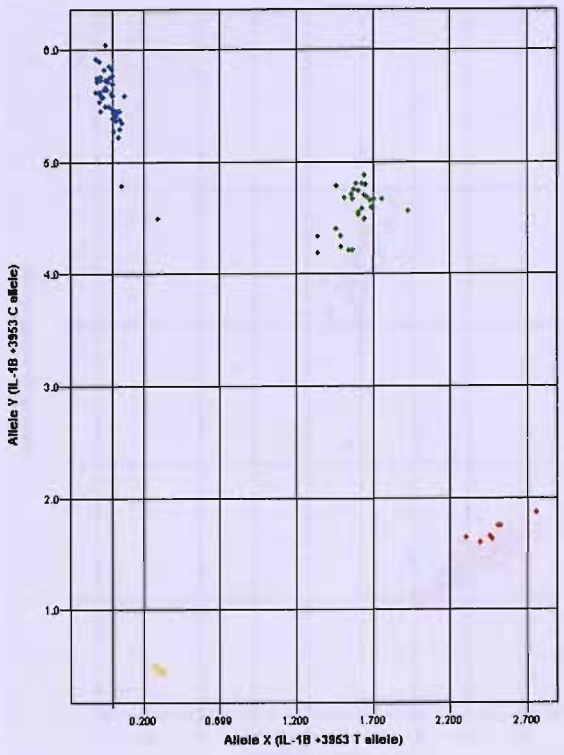
1) IL1B-511 assay.



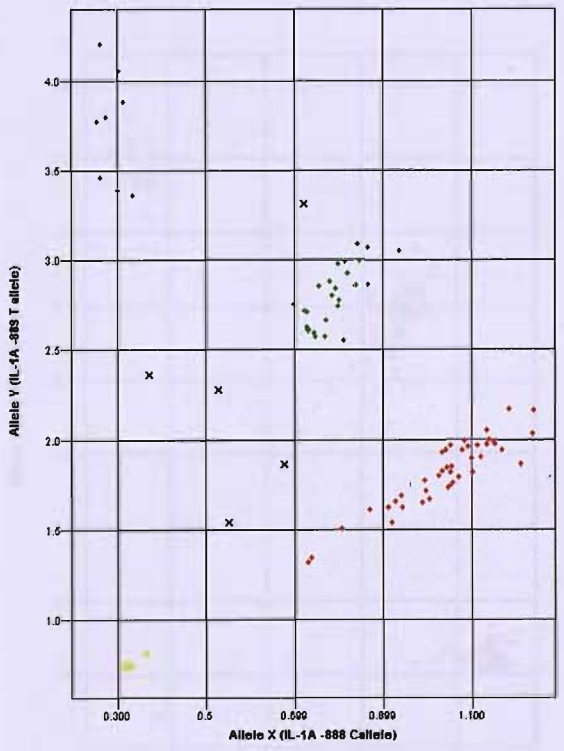
2) IL1B-31 assay.



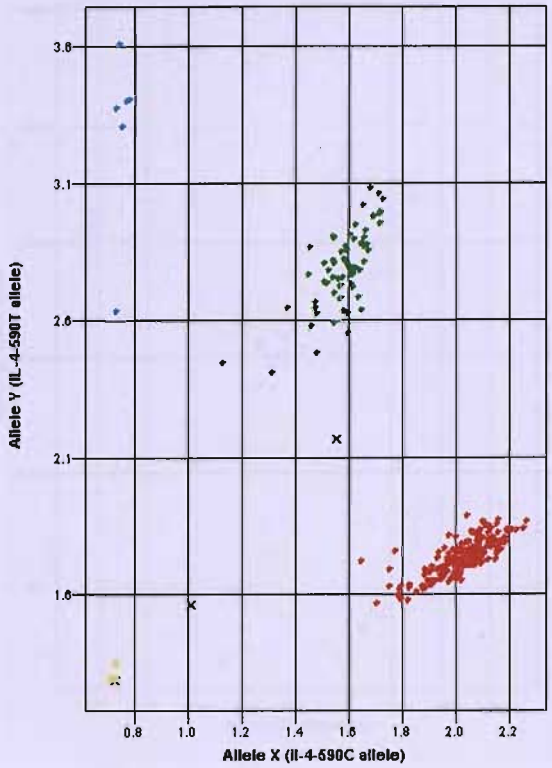
3) IL1+3953 assay.



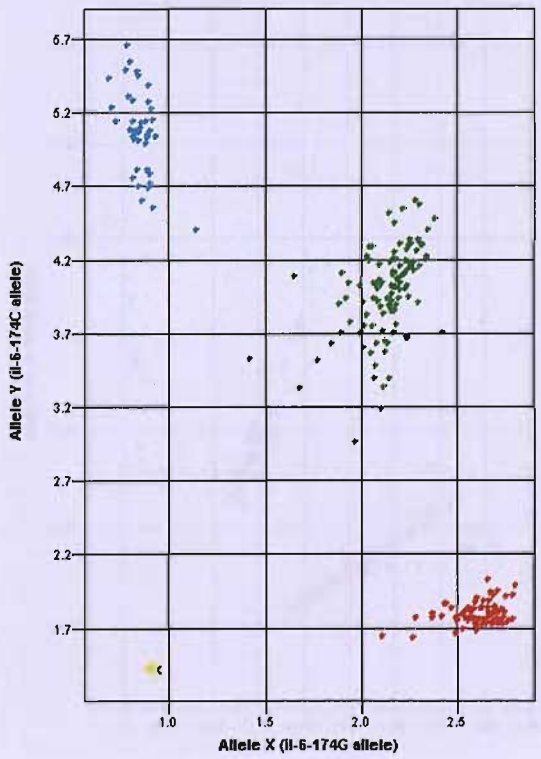
4) IL1A-889 assay.



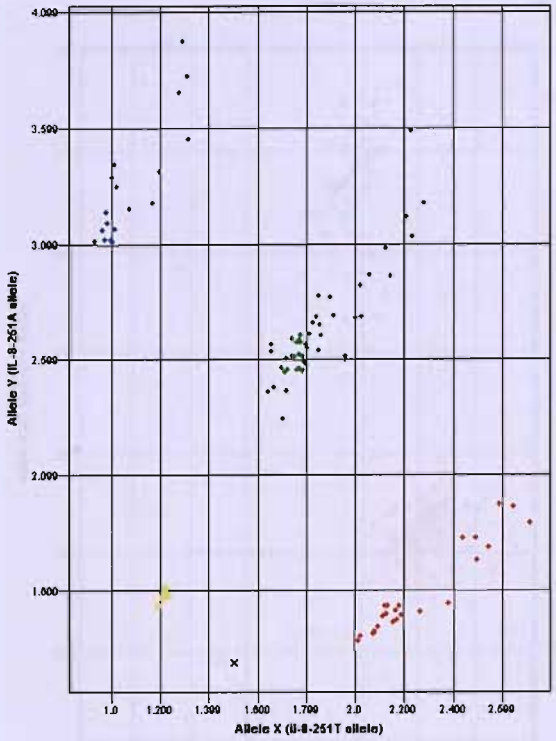
6) IL4-590 assay.



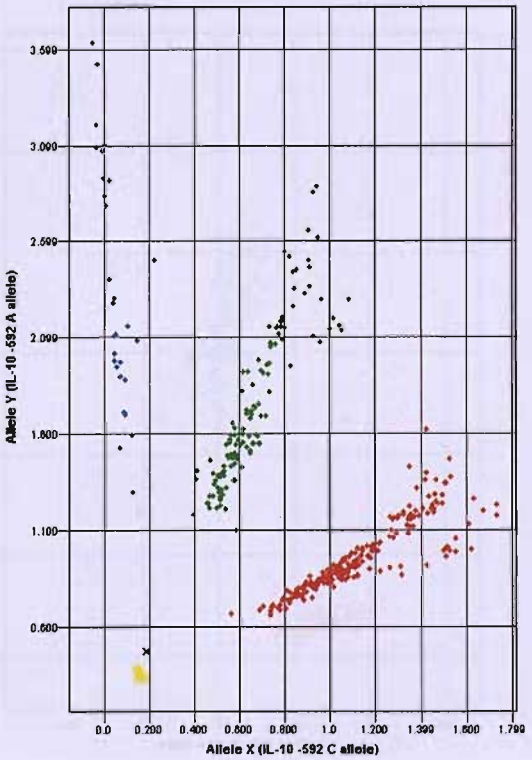
7) IL6-174 assay.



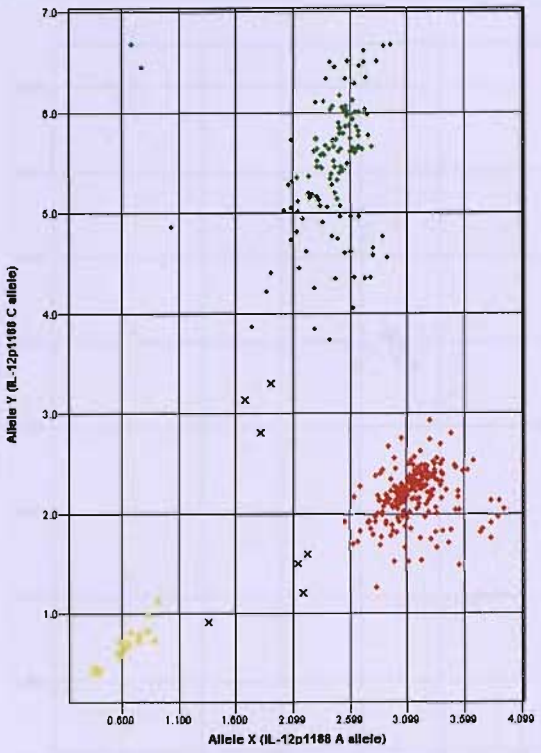
8) IL8-251 assay.



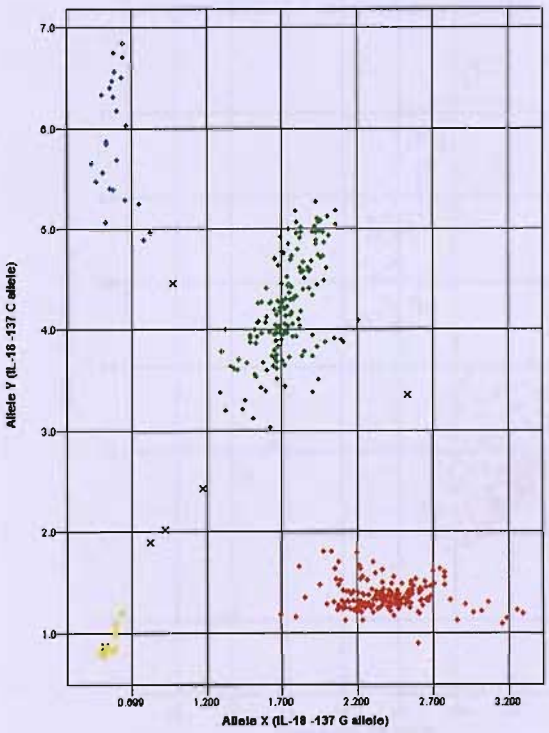
9) IL10-592 assay.



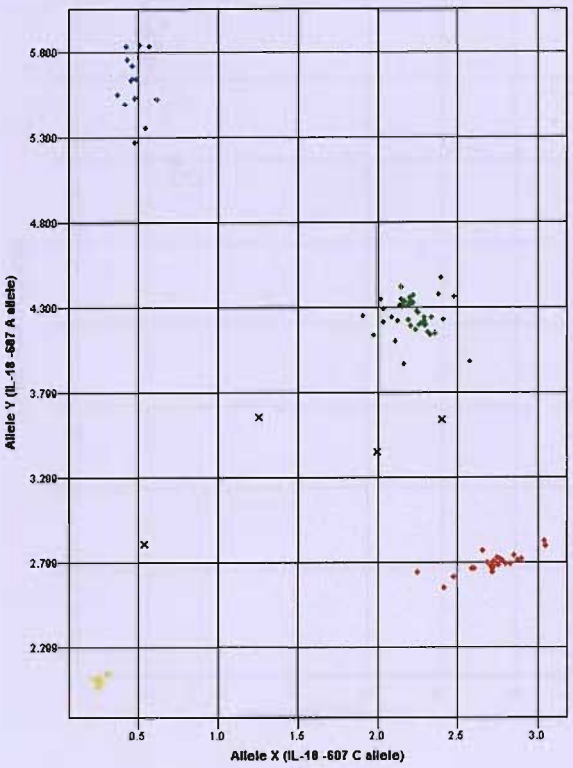
10) IL12B+1188 assay.



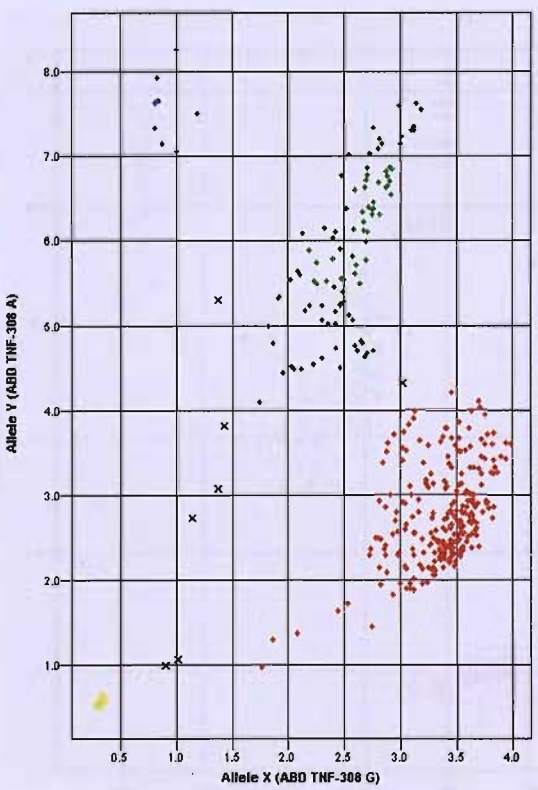
11) IL18-137 assay.



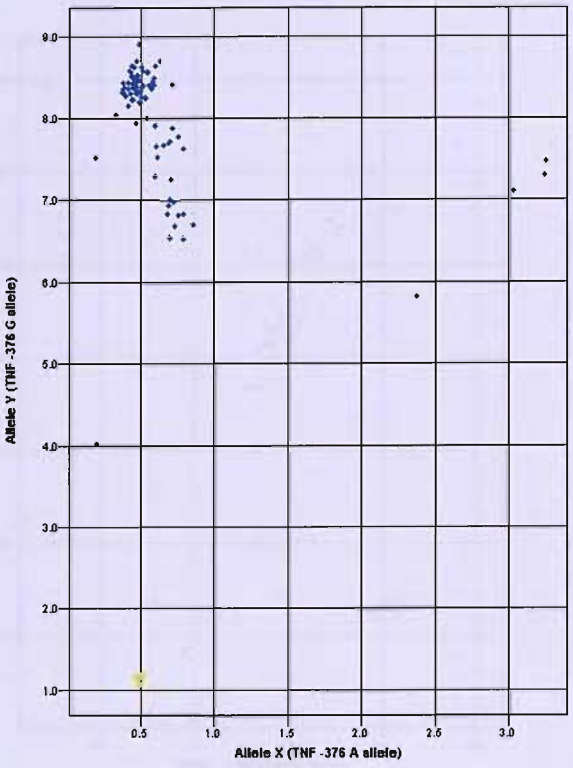
12) IL18-607 assay.



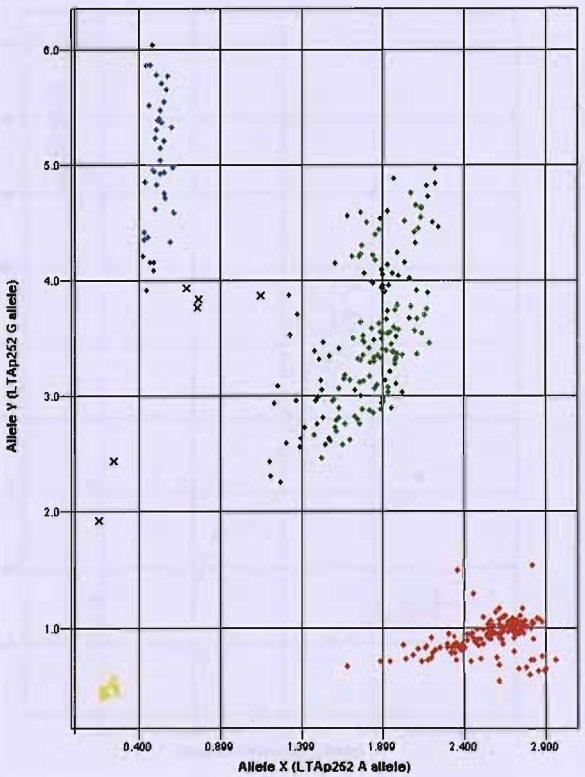
13) TNF-308 assay.



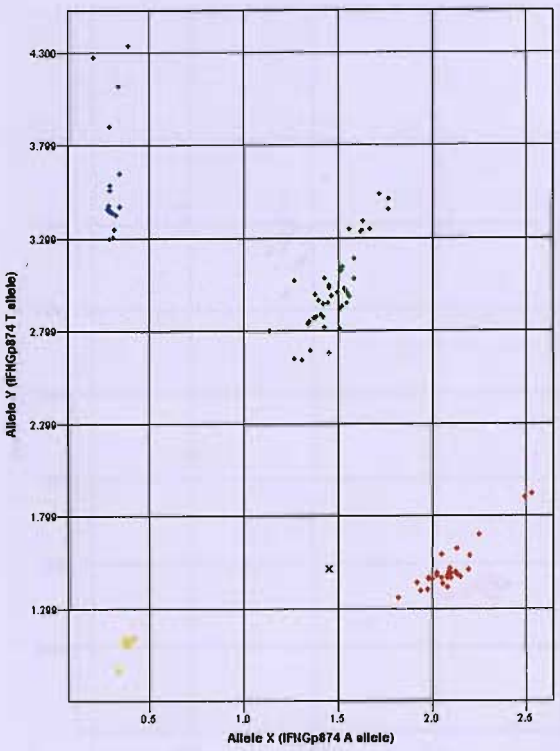
14) TNF-376 assay.



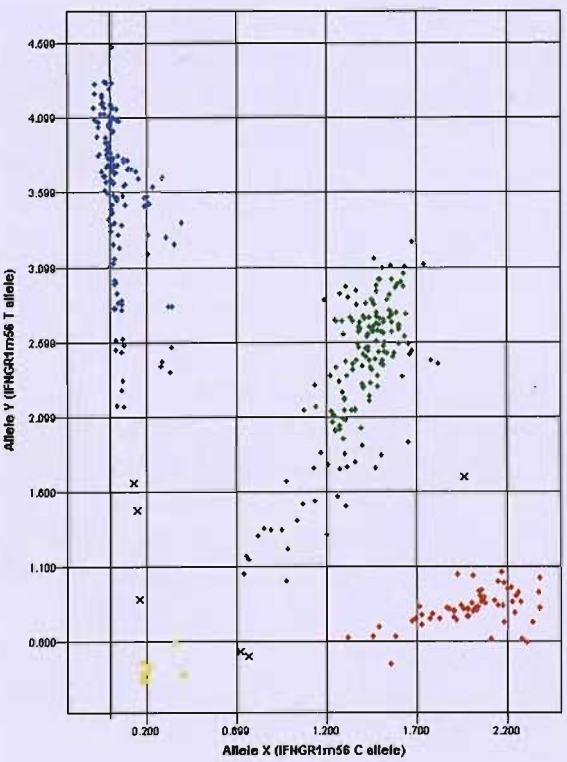
15) LTA+252 assay.



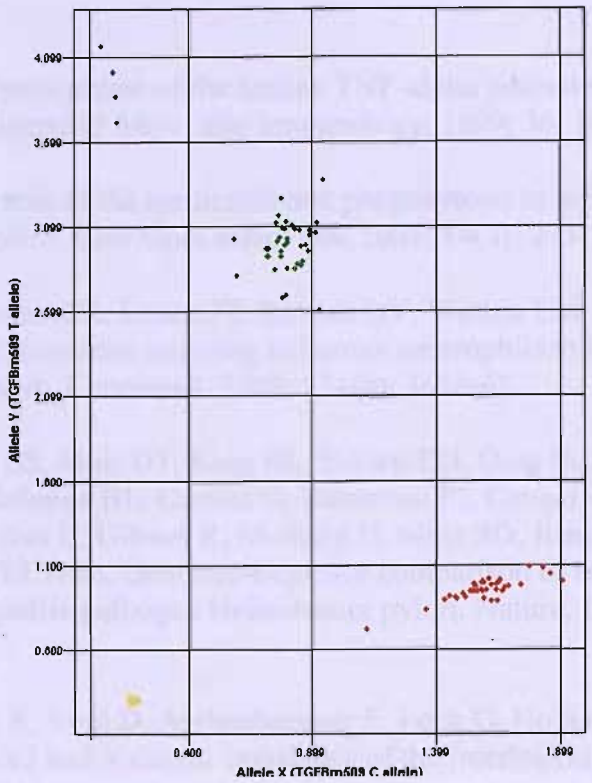
16) IFNG+874 assay.



17) IFNGR1-56 assay.



18) TGFB-509 assay.



References.

Akira S. The role of IL-18 in innate immunity. Curr Opin Immunol. 2000; 12(1): 59-63.

Allen, RD. Polymorphism of the human TNF-alpha promoter- random variation or functional diversity? Molecular immunology, 1999; 36: 1017-1027.

Allen LA. The role of the neutrophil and phagocytosis in infection caused by Helicobacter pylori. Curr Opin Infect Dis. 2001; 14(3): 273-7.

Allen LA, Beecher BR, Lynch JT, Rohner OV, Wittine LM. Helicobacter pylori disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide release. J Immunol. 2005; 174(6): 3658-67.

Alm RA, Ling LS, Moir DT, King BL, Brown ED, Doig PC, Smith DR, Noonan B, Guild BC, deJonge BL, Carmel G, Tummino PJ, Caruso A, Uria-Nickelsen M, Mills DM, Ives C, Gibson R, Merberg D, Mills SD, Jiang Q, Taylor DE, Vovis GF and TJ Trust. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen Helicobacter pylori. Nature, 1999; 397(6715): 176-80.

Andus T, Daig R, Vogl D, Aschenbrenner E, Lock G, Hollerbach S, Kollinger M, Scholmerich J and V Gross. Imbalance of the interleukin 1 system in colonic mucosa--association with intestinal inflammation and interleukin 1 receptor antagonist [corrected] genotype 2. Gut. 1997; 41(5): 651-7.

Appelmelk BJ, Vandenbroucke-Grauls CM. H pylori and Lewis antigens. Gut. 2000; 47(1): 10-1.

Asahi M, Azuma T, Ito S, Ito Y, Suto H, Nagai Y, Tsubokawa M, Tohyama Y, Maeda S, Omata M, Suzuki T and C Sasakawa. Helicobacter pylori CagA protein can be tyrosine phosphorylated in gastric epithelial cells. J Exp Med. 2000; 191(4): 593-602.

Aras RA, Lee Y, Kim SK, Israel D, Peek RM Jr and MJ Blaser. Natural variation in populations of persistently colonizing bacteria affect human host cell phenotype. J Infect Dis. 2003a; 188(4): 486-96.

Aras RA, Fischer W, Perez-Perez GI, Crosatti M, Ando T, Haas R and MJ Blaser. Plasticity of repetitive DNA sequences within a bacterial (Type IV) secretion system component. J Exp Med. 2003b; 198(9): 1349-60.

Argent RH, Kidd M, Owen RJ, Thomas RJ, Limb MC, Atherton JC. Determinants and consequences of different levels of CagA phosphorylation for clinical isolates of Helicobacter pylori. Gastroenterology. 2004; 127(2): 514-23.

Atherton JC, Cao P, Peek RM Jr, Tummuru MK, Blaser MJ and TL Cover. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. J Biol Chem. 1995; 270(30): 17771-7.

Atherton JC. The chips are down for Helicobacter pylori. Gut, 2002; 50: 293-294.

Azuma T, Kato S, Zhou W, Yamazaki S, Yamakawa A, Ohtani M, Fujiwara S, Minoura T, Iinuma K, and T Kato. Diversity of vacA and cagA genes of Helicobacter pylori in Japanese children. Aliment Pharmacol Ther. 2004; 20 Suppl 1: 7-12.

Backert S, Schwarz T, Miehlke S, Kirsch C, Sommer C, Kwok T, Gerhard M, Goebel UB, Lehn N, Koenig W and TF Meyer. Functional analysis of the cag pathogenicity island in Helicobacter pylori isolates from patients with gastritis, peptic ulcer, and gastric cancer. Infect Immun. 2004; 72(2): 1043-56.

Baldari CT, Lanzavecchia A and JL Telford. Immune subversion by Helicobacter pylori. Trends in Immunology, 2005; 26(4): 199-207.

Bamford KB, Fan X, Crowe SE, Leary JF, Gourley WK, Luthra GK, Brooks EG, Graham DY, Reyes VE and PB Ernst. Lymphocytes in the human gastric mucosa during Helicobacter pylori have a T helper cell 1 phenotype. Gastroenterology, 1998; 114(3): 482-92.

Banatvala N, Mayo K, Megraud F, Jennings R, Deeks JJ and RA Feldman. The cohort effect and Helicobacter pylori. J Infect Dis. 1993; 168(1): 219-21.

Bauditz J, Ortner M, Bierbaum M, Niedobitek G, Lochs H and S Schreiber. Production of IL-12 in gastritis relates to infection with *Helicobacter pylori.* Clin Exp Immunol. 1999; 117(2): 316 –23.

Benedict CL, Kearney JF. Increased junctional diversity in fetal B cells results in a loss of protective anti-phosphorylcholine antibodies in adult mice. Immunity. 1999; 10(5): 607-17.

Bergman MP, Engering A, Smits HH, van Vliet SJ, van Bodegraven AA, Wirth HP, Kapsenberg ML, Vandenbroucke-Grauls CM, van Kooyk Y and BJ Appelmelk. Helicobacter pylori modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN. J Exp Med. 2004; 200(8): 979-90.

Berkowicz J and A Lee. Person-to-person transmission of Campylobacter pylori. Lancet, 1987; 2(8560): 680-1.

Bertoni F and E Zucca. Delving deeper into MALT lymphoma biology. Journal clinical investigations, 2006; 116; 22-26.

Bidwell J, Keen L, Gallagher G, Kimberly R, Huizinga T, McDermott MF, Oksenberg J, McNicholl J, Pociot F, Hardt C and S D'Alfonso. Cytokine gene

polymorphism in human disease: on-line databases. Genes Immun. 1999; 1(1): 3-19.

Bidwell J, Keen L, Gallagher G, Kimberly R, Huizinga T, McDermott MF, Oksenberg J, McNicholl J, Pociot F, Hardt C and S D'Alfonso. Cytokine gene polymorphism in human disease: on-line databases, supplement 1. Genes Immun. 2001; 2(2): 61-70.

Bjorkholm B, Lundin A, Sillen A, Guillemin K, Salama N, Rubio C, Gordon JI, Falk P and L Engstrand. Comparison of genetic divergence and fitness between two subclones of Helicobacter pylori. Infect Immun. 2001; 69(12): 7832-8.

Blaser MJ. Malaria and the natural history of Helicobacter pylori infection. Lancet. 1993; 342(8870): 551.

Blaser MJ, Perez-Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH, Stemmermann GN and A Nomura. Infection with Helicobacter pylori strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res. 1995; 55(10): 2111-5.

Blaser MJ. Helicobacter pylori and gastric diseases. BMJ. 1998; 316(7143): 1507-10.

Bode G, Rothenbacher D, Brenner H and G Adler. Helicobacter pylori and abdominal symptoms: a population-based study among preschool children in southern Germany. Pediatrics. 1998; 101(4 Pt 1): 634-7.

Boncristiano M, Paccani SR, Barone S, Ulivieri C, Patrussi L, Ilver D, Amedei A, D'Elios MM, Telford JL, Baldari CT. The Helicobacter pylori vacuolating toxin inhibits T cell activation by two independent mechanisms. J Exp Med. 2003; 198(12): 1887-97.

Boren T, Falk P, Roth KA, Larson G and S Normark. Attachment of Helicobacter pylori to human gastric epithelium mediated by blood group antigens. Science 1993; 262(5141): 1892-5.

Bours V, Bentires-Alj M, Hellin AC, Viatour P, Robe P, Delhalle S, Benoit V and MP Merville. Nuclear factor-kappa B, cancer, and apoptosis. Biochem Pharmacol. 2000; 60(8): 1085-9.

Brinkman BM, Huizinga TW, Kurban SS, van der Velde EA, Schreuder GM, Hazes JM, Breedveld FC and CL Verweij. Tumour necrosis factor alpha gene polymorphisms in rheumatoid arthritis: association with susceptibility to, or severity of, disease? Br J Rheumatol. 1997; 36(5): 516-21.

Bussiere FI, Chaturvedi R, Cheng Y, Gobert AP, Asim M, Blumberg DR, Xu H, Kim PY, Hacker A, Casero RA Jr, Wilson KT. Spermine causes loss of innate immune response to Helicobacter pylori by inhibition of inducible nitric-oxide synthase translation. J Biol Chem. 2005; 280(4): 2409-12.

Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, Rappuoli R and A Covacci. Cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci U S A. 1996; 93(25): 14648-53.

Censini S, Stein M and A Covacci. Cellular responses induced after contact with Helicobacter pylori. Curr Opin Microbiol. 2001; 4(1): 41-6.

Chaturvedi R, Cheng Y, Asim M, Bussiere FI, Xu H, Gobert AP, Hacker A, Casero RA Jr, Wilson KT. Induction of polyamine oxidase 1 by Helicobacter pylori causes macrophage apoptosis by hydrogen peroxide release and mitochondrial membrane depolarization. J Biol Chem. 2004; 279(38): 40161-73.

Chen W, Shu D and VS Chadwick. 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. 2001; 16(4): 377-83.

Cheung VG, Conlin LK, Weber TM, Arcaro M, Jen KY, Morley M, Spielman RS. Natural variation in human gene expression assessed in lymphoblastoid cells. Nat Genet. 2003; 33(3): 422-5.

Chouchane L, Ahmed SB, Baccouche S and S Remadi. Polymorphism in the tumor necrosis factor-alpha promotor region and in the heat shock protein 70 genes associated with malignant tumors. Cancer, 1997; 80(8): 1489-96.

Clegg RM. Fluorescence resonance energy transfer. Curr Opin Biotechnol. 1995; 6(1): 103-10.

Correa P, Cuello C, Duque E, Burbano LC, Garcia FT, Bolanos O, Brown C and W Haenszel. Gastric cancer in Colombia. III. Natural history of precursor lesions. J Natl Cancer Inst. 1976; 57(5): 1027-35.

Correa P, Fox J, Fontham E, Ruiz B, Lin YP, Zavala D, Taylor N, Mackinley D, de Lima E and H Portilla. Helicobacter pylori and gastric carcinoma. Serum antibody prevalence in populations with contrasting cancer risks. Cancer, 1990; 66(12): 2569-74.

Correa P. Does Helicobacter pylori cause gastric cancer via oxidative stress? Biol Chem. 2006; 387(4): 361-4.

Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002; 420(6917): 860-7.

Covacci A, Censini S, Bugnoli M, Petracca R, Burroni D, Macchia G, Massone A, Papini E, Xiang Z, and N Figura. Molecular characterization of the 128-kDa immunodominant antigen of Helicobacter pylori associated with cytotoxicity and duodenal ulcer. Proc Natl Acad Sci U S A. 1993; 90(12): 5791-5.

Cover TL, Dooley CP, and MJ Blaser. Characterization of and human serologic response to proteins in Helicobacter pylori broth culture supernatants with vacuolizing cytotoxin activity. Infect Immun. 1990; 58(3): 603-10.

Cover TL, Tummuru MK, Cao P, Thompson SA, Blaser MJ. Divergence of genetic sequences for the vacuolating cytotoxin among Helicobacter pylori strains. J Biol Chem. 1994; 269(14): 10566-73.

Cover TL, Krishna US, Israel DA, Peek RM Jr. Induction of gastric epithelial cell apoptosis by Helicobacter pylori vacuolating cytotoxin. Cancer Res. 2003; 63(5): 951-7.

Cox A, Camp NJ, Nicklin MJ, di Giovine FS and GW Duff. An analysis of linkage disequilibrium in the interleukin-1 gene cluster, using a novel grouping method for multiallelic markers. Am J Hum Genet. 1998; 62(5): 1180-8.

Crabtree JE, Shallcross TM, Heatley RV and JI Wyatt. Mucosal tumour necrosis factor alpha and interleukin-6 in patients with Helicobacter pylori associated gastritis. Gut, 1991a; 32(12): 1473-7.

Crabtree JE, Taylor JD, Wyatt JI, Heatley RV, Shallcross TM, Tompkins DS and BJ Rathbone. Mucosal IgA recognition of Helicobacter pylori 120 kDa protein, peptic ulceration, and gastric pathology. Lancet, 1991b; 338(8763): 332-5.

Crabtree JE, Peichl P, Wyatt JI, Stachl U and IJ Lindley. Gastric interleukin-8 and IgA IL-8 autoantibodies in Helicobacter pylori infection. Scand J Immunol. 1993; 37(1): 65-70.

Cullen DJ, Collins BJ, Christiansen KJ, Epis J, Warren JR, Surveyor I and KJ Cullen. When is Helicobacter pylori infection acquired? Gut, 1993; 34(12): 1681-2.

Dammers PM and FG Kroese. Recruitment and selection of marginal zone B cells is independent of exogenous antigens. Eur J Immunol. 2005; 35(7): 2089-99.

Daniels J, Holmans P, Williams N, Turic D, McGuffin P, Plomin R, Owen MJ. A simple method for analyzing microsatellite allele image patterns generated from DNA pools and its application to allelic association studies. Am J Hum Genet. 1998; 62(5): 1189-97.

Del Giudice G, Covacci A, Telford JL, Montecucco C and R Rappuoli. The design of vaccines against Helicobacter pylori and their development. Annu Rev Immunol. 2001; 19: 523-63.

D'Elios MM, Manghetti M, De Carli M, Costa F, Baldari CT, Burroni D, Telford JL, Romagnani S and G Del Prete. T helper 1 effector cells specific for Helicobacter pylori in the gastric antrum of patients with peptic ulcer disease. J Immunol. 1997; 158(2): 962-7.

Demeter J, Porzsolt F, Ramisch S, Schmidt D, Schmid M and G Messer. Polymorphism of the tumour necrosis factor-alpha and lymphotoxin-alpha genes in chronic lymphocytic leukaemia. Br J Haematol. 1997; 97(1): 107-12.

Dierlamm J, Baens M, Wlodarska I, Stefanova-Ouzounova M, Hernandez JM, Hossfeld DK, De Wolf-Peeters C, Hagemeijer A, Van den Berghe H and P Marynen. The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21)p6ssociated with mucosa-associated lymphoid tissue lymphomas. Blood, 1999; 93(11): 3601-9.

Dominici R, Cattaneo M, Malferrari G, Archi D, Mariani C, Grimaldi LM and I Biunno. Cloning and functional analysis of the allelic polymorphism in the transcription regulatory region of interleukin-1 alpha. Immunogenetics. 2002 ; 54(2): 82-6.

Drumm B, Perez-Perez GI, Blaser MJ and PM Sherman. Intrafamilial clustering of Helicobacter pylori infection. N Engl J Med. 1990; 322(6): 359-63.

Dubois A, Berg DE, Incecik ET, Fiala N, Heman-Ackah LM, Perez-Perez GI, Blaser MJ. Transient and persistent experimental infection of nonhuman primates with Helicobacter pylori: implications for human disease. Infect Immun. 1996; 64(8): 2885-91.

Dundon WG, Nishioka H, Polenghi A, Papinutto E, Zanotti G, Montemurro P, Del GG, Rappuoli R, Montecucco C. The neutrophil-activating protein of Helicobacter pylori. Int J Med Microbiol. 2002; 291(6-7): 545-50.

Ebert MP, Yu J, Miehlke S, Fei G, Lendeckel U, Ridwelski K, Stolte M, Bayerdorffer E, Malfertheiner P. Expression of transforming growth factor beta-1 in gastric cancer and in the gastric mucosa of first-degree relatives of patients with gastric cancer. Br J Cancer. 2000; 82(11): 1795-800.

El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr and CS Rabkin. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature, 2000; 404: 398-402.

El-Omar EM, Rabkin CS, Gammon MD, Vaughan TL, Risch HA, Schoenberg JB, Stanford JL, Mayne ST, Goedert J, Blot WJ, Fraumeni JF Jr and WHChow. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. Gastroenterology, 2003; 124(5): 1193-201.

Eskdale J, Gallagher G, Verweij CL, Keijsers V, Westendorp RG and TW Huizinga. Interleukin 10 secretion in relation to human IL-10 locus haplotypes. Proc Natl Acad Sci U S A. 1998; 95(16): 9465-70.

Evans DJ Jr, Evans DG, Takemura T, Nakano H, Lampert HC, Graham DY, Granger DN, Kvietys PR. Characterization of a Helicobacter pylori neutrophil-activating protein. Infect Immun. 1995; 63(6): 2213-20.

Evans DJ Jr and DG Evans. Helicobacter pylori adhesins: review and perspectives. Helicobacter, 2000; 5(4): 183-95.

Faller G, Steininger H, Kranzlein J, Maul H, Kerkau T, Hensen J, Hahn EG and T Kirchner. Antigastric autoantibodies in Helicobacter pylori infection: implications of histological and clinical parameters of gastritis. Gut, 1997; 41(5): 619-23.

Fengoglio-Preiser CM, Noffsinger AE, Stemmermann GN, Lantz PE, Listrom MB and FO Rilke, eds. (1997) Gastrointestinal Pathology: An atlas and text, 2nd Edition. Lippincott, Williams and Wilkins.

Fiedorek SC, Malaty HM, Evans DL, Pumphrey CL, Casteel HB, Evans DJ Jr and DY Graham. Factors influencing the epidemiology of Helicobacter pylori infection in children. Pediatrics. 1991; 88(3): 578-82.

Fischer W, Puls J, Buhrdorf R, Gebert B, Odenbreit S and R Haas. Systematic mutagenesis of the Helicobacter pylori cag pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. Mol Microbiol. 2001; 42(5): 1337-48.

Fishman D, Faulds G, Jeffery R, Mohamed-Ali V, Yudkin J, Humphries S and P Woo. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. J Clin Invest. 1998; 102(7): 1369-76.

Foryst-Ludwig A and M Naumann. p21-activated kinase 1 activates the nuclear factor kappa B (NF-kappa B)-inducing kinase-Ikappa B kinases NF-kappa B pathway and proinflammatory cytokines in Helicobacter pylori infection. J Biol Chem. 2000; 275(50): 39779-85.

Fox JG, Beck P, Dangler CA, Whary MT, Wang TC, Shi HN and C Nagler-Anderson. Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. Nat Med. 2000; 6(5): 536-42.

Furuta T, El-Omar EM, Xiao F, Shirai N, Takashima M, Sugimura H and H Sugimurra. Interleukin 1beta polymorphisms increase risk of hypochlorhydria and atrophic gastritis and reduce risk of duodenal ulcer recurrence in Japan. Gastroenterology, 2002; 123: 92-105.

Gebert B, Fischer W, Weiss E, Hoffmann R and R Haas. Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. Science, 2003; 301: 1099-1102.

Gerhard M, Lehn N, Neumayer N, Boren T, Rad R, Schepp W, Miehlke S, Classen M and C Prinz. Clinical relevance of the Helicobacter pylori gene for blood-group antigen-binding adhesin. Proc Natl Acad Sci U S A. 1999; 96(22): 12778-83.

Gerhard M, Schmees C, Voland P, Endres N, Sander M, Reindl W, Rad R, Oelsner M, Decker T, Mempel M, Hengst L, Prinz C. A secreted low-molecular-weight protein from Helicobacter pylori induces cell-cycle arrest of T cells. Gastroenterology. 2005; 128(5): 1327-39.

Gewirtz AT, Yu Y, Krishna US, Israel DA, Lyons SL and Peek RM Jr. Helicobacter pylori flagellin evades toll-like receptor 5-mediated innate immunity. J Infect Dis. 2004; 189(10): 1914-20.

Ghose C, Perez-Perez GI, Dominguez-Bello MG, Pride DT, Bravi CM and MJ Blaser. East Asian genotypes of Helicobacter pylori strains in Amerindians provide evidence for its ancient human carriage. Proc Natl Acad Sci U S A. 2002; 99(23): 15107-11.

Gibson AW, Edberg JC, Wu J, Westendorp RG, Huizinga TW and RP Kimberly. Novel single nucleotide polymorphisms in the distal IL-10 promoter affect IL-10 production and enhance the risk of systemic lupus erythematosus. J Immunol. 2001; 166(6): 3915-22.

Giedraitis V, He B, Huang WX and J Hillert. Cloning and mutation analysis of the human IL-18 promoter: a possible role of polymorphisms in expression regulation. J Neuroimmunol. 2001; 112(1-2): 146-52.

Goossens T, Klein U, Kuppers R. Frequent occurrence of deletions and duplications during somatic hypermutation: implications for oncogene translocations and heavy chain disease. Proc Natl Acad Sci U S A. 1998; 95(5): 2463-8.

Graham DY, Adam E, Reddy GT, Agarwal JP, Agarwal R, Evans DJ Jr, Malaty HM and DG Evans. Seroepidemiology of Helicobacter pylori infection in India. Comparison of developing and developed countries. Dig Dis Sci. 1991; 36(8): 1084-8.

Grainger DJ, Heathcote K, Chiano M, Snieder H, Kemp PR, Metcalfe JC, Carter ND and TD Spector. Genetic control of the circulating concentration of transforming growth factor type beta1. Hum Mol Genet. 1999; 8(1): 93-7.

Gray D, McConnell I, Kumararatne DS, MacLennan IC, Humphrey JH, Bazin H. Marginal zone B cells express CR1 and CR2 receptors. Eur J Immunol. 1984; 14(1): 47-52.

Greeve J, Philipsen A, Krause K, Klapper W, Heidorn K, Castle BE, Janda J, Marcu KB, Parwaresch R. Expression of activation-induced cytidine deaminase in human B-cell non-Hodgkin lymphomas. Blood. 2003; 101(9): 3574-80.

Greiner A, Knorr C, Qin Y, Sebald W, Schimpl A, Banchereau J and HK Muller-Hermelink. Low-grade B cell lymphomas of mucosa-associated lymphoid tissue (MALT-type) require CD40-mediated signaling and Th2-type cytokines for in vitro growth and differentiation. Am J Pathol. 1997; 150(5): 1583-93.

Greiner A, Knorr C, Qin Y, Schultz A, Marx A, Kroczek RA and HK Muller-Hermelink. CD40 ligand and autoantigen are involved in the pathogenesis of low-grade B-cell lymphomas of mucosa-associated lymphoid tissue. Dev Immunol. 1998; 6(3-4): 187-95.

Guasch JF, Bertina RM and PH Reitsma. Five novel intragenic dimorphisms in the human interleukin-1 genes combine to high informativity. Cytokine 1996; 8: 598-602.

Guinamard R, Okigaki M, Schlessinger J, Ravetch JV. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. Nat Immunol. 2000; 1(1): 31-6.

Guo WX, Burger AM, Fischer RT, Sieckmann DG, Longo DL, Kenny JJ. Sequence changes at the V-D junction of the VH1 heavy chain of anti-phosphocholine antibodies alter binding to and protection against Streptococcus pneumoniae. Int Immunol. 1997; 9(5): 665-77.

Hajeer AH and IV Hutchinson. Influence of TNFalpha gene polymorphisms on TNFalpha production and disease. Hum Immunol. 2001; 62(11): 1191-9.

Hansson LE, Nyren O, Hsing AW, Bergstrom R, Josefsson S, Chow WH, Fraumeni JF Jr and HO Adami. The risk of stomach cancer in patients with gastric or duodenal ulcer disease. N Engl J Med. 1996; 335(4): 242-9.

Hauer AC, Finn TM, MacDonald TT, Spencer J and PG Isaacson. Analysis of TH1 and TH2 cytokine production in low grade B cell gastric MALT-type lymphomas stimulated in vitro with Helicobacter pylori. J Clin Pathol. 1997; 50(11): 957-9.

Haukim N, Bidwell JL, Smith AJ, Keen LJ, Gallagher G, Kimberly R, Huizinga T, McDermott MF, Oksenberg J, McNicholl J, Pociot F, Hardt C and S D'Alfonso. Cytokine gene polymorphism in human disease: on-line databases, supplement 2. Genes Immun. 2002; 3(6): 313-30.

Heid CA, Stevens J, Livak KJ and PM Williams. Real time quantitative PCR. . Genome Res. 1996; 6: 986-94.

Hellmig S, Vollenberg S, Goebeler-Kolve ME, Fischbach W, Hampe J, Folsch UR and S Schreiber. IL-1 gene cluster polymorphisms and development of primary gastric B-cell lymphoma in Helicobacter pylori infection. Blood, 2004; 104(9): 2994-5.

Higashi H, Tsutsumi R, Muto S, Sugiyama T, Azuma T, Asaka M, Hatakeyama M. SHP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein. Science. 2002; 295(5555): 683-6.

Higashi H, Nakaya A, Tsutsumi R, Yokoyama K, Fujii Y, Ishikawa S, Higuchi M, Takahashi A, Kurashima Y, Teishikata Y, Tanaka S, Azuma T, Hatakeyama M. Helicobacter pylori CagA induces Ras-independent morphogenetic response through SHP-2 recruitment and activation. J Biol Chem. 2004; 279(17): 17205-16.

Holcombe C, Omotara BA, Eldridge J and DM Jones. H. pylori, the most common bacterial infection in Africa: a random serological study. Am J Gastroenterol. 1992; 87(1): 28-30.

Hoogendoorn B, Coleman SL, Guy CA, Smith K, Bowen T, Buckland PR, O'Donovan MC. Functional analysis of human promoter polymorphisms. Hum Mol Genet. 2003; 12(18): 2249-54.

Horuk R, Chitnis CE, Darbonne WC, Colby TJ, Rybicki A, Hadley TJ, Miller LH. A receptor for the malarial parasite Plasmodium vivax: the erythrocyte chemokine receptor. Science. 1993; 261(5125): 1182-4.

Howell WM, Turner SJ, Bateman AC and JM Theaker. IL-10 promoter polymorphisms influence tumour development in cutaneous malignant melanoma. Genes Immun. 2001; 2(1): 25-31.

Howell WM, Turner SJ, Collins A, Bateman AC and JM Theaker. Influence of TNFalpha and LTalpha single nucleotide polymorphisms on susceptibility to and prognosis in cutaneous malignant melanoma in the British population. Eur J Immunogenet. 2002; 29(1): 17-23.

Howell WM, Turner SJ, Theaker JM and AC Bateman. Cytokine gene single nucleotide polymorphisms and susceptibility to and prognosis in cutaneous malignant melanoma. Eur J Immunogenet. 2003; 30(6): 409-14.

Hull J, Thomson A, Kwiatkowski D. Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families. Thorax. 2000; 55(12): 1023-7.

Hull J, Ackerman H, Isles K, Usen S, Pinder M, Thomson A, Kwiatkowski D. Unusual haplotypic structure of IL8, a susceptibility locus for a common respiratory virus. Am J Hum Genet. 2001; 69(2): 413-9.

Hurme M and S Santtila. IL-1 receptor antagonist (IL-1Ra) plasma levels are co-ordinately regulated by both IL-1Ra and IL-1beta genes. Eur J Immunol. 1998; 28(8): 2598-602.

Hussell T, Isaacson PG, Crabtree JE and J Spencer. The response of cells from low-grade B-cell gastric lymphomas of mucosa-associated lymphoid tissue to Helicobacter pylori. Lancet, 1993; 342: 571-4.

Hussell T, Isaacson PG, Crabtree JE and J Spencer. Helicobacter pylori-specific tumour-infiltrating T cells provide contact dependent help for the growth of malignant B cells in low-grade gastric lymphoma of mucosa-associated lymphoid tissue. J Pathol. 1996; 178(2): 122-7.

Hwang IR, Kodama T, Kikuchi S, Sakai K, Peterson LE, Graham DY, Yamaoka Y. Effect of interleukin 1 polymorphisms on gastric mucosal interleukin 1beta production in Helicobacter pylori infection. Gastroenterology. 2002; 123(6): 1793-803.

Inoue T, Nabeshima K, Kataoka H and M Koono. Feasibility of archival non-buffered formalin-fixed and paraffin-embedded tissues for PCR amplification: an analysis of resected gastric carcinoma. Pathol Int. 1996; 46(12): 997-1004.

Isaacson PG and MQ Du. Gastrointestinal lymphoma: where morphology meets molecular biology. J Pathol. 2005; 205(2): 255-74.

Ismail HF, Fick P, Zhang J, Lynch RG and DJ Berg. Depletion of neutrophils in IL-10(-/-) mice delays clearance of gastric Helicobacter infection and decreases the Th1 immune response to Helicobacter. J Immunol. 2003; 170(7): 3782-9.

Israel DA, Salama N, Krishna U, Rieger UM, Atherton JC, Falkow S and RM Peek Jr. Helicobacter pylori genetic diversity within the gastric niche of a single human host. Proc Natl Acad Sci U S A. 2001; 98(25): 14625-30.

Jaffe ES, Harris NL, Stein H and JW Vardiman, eds. (2001) World Health Organisation classification of tumours: pathology and genetics of tumours of haematopoietic and lymphoma tissues. IARC press, Lyon.

Juszczynski P, Kalinka E, Bienvenu J, Woszczek G, Borowiec M, Robak T, Kowalski M, Lech-Maranda E, Baseggio L, Coiffier B, Salles G and K Warzocha. Human leukocyte antigens class II and tumor necrosis factor genetic polymorphisms are independent predictors of non-Hodgkin lymphoma outcome. Blood, 2002; 100(8): 3037-40.

Kaijzel EL, van Dongen H, Bakker AM, Breedveld FC, Huizinga TW and CL Verweij. Relationship of polymorphisms of the Interleukin-1 gene cluster to occurrence and severity of rheumatoid arthritis. Tissue Antigens, 2002; 59: 122-6.

Karasneh J, Hajeer AH, Barrett J, Ollier WE, Thornhill M and A Gul. Association of specific interleukin 1 gene cluster polymorphisms with increased susceptibility for Behcet's disease. Rheumatology (Oxford), 2003; 42(7): 860-4.

Karttunen R, Karttunen T, Ekre HP and TT MacDonald. Interferon gamma and interleukin 4 secreting cells in the gastric antrum in Helicobacter pylori positive and negative gastritis. Gut, 1995; 36(3): 341-5.

Karttunen RA, Karttunen TJ, Yousfi MM, el-Zimaity HM, Graham DY and FA el-Zaatari. 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. 1997; 32(1): 22-7.

Kersulyte D, Chalkauskas H, and DE Berg. Emergence of recombinant strains of Helicobacter pylori during human infection. Mol Microbiol. 1999; 31(1): 31-43.

Kidd M, Lastovica AJ, Atherton JC, Louw JA. Heterogeneity in the Helicobacter pylori vacA and cagA genes: association with gastroduodenal disease in South Africa? Gut. 1999; 45(4): 499-502.

Knight JC, Udalova I, Hill AV, Greenwood BM, Peshu N, Marsh K and D Kwiatkowski. A polymorphism that affects OCT-1 binding to the TNF promoter region is associated with severe malaria. Nat Genet. 1999; 22(2): 145-50.

Knight JC, Keating BJ, Rockett KA, Kwiatkowski DP. In vivo characterization of regulatory polymorphisms by allele-specific quantification of RNA polymerase loading. Nat Genet. 2003; 33(4): 469-75.

Knight JC. Regulatory polymorphisms underlying complex disease traits. J Mol Med. 2005; 83(2): 97-109.

Kornman KS, di Giovine FS. Genetic variations in cytokine expression: a risk factor for severity of adult periodontitis. Ann Periodontol. 1998; 3(1): 327-38.

Kornman KS, Pankow J, Offenbacher S, Beck J, di Giovine F, Duff GW. Interleukin-1 genotypes and the association between periodontitis and cardiovascular disease. J Periodontal Res. 1999; 34(7): 353-7.

Kosel S, Grasbon-Frodi EM, Arima K, Chimelli L, Hahn M, Hashizume Y, Hulette C, Ikeda K, Jacobsen PF, Jones M, Kobayashi M, Love S, Mizutani T, Rosemberg S, Sasaki A, Smith TW, Takahashi H, Vortmeyer AO and MB Greaber. Inter-laboratory comparison of DNA preservation in archival paraffin-embedded human brain tissue from participating centres on four continents. Neurogenetics, 2001; 3: 163-170.

Krausa P, Bodmer JG and MJ Browning. Defining the common subtypes of HLA A9, A10, A28 and A19 by use of ARMS/PCR. Tissue Antigens, 1993; 42: 91-9.

Kroeger KM, Steer JH, Joyce DA and LJ Abraham. Effects of stimulus and cell type on the expression of the -308 tumour necrosis factor promoter polymorphism. Cytokine, 2000; 12(2): 110-9.

Kuipers EJ, Pena AS, van Kamp G, Uyterlinde AM, Pals G, Pels NF, Kurz-Pohlmann E and SG Meuwissen. Seroconversion for Helicobacter pylori. Lancet, 1993; 342 (8867): 328-31.

Kuipers EJ, Israel DA, Kusters JG, Gerrits MM, Weel J, van Der Ende A, van Der Hulst RW, Wirth HP, Hook-Nikanne J, Thompson SA and MJ Blaser. Quasispecies development of Helicobacter pylori observed in paired isolates obtained years apart from the same host. J Infect Dis. 2000; 181(1): 273-82.

Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C and JJ Sninsky. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res. 1990; 18(4): 999-1005.

Lazarus M, Hajeer AH, Turner D, Sinnott P, Worthington J, Ollier WE and IV Hutchinson. Genetic variation in the interleukin 10 gene promoter and systemic lupus erythematosus. J Rheumatol. 1997; 24(12): 2314-7.

Lee SK, Stack A, Katzowitsch E, Aizawa SI, Suerbaum S, Josenhans C. Helicobacter pylori flagellins have very low intrinsic activity to stimulate human gastric epithelial cells via TLR5. Microbes Infect. 2003; 5(15): 1345-56.

Lehmann FS, Terracciano L, Carena I, Baeriswyl C, Drewe J, Tornillo L, De Libero G and C Beglinger. In situ correlation of cytokine secretion and apoptosis in Helicobacter pylori-associated gastritis. Am J Physiol Gastrointest Liver Physiol. 2002; 283(2): G481-8.

Lehours P, Dupouy S, Bergey B, Ruskone-Foumestraux A, Delchier JC, Rad R, Richy F, Tankovic J, Zerbib F, Megraud F and A Menard. Identification of a genetic marker of Helicobacter pylori strains involved in gastric extranodal marginal zone B cell lymphoma of the MALT-type. Gut, 2004; 53(7): 931-7.

Letley DP, Lastovica A, Louw JA, Hawkey CJ and JC Atherton. 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. 1999; 37(4): 1203-5.

Letley DP, Rhead JL, Twells RJ, Dove B and JC Atherton. Determinants of non-toxicity in the gastric pathogen Helicobacter pylori. J Biol Chem. 2003; 278(29): 26734-41.

Lindholm C, Quiding-Jarbrink M, Lonroth H and AM Svennerholm. Induction of chemokine and cytokine responses by Helicobacter pylori in human stomach explants. Scand J Gastroenterol. 2001; 36(10): 1022-9.

Lindkvist P, Asrat D, Nilsson I, Tsega E, Olsson GL, Wretlind B and J Giesecke. Age at acquisition of Helicobacter pylori infection: comparison of a high and a low prevalence country. Scand J Infect Dis. 1996; 28(2): 181-4.

Liu H, Ruskon-Fourmestraux A, Lavergne-Slove A, Ye H, Molina T, Bouhnik Y, Hamoudi RA, Diss TC, Dogan A, Megraud F, Rambaud JC, Du MQ and PG Isaacson. Resistance of t(11;18) positive gastric mucosa-associated lymphoid tissue lymphoma to Helicobacter pylori eradication therapy. Lancet, 2001; 357(9249): 39-40.

Liu H, Ye H, Ruskone-Fourmestraux A, De Jong D, Pileri S, Thiede C, Lavergne A, Boot H, Caletti G, Wundisch T, Molina T, Taal BG, Elena S, Thomas T, Zinzani PL, Neubauer A, Stolte M, Hamoudi RA, Dogan A, Isaacson PG and MQ Du. T(11;18) is a marker for all stage gastric MALT lymphomas that will not respond to H. pylori eradication. Gastroenterology, 2002a; 122(5): 1286-94.

Liu H, Huang X, Zhang Y, Ye H, Hamidi AE, Kocjan G, Dogan A, Isaacson PG and MQ Du. Archival fixed histologic and cytologic specimens including stained and unstained materials are amenable to RT-PCR. Diagn Mol Pathol. 2002b; 11(4): 222-7.

Loffeld RJ, Werdmuller BF, Kusters JG, and EJ Kuipers. Functional dyspepsia is associated with cagA-positive Helicobacter pylori strains. Scand J Gastroenterol. 2001; 36(4): 351-5.

Lu H, Hsu PI, Graham DY and Y Yamaoka. Duodenal ulcer promoting gene of Helicobacter pylori. Gastroenterology, 2005; 128(4): 833-48.

Lucas PC, Yonezumi M, Inohara N, McAllister-Lucas LM, Abazeed ME, Chen FF, Yamaoka S, Seto M and G Nunez. Bcl10 and MALT1, independent targets of chromosomal translocation in malt lymphoma, cooperate in a novel NF-kappa B signaling pathway. J Biol Chem. 2001; 276(22): 19012-9.

Machado JC, Pharoah P, Sousa S, Carvalho R, Oliveira C, Figueiredo C, Amorim A, Seruca R, Caldas C, Carneiro F and M Sobrinho-Simoes. Interleukin 1B and interleukin 1RN polymorphisms are associated with increased risk of gastric carcinoma. Gastroenterology, 2001; 121: 823-9.

Mahdavi J, Sonden B, Hurtig M, Olfat FO, Forsberg L, Roche N, Angstrom J, Larsson T, Teneberg S, Karlsson KA, Altraja S, Wadstrom T, Kersulyte D, Berg DE, Dubois A, Petersson C, Magnusson KE, Norberg T, Lindh F, Lundskog BB, Arnqvist A, Hammarstrom L and T Boren. Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. Science, 2002; 297(5581): 573-8.

Mai UE, Perez-Perez GI, Allen JB, Wahl SM, Blaser MJ and PD Smith. Surface proteins from Helicobacter pylori exhibit chemotactic activity for human leukocytes and are present in gastric mucosa. J Exp Med. 1992; 175(2): 517-25.

Marshall BJ and JR Warren. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet, 1984; 1: 1311-15.

Mattsson A, Quiding-Jarbrink M, Lonroth H, Hamlet A, Ahlstedt I and A Svennerholm. Antibody-secreting cells in the stomachs of symptomatic and asymptomatic Helicobacter pylori-infected subjects. Infect Immun. 1998; 66(6): 2705-12.

McCallion WA, Murray LJ, Bailie AG, Dalzell AM, O'Reilly DP and KB Bamford. Helicobacter pylori infection in children: relation with current household living conditions. Gut, 1996; 39(1): 18-21.

McDowell TL, Symons JA, Ploski R, Forre O and GW Duff. A genetic association between juvenile rheumatoid arthritis and a novel interleukin-1 alpha polymorphism. Arthritis Rheum. 1995; 38(2): 221-8.

McGuire W, Hill AV, Allsopp CE, Greenwood BM and D Kwiatkowski. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. Nature, 1994; 371(6497): 508-10.

McGuire W, Knight JC, Hill AV, Allsopp CE, Greenwood BM and D Kwiatkowski. Severe malarial anemia and cerebral malaria are associated with different tumor necrosis factor promoter alleles. J Infect Dis. 1999; 179(1): 287-90.

Meining A, Morgner A, Miehlke S, Bayerdorffer E and M Stolte. Atrophy-metaplasia-dysplasia-carcinoma sequence in the stomach: a reality or merely an hypothesis? Best Pract Res Clin Gastroenterol. 2001; 15(6): 983-98.

Messer G, Spengler U, Jung MC, Honold G, Blomer K, Pape GR, Riethmuller G and EH Weiss. Polymorphic structure of the tumor necrosis factor (TNF) locus: an NcoI polymorphism in the first intron of the human TNF-beta gene correlates with a variant amino acid in position 26 and a reduced level of TNF-beta production. J Exp Med. 1991; 173(1): 209-19.

Miehlke S, Kirsch C, Agha-Amiri K, Gunther T, Lehn N, Malfertheiner P, Stolte M, Ehninger G, Bayerdorffer E. The Helicobacter pylori vacA s1, m1 genotype and cagA is associated with gastric carcinoma in Germany. Int J Cancer. 2000; 87(3): 322-7.

Miller RD, Phillips MS, Jo I, Donaldson MA, Studebaker JF, Addleman N, Alfisi SV, Ankener WM, Bhatti HA, Callahan CE, Carey BJ, Conley CL, Cyr JM, Derohannessian V, Donaldson RA, Elosua C, Ford SE, Forman AM, Gelfand CA, Grecco NM, Gutendorf SM, Hock CR, Hozza MJ, Hur S, In SM, Jackson DL, Jo SA, Jung SC, Kim S, Kimm K, Kloss EF, Koboldt DC, Kuebler JM, Kuo FS, Lathrop JA, Lee JK, Leis KL, Livingston SA, Lovins EG, Lundy ML, Maggan S, Minton M, Mockler MA, Morris DW, Nachtman EP, Oh B, Park C, Park CW, Pavelka N, Perkins AB, Restine SL, Sachidanandam R, Reinhart AJ, Scott KE, Shah GJ, Tate JM, Varde SA, Walters A, White JR, Yoo YK, Lee JE, Boyce-Jacino MT and PY Kwok. The SNP Consortium Allele Frequency Project. High-density single-nucleotide polymorphism maps of the human genome. Genomics, 2005; 86(2): 117-26.

Mitchell HM, Li YY, Hu PJ, Liu Q, Chen M, Du GG, Wang ZJ, Lee A and SL Hazell. Epidemiology of Helicobacter pylori in southern China: identification of early childhood as the critical period for acquisition. J Infect Dis.1992; 166(1): 149-53.

Mizushima T, Sugiyama T, Komatsu Y, Ishizuka J, Kato M, Asaka M. Clinical relevance of the babA2 genotype of Helicobacter pylori in Japanese clinical isolates. J Clin Microbiol. 2001; 39(7): 2463-5.

Mohammadi M, Nedrud J, Redline R, Lycke N, Czinn SJ. Murine CD4 T-cell response to Helicobacter infection: TH1 cells enhance gastritis and TH2 cells reduce bacterial load. Gastroenterology. 1997; 113(6): 1848-57.

Molinari M, Salio M, Galli C, Norais N, Rappuoli R, Lanzavecchia A, Montecucco C. Selective inhibition of Ii-dependent antigen presentation by Helicobacter pylori toxin VacA. J Exp Med. 1998; 187(1): 135-40.

Montecucco C, de Bernard M. Molecular and cellular mechanisms of action of the vacuolating cytotoxin (VacA) and neutrophil-activating protein (HP-NAP) virulence factors of Helicobacter pylori. Microbes Infect. 2003; 5(8): 715-21.

Monteleone G, Trapasso F, Parrello T, Biancone L, Stella A, Iuliano R, Luzza F, Fusco A and F Pallone. Bioactive IL-18 expression is up-regulated in Crohn's disease. J Immunol. 1999; 163(1): 143-7.

Monteleone G, Del Vecchio Blanco G, Palmieri G, Vavassori P, Monteleone I, Colantoni A, Battista S, Spagnoli LG, Romano M, Borrelli M, MacDonald TT and F Pallone. Induction and regulation of Smad7 in the gastric mucosa of patients with Helicobacter pylori infection. Gastroenterology. 2004; 126(3): 674-82.

Morahan G, Huang D, Ymer SI, Cancilla MR, Stephen K, Dabadghao P, Werther G, Tait BD, Harrison LC and PG Colman. Linkage disequilibrium of a type 1 diabetes susceptibility locus with a regulatory IL12B allele. Nat Genet. 2001; 27(2): 218-21.

Morahan G, Boutlis CS, Huang D, Pain A, Saunders JR, Hobbs MR, Granger DL, Weinberg JB, Peshu N, Mwaikambo ED, Marsh K, Roberts DJ and NM Anstey. A promoter polymorphism in the gene encoding interleukin-12 p40 (IL12B) is associated with mortality from cerebral malaria and with reduced nitric oxide production. Genes Immun. 2002; 3(7): 414-8.

Morley M, Molony CM, Weber TM, Devlin JL, Ewens KG, Spielman RS, Cheung VG. Genetic analysis of genome-wide variation in human gene expression. Nature. 2004; 430(7001): 743-7.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA and RL Coffman. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol. 1986; 136(7): 2348-57.

Mullen AC, Hutchins AS, High FA, Lee HW, Sykes KJ, Chodosh LA and SL Reiner. Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. Nat Immunol. 2002; 3(7): 652-8.

Murphy KM and SL Reiner. The lineage decisions of helper T cells. Nature reviews in Immunology, 2002; 2: 933-944.

Negrini R, Savio A, Poiesi C, Appelmelk BJ, Buffoli F, Paterlini A, Cesari P, Graffeo M, Vaira D and G Franzin. Antigenic mimicry between Helicobacter pylori and gastric mucosa in the pathogenesis of body atrophic gastritis. Gastroenterology, 1996; 111(3): 655-65.

Noach LA, Bosma NB, Jansen J, Hoek FJ, van Deventer SJ and GN Tytgat. Mucosal tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 production in patients with Helicobacter pylori infection. Scand J Gastroenterol. 1994; 29(5): 425-9.

Nomura AM, Perez-Perez GI, Lee J, Stemmermann G, and MJ Blaser. Relation between Helicobacter pylori cagA status and risk of peptic ulcer disease. Am J Epidemiol. 2002a; 155(11): 1054-9.

Nomura AM, Lee J, Stemmermann GN, Nomura RY, Perez-Perez GI, and MJ Blaser. Helicobacter pylori CagA seropositivity and gastric carcinoma risk in a Japanese American population. J Infect Dis. 2002b; 186(8): 1138-44.

Odenbreit S, Puls J, Sedlmaier B, Gerland E, Fischer W and R Haas. Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. Science, 2000; 287(5457): 1497-500.

Odenbreit S, Gebert B, Puls J, Fischer W, Haas R. Interaction of Helicobacter pylori with professional phagocytes: role of the cag pathogenicity island and translocation, phosphorylation and processing of CagA. Cell Microbiol. 2001; 3(1): 21-31.

Olfat FO, Zheng Q, Oleastro M, Voland P, Boren T, Karttunen R, Engstrand L, Rad R, Prinz C, Gerhard M. Correlation of the Helicobacter pylori adherence factor BabA with duodenal ulcer disease in four European countries. FEMS Immunol Med Microbiol. 2005; 44(2): 151-6.

Oliver AM, Martin F, Kearney JF. IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. J Immunol. 1999; 162(12): 7198-207.

Papini E, Satin B, Norais N, de Bernard M, Telford JL, Rappuoli R, Montecucco C. Selective increase of the permeability of polarized epithelial cell monolayers by Helicobacter pylori vacuolating toxin. J Clin Invest. 1998; 102(4): 813-20.

Parsonnet J, Hansen S, Rodriguez L, Gelb AB, Warnke RA, Jellum E, Orentreich N, Vogelman JH and GD Friedman. Helicobacter pylori infection and gastric lymphoma. N Engl J Med. 1994; 330(18): 1267-71.

Parsonnet J. Helicobacter pylori: the size of the problem. Gut, 1998; 43 Suppl 1: S6-9.

Pasparakis M, Alexopoulou L, Episkopou V and G Kollias. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J Exp Med. 1996; 184(4): 1397-411.

Peek RM Jr, Miller GG, Tham KT, Perez-Perez GI, Zhao X, Atherton JC, and MJ Blaser. Heightened inflammatory response and cytokine expression in vivo to cagA+ Helicobacter pylori strains. Lab Invest.1995; 73(6): 760-70.

Peek RM Jr. Events at the host-microbial interface of the gastrointestinal tract IV. The pathogenesis of Helicobacter pylori persistence. Am J Physiol Gastrointest Liver Physiol. 2005; 289(1): G8-12.

Pelser HH, Household KC, Joubert G, van der Linde G, Kraaij P, Meinardi M, McLeod A, and M Anthony. Prevalence of Helicobacter pylori antibodies in

children in Bloemfontein, South Africa. J Pediatr Gastroenterol Nutr.1997; 24(2): 135-9.

Perez-Perez GI, Taylor DN, Bodhidatta L, Wongsrichanalai J, Baze WB, Dunn BE, Echeverria PD, and MJ Blaser. Seroprevalence of Helicobacter pylori infections in Thailand. J Infect Dis. 1990; 161(6): 1237-41.

Perez-Perez GI, Sack RB, Reid R, Santosham M, Croll J and MJ Blaser. Transient and persistent Helicobacter pylori colonization in Native American children. J Clin Microbiol. 2003; 41(6): 2401-7.

Pociot F, Molvig J, Wogensen L, Worsaae H and J Nerup. A TaqI polymorphism in the human interleukin-1 beta (IL-1 beta) gene correlates with IL-1 beta secretion in vitro. Eur J Clin Invest. 1992; 22(6): 396-402.

Pravica V, Asderakis A, Perrey C, Hajeer A, Sinnott PJ and I.V. Hutchinson. In vitro production of IFN-γ correlates with CA repeat polymorphism in the human IFN-γ gene. Eur J Immunogenet. 1999; 26(1): 1-3.

Pravica V, Perrey C, Stevens A, Lee JH and IV Hutchinson. A single nucleotide polymorphism in the first intron of the human IFN-gamma gene: absolute correlation with a polymorphic CA microsatellite marker of high IFN-gamma production. Hum Immunol. 2000; 61(9): 863-6.

Rad R, Gerhard M, Lang R, Schoniger M, Rosch T, Schepp W, Becker I, Wagner H, Prinz C. The Helicobacter pylori blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. J Immunol. 2002; 168(6): 3033-41.

Rad R, Prinz C, Neu B, Neuhofer M, Zeitner M, Voland P, Becker I, Schepp W and M Gerhard. Synergistic effect of Helicobacter pylori virulence factors and interleukin-1 polymorphisms for the development of severe histological changes in the gastric mucosa. J Infect Dis. 2003; 188(2): 272-81.

Rad R, Dossumbekova A, Neu B, Lang R, Bauer S, Saur D, Gerhard M and C Prinz. Cytokine gene polymorphisms influence mucosal cytokine expression, gastric inflammation, and host specific colonisation during Helicobacter pylori infection. Gut. 2004; 53(8): 1082-9.

Reuss E, Fimmers R, Kruger A, Becker C, Rittner C and T Hohler. Differential regulation of interleukin-10 production by genetic and environmental factors--a twin study. Genes Immun. 2002; 3(7): 407-13.

Reynard MP, Turner D and CV Navarrette. Allele frequencies of polymorphisms of the TNFα, IL-10, interferon-γ and IL-2 genes in a North European Caucasoid group from the UK. Eur J Immunogenet. 2000; 27: 241-249.

Rockman MV, Wray GA. Abundant raw material for cis-regulatory evolution in humans. Mol Biol Evol. 2002; 19(11): 1991-2004.

Rockman MV, Hahn MW, Soranzo N, Goldstein DB, Wray GA. Positive selection on a human-specific transcription factor binding site regulating IL4 expression. Curr Biol. 2003; 13(23): 2118-23.

Rollinson S, Levene AP, Mensah FK, Roddam PL, Allan JM, Diss TC, Roman E, Jack A, MacLennan K, Dixon MF and GJ Morgan. Gastric marginal zone lymphoma is associated with polymorphisms in genes involved in inflammatory response and antioxidative capacity. Blood, 2003; 102: 1007-11.

Rosenzweig SD, Schaffer AA, Ding L, Sullivan R, Enyedi B, Yim JJ, Cook JL, Musser JM and SM Holland. Interferon-gamma receptor 1 promoter polymorphisms: population distribution and functional implications. Clin Immunol. 2004; 112(1): 113-9.

Rothman N, Skibola CF, Wang SS, Morgan G, Lan Q, Smith MT, Spinelli JJ, Willett E, De Sanjose S, Cocco P, Berndt SI, Brennan P, Brooks-Wilson A, Wacholder S, Becker N, Hartge P, Zheng T, Roman E, Holly EA, Boffetta P, Armstrong B, Cozen W, Linet M, Bosch FX, Ennas MG, Holford TR, Gallagher RP, Rollinson S, Bracci PM, Cerhan JR, Whitby D, Moore PS, Leaderer B, Lai A, Spink C, Davis S, Bosch R, Scarpa A, Zhang Y, Severson RK, Yeager M, Chanock S and A Nieters. Genetic variation in TNF and IL10 and risk of non-Hodgkin lymphoma: a report from the InterLymph Consortium. Lancet Oncol. 2006; 7(1): 27-38.

Ruland J, Duncan GS, Elia A, del Barco Barrantes I, Nguyen L, Plyte S, Millar DG, Bouchard D, Wakeham A, Ohashi PS and TW Mak. Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. Cell, 2001; 104(1): 33-42.

Salama N, Guillemin K, McDaniel TK, Sherlock G, Tompkins L, and S Falkow. A whole-genome microarray reveals genetic diversity among Helicobacter pylori strains. Proc Natl Acad Sci U S A. 2000; 97(26): 14668-73.

Sambrook J, Fritsch EF and T Maniatis. Molecular cloning – A laboratory manual. 2nd Edition, 1989. Cold Spring Harbor Laboratory Press.

Santtila S, Savinainen K, Hurme M. Presence of the IL-1RA allele 2 (IL1RN*2) is associated with enhanced IL-1beta production in vitro. Scand J Immunol. 1998; 47(3): 195-8.

Satin B, Del Giudice G, Della Bianca V, Dusi S, Laudanna C, Tonello F, Kelleher D, Rappuoli R, Montecucco C, Rossi F. The neutrophil-activating protein (HP-NAP) of Helicobacter pylori is a protective antigen and a major virulence factor. J Exp Med. 2000; 191(9): 1467-76.

Seegers D, Zwiers A, Strober W, Pena AS and G Bouma. A TaqI polymorphism in the 3'UTR of the IL-12 p40 gene correlates with increased IL-12 secretion. Genes Immun. 2002; 3(7): 419-23.

Segal ED, Falkow S and LS Tompkins. Helicobacter pylori attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins. Proc Natl Acad Sci U S A. 1996; 93(3): 1259-64.

Segal ED, Cha J, Lo J, Falkow S and LS Tompkins. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. Proc Natl Acad Sci U S A. 1999; 96(25): 14559-64.

Selbach M, Moese S, Hauck CR, Meyer TF, Backert S. Src is the kinase of the Helicobacter pylori CagA protein in vitro and in vivo. J Biol Chem. 2002; 277(9): 6775-8.

Severson RK and S Davis. Increasing incidence of primary gastric lymphoma. Cancer, 1990; 66(6): 1283-7.

Shapiro-Shelef M and K Calame. Regulation of plasma-cell development. Nat Rev Immunol. 2005; 5(3): 230-42.

Sharma SA, Tummuru MK, Blaser MJ, and LD Kerr. Activation of IL-8 gene expression by Helicobacter pylori is regulated by transcription factor nuclear factor-kappa B in gastric epithelial cells. J Immunol. 1998; 160(5): 2401-7.

Shimizu T, Haruna H, Ohtsuka Y, Kaneko K, Gupta R and Y Yamashiro. Cytokines in the gastric mucosa of children with Helicobacter pylori infection. Acta Paediatr. 2004; 93(3): 322-6.

Shin HD, Winkler C, Stephens JC, Bream J, Young H, Goedert JJ, O'Brien TR, Vlahov D, Buchbinder S, Giorgi J, Rinaldo C, Donfield S, Willoughby A, O'Brien SJ and MW Smith. Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10. Proc Natl Acad Sci U S A. 2000; 97(26): 14467-72.

Silverman EK, Palmer LJ. Case-control association studies for the genetics of complex respiratory diseases. Am J Respir Cell Mol Biol. 2000; 22(6): 645-8.

Sitas F, Forman D, Yarnell JW, Burr ML, Elwood PC, Pedley S, and KJ Marks. Helicobacter pylori infection rates in relation to age and social class in a population of Welsh men. Gut. 1991; 32(1): 25-8.

Smith AJ, Keen LJ, Billingham MJ, Perry MJ, Elson CJ, Kirwan JR, Sims JE, Doherty M, Spector TD and JL Bidwell. Extended haplotypes and linkage disequilibrium in the IL1R1-IL1A-IL1B-IL1RN gene cluster: association with knee osteoarthritis. Genes Immun. 2004; 5(6): 451-60.

Smythies LE, Waites KB, Lindsey JR, Harris PR, Ghiara P, Smith PD. Helicobacter pylori-induced mucosal inflammation is Th1 mediated and

exacerbated in IL-4, but not IFN-gamma, gene-deficient mice. J Immunol. 2000; 165(2): 1022-9.

Snapper CM, Yamada H, Smoot D, Sneed R, Lees A, Mond JJ. Comparative in vitro analysis of proliferation, Ig secretion, and Ig class switching by murine marginal zone and follicular B cells. J Immunol. 1993; 150(7): 2737-45.

Sorensen TI, Nielsen GG, Andersen PK and TW Teasdale. Genetic and environmental influences on premature death in adult adoptees. N Engl J Med. 1988; 318(12): 727-32.

Spink CF, Keen LJ, Mensah FK, Law GR, Bidwell JL and GJ Morgan. Association between non-Hodgkin lymphoma and haplotypes in the TNF region. Br J Haematol. 2006; 133(3): 293-300.

Stein M, Rappuoli R and A Covacci. Tyrosine phosphorylation of the Helicobacter pylori CagA antigen after cag-driven host cell translocation. Proc Natl Acad Sci U S A. 2000; 97(3): 1263-8.

Streubel B, Lamprecht A, Dierlamm J, Cerroni L, Stolte M, Ott G, Raderer M and A Chott. T(14;18)(q32;q21) involving IGH and MALT1 is a frequent chromosomal aberration in MALT lymphoma. Blood, 2003; 101(6): 2335-9.

Suarez F, Lortholary O, Hermine O, and M Lecuit. Infection-associated lymphomas derived from marginal zone B cells: a model of antigen-driven lymphoproliferation. Blood, 2006; 107(8): 3034-44.

Suerbaum S, Smith JM, Bapumia K, Morelli G, Smith NH, Kunstmann E, Dyrek I, and M Achtman. Free recombination within Helicobacter pylori. Proc Natl Acad Sci U S A. 1998; 95(21): 12619-24.

Sundrud MS, Torres VJ, Unutmaz D, Cover TL. Inhibition of primary human T cell proliferation by Helicobacter pylori vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. Proc Natl Acad Sci U S A. 2004; 101(20): 7727-32.

Szabo I, Brutsche S, Tombola F, Moschioni M, Satin B, Telford JL, Rappuoli R, Montecucco C, Papini E and M Zoratti. Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of Helicobacter pylori is required for its biological activity. EMBO J. 1999; 18(20): 5517-27.

Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, and LH Glimcher. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000;100(6):655-69.

Szabo SJ, Sullivan BM, Peng SL and LH Glimcher. Molecular mechanisms regulating Th1 immune responses. Annu. Rev. Immunol. 2003; 21; 713-58.

Telford JL, Ghiara P, Dell'Orco M, Comanducci M, Burroni D, Bugnoli M, Tecce MF, Censini S, Covacci A, Xiang Z, Papini E, Montecucco C, Luca P and

R Rappuoli. Gene structure of the Helicobacter pylori cytotoxin and evidence of its key role in gastric disease. J Exp Med. 1994; 179(5): 1653-58.

Terry CF, Loukaci V, Green FR. Cooperative influence of genetic polymorphisms on interleukin 6 transcriptional regulation. J Biol Chem. 2000; 275(24): 18138-44.

Thome M. CARMA1, BCL-10 and MALT1 in lymphocyte development and activation. Nat Rev Immunol. 2004; 4(5): 348-59.

Thye T, Burchard GD, Nilius M, Muller-Myhsok B, Horstmann RD. Genomewide linkage analysis identifies polymorphism in the human interferon-gamma receptor affecting Helicobacter pylori infection. Am J Hum Genet. 2003; 72(2): 448-53.

Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzegerald LM, Lee N, Adams MD, Hickey EK, Berg DE, Gocayne JD, Utterback TR, Peterson JD, Kelley JM, Cotton MD, Weidman JM, Fujii C, Bowman C, Watthey L, Wallin E, Hayes WS, Borodovsky M, Karp PD, Smith HO, Fraser CM, and VC Venter. The complete genome sequence of the gastric pathogen Helicobacter pylori. Nature, 1997; 388(6642): 539-47.

Tombola F, Morbiato L, Del Giudice G, Rappuoli R, Zoratti M, Papini E. The Helicobacter pylori VacA toxin is a urea permease that promotes urea diffusion across epithelia. J Clin Invest. 2001; 108(6): 929-37.

Tomita T, Jackson AM, Hida N, Hayat M, Dixon MF, Shimoyama T, Axon AT, Robinson PA and JE Crabtree. Expression of Interleukin-18, a Th1 cytokine, in human gastric mucosa is increased in Helicobacter pylori infection. J Infect Dis. 2001; 183(4): 620-7.

Tournamille C, Colin Y, Cartron JP, Le Van Kim C. Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. Nat Genet. 1995; 10(2): 224-8.

Tran AX, Karbarz MJ, Wang X, Raetz CR, McGrath SC, Cotter RJ, Trent MS. Periplasmic cleavage and modification of the 1-phosphate group of Helicobacter pylori lipid A. J Biol Chem. 2004; 279(53): 55780-91.

Trinchieri G. Regulatory role of T-cells producing both IFNγ and IL-10 in persistent infection. J. Exp. Med. 2001:194(10);53-57.

Trinchieri G, Pflanz S, and RA Kastelein. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. Immunity. 2003; 19(5): 641-4.

Tsutsumi R, Higashi H, Higuchi M, Okada M, Hatakeyama M. Attenuation of Helicobacter pylori CagA x SHP-2 signaling by interaction between CagA and C-terminal Src kinase. J Biol Chem. 2003; 278(6): 3664-70.

Tummuru MK, Cover TL, and MJ Blaser. Cloning and expression of a high-molecular-mass major antigen of Helicobacter pylori: evidence of linkage to cytotoxin production. Infect Immun. 1993; 61(5): 1799-809.

Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ and IV Hutchinson. An investigation of polymorphism in the interleukin-10 gene promoter. Eur J Immunogenet. 1997; 24(1): 1-8.

Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N and RJ Schlemper. Helicobacter pylori infection and the development of gastric cancer. N Engl J Med. 2001; 345(11): 784-9.

Uren AG, O'Rourke K, Aravind LA, Pisabarro MT, Seshagiri S, Koonin EV and VM Dixit. Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol Cell. 2000; 6(4): 961-7.

van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, Delabesse E, Davi F, Schuuring E, Garcia-Sanz R, van Krieken JH, Droese J, Gonzalez D, Bastard C, White HE, Spaargaren M, Gonzalez M, Parreira A, Smith JL, Morgan GJ, Kneba M and EA Macintyre. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations. Leukemia, 2003; 17(12): 2257-317.

Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, Athman R, Memet S, Huerre MR, Coyle AJ, DiStefano PS, Sansonetti PJ, Labigne A, Bertin J, Philpott DJ, Ferrero RL. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat Immunol. 2004; 5(11): 1166-74.

Waldschmidt TJ, Kroese FG, Tygrett LT, Conrad DH, Lynch RG. The expression of B cell surface receptors. III. The murine low-affinity IgE Fc receptor is not expressed on Ly 1 or 'Ly 1-like' B cells. Int Immunol. 1991; 3(4): 305-15.

Wang J, Brooks EG, Bamford KB, Denning TL, Pappo J, Ernst PB. Negative selection of T cells by Helicobacter pylori as a model for bacterial strain selection by immune evasion. J Immunol. 2001; 167(2): 926-34.

Wang G, Maier RJ. An NADPH quinone reductase of Helicobacter pylori plays an important role in oxidative stress resistance and host colonization. Infect Immun. 2004; 72(3): 1391-6.

Wang G, Olczak AA, Walton JP, Maier RJ. Contribution of the Helicobacter pylori thiol peroxidase bacterioferritin comigratory protein to oxidative stress

resistance and host colonization. Infect Immun. 2005; 73(1): 378-84.

Warzocha K, Ribeiro P, Bienvenu J, Roy P, Charlot C, Rigal D, Coiffier B and G Salles. Genetic polymorphisms in the tumor necrosis factor locus influence non-Hodgkin's lymphoma outcome. Blood, 1998; 91(10): 3574-81.

Webb PM, Knight T, Greaves S, Wilson A, Newell DG, Elder J, and D Forman. Relation between infection with Helicobacter pylori and living conditions in childhood: evidence for person to person transmission in early life. BMJ. 1994; 308(6931): 750-3.

Weeks DL, Eskandari S, Scott DR, and G Sachs. A H+-gated urea channel: the link between Helicobacter pylori urease and gastric colonization. Science. 2000; 287(5452): 482-5.

Weller S, Reynaud CA, Weill JC. Splenic marginal zone B cells in humans: where do they mutate their Ig receptor? Eur J Immunol. 2005; 35(10): 2789-92.

Wen L, Brill-Dashoff J, Shinton SA, Asano M, Hardy RR, Hayakawa K. Evidence of marginal-zone B cell-positive selection in spleen. Immunity. 2005; 23(3): 297-308.

Westendorp RG, Langermans JA, Huizinga TW, Elouali AH, Verweij CL, Boomsma DI, Vandenbroucke JP. Genetic influence on cytokine production and fatal meningococcal disease. Lancet. 1997; 349(9046): 170-3.

Whary MT and JG Fox. Th1-mediated pathology in mouse models of human disease is ameliorated by concurrent Th2 responses to parasite antigens. Curr Top Med Chem. 2004; 4(5): 531-8.

Whary MT, Sundina N, Bravo LE, Correa P, Quinones F, Caro F and JG Fox. Intestinal helminthiasis in Colombian children promotes a Th2 response to Helicobacter pylori: possible implications for gastric carcinogenesis. Cancer Epidemiol Biomarkers Prev. 2005; 14(6): 1464-9.

Willenbrock K, Jungnickel B, Hansmann ML, Kuppers R. Human splenic marginal zone B cells lack expression of activation-induced cytidine deaminase. Eur J Immunol. 2005; 35(10): 3002-7.

Wilson AG, di Giovine FS, Blakemore AI, Duff GW. Single base polymorphism in the human tumour necrosis factor alpha (TNF alpha) gene detectable by NcoI restriction of PCR product. Hum Mol Genet.1992; 1(5): 353.

Wilson AG, Symons JA, McDowell TL, McDevitt HO and GW Duff. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. Proc Natl Acad Sci U S A. 1997; 94(7): 3195-9.

Wotherspoon AC, Ortiz-Hidalgo C, Falzon MR and PG Isaacson. Helicobacter pylori-associated gastritis and primary B-cell gastric lymphoma. Lancet, 1991; 338: 1175-6.

Wotherspoon AC, Doglioni C, Diss TC, Pan L, Moschini A, de Boni M and PG Isaacson. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of Helicobacter pylori. Lancet, 1993; 342: 575-7.

Wray GA, Hahn MW, Abouheif E, Balhoff JP, Pizer M, Rockman MV, Romano LA. The evolution of transcriptional regulation in eukaryotes. Mol Biol Evol. 2003; 20(9): 1377-419.

Xiang Z, Censini S, Bayeli PF, Telford JL, Figura N, Rappuoli R, and A Covacci. Analysis of expression of CagA and VacA virulence factors in 43 strains of Helicobacter pylori reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. Infect Immun. 1995; 63(1): 94-8.

Yamamoto T, Kita M, Ohno T, Iwakura Y, Sekikawa K, Imanishi J. Role of tumor necrosis factor-alpha and interferon-gamma in Helicobacter pylori infection. Microbiol Immunol. 2004; 48(9): 647-54.

Yamaoka Y, Kita M, Kodama T, Sawai N, Kashima K, and J Imanishi. Induction of various cytokines and development of severe mucosal inflammation by cagA gene positive Helicobacter pylori strains. Gut. 1997; 41(4): 442-51.

Yamaoka Y, Kwon DH, Graham DY. A M(r) 34,000 proinflammatory outer membrane protein (oipA) of Helicobacter pylori. Proc Natl Acad Sci U S A. 2000; 97(13): 7533-8.

Yamaoka Y, Souchek J, Odenbreit S, Haas R, Arnqvist A, Boren T, Kodama T, Osato MS, Gutierrez O, Kim JG, and DY Graham. Discrimination between cases of duodenal ulcer and gastritis on the basis of putative virulence factors of Helicobacter pylori. J Clin Microbiol. 2002; 40(6): 2244-6.

Yamaoka Y, Kudo T, Lu H, Casola A, Brasier AR, Graham DY. Role of interferon-stimulated responsive element-like element in interleukin-8 promoter in Helicobacter pylori infection. Gastroenterology. 2004; 126(4): 1030-43.

Yamazaki S, Yamakawa A, Ito Y, Ohtani M, Higashi H, Hatakeyama M, and T Azuma. The CagA protein of Helicobacter pylori is translocated into epithelial cells and binds to SHP-2 in human gastric mucosa. J Infect Dis. 2003; 187(2): 334-7.

Ye H, Liu H, Attygalle A, Wotherspoon AC, Nicholson AG, Charlotte F, Leblond V, Speight P, Goodlad J, Lavergne-Slove A, Martin-Subero JI, Siebert R, Dogan A, Isaacson PG and MQ Du. Variable frequencies of t(11;18)(q21;q21) in MALT lymphomas of different sites: significant association with CagA strains of H pylori in gastric MALT lymphoma. Blood, 2003; 102(3): 1012-8.

Zambon CF, Basso D, Navaglia F, Germano G, Gallo N, Milazzo M, Greco E, Fogar P, Mazza S, Di Mario F, Basso G, Rugge M and M Plebani. Helicobacter pylori virulence genes and host IL-1RN and IL-1beta genes interplay in favouring the development of peptic ulcer and intestinal metaplasia. Cytokine, 2002; 18: 242-51.

Zambon CF, Navaglia F, Basso D, Rugge M and M Plebani. Helicobacter pylori babA2, cagA, and s1 vacA genes work synergistically in causing intestinal metaplasia. J Clin Pathol. 2003; 56(4): 287-91.

Zavros Y, Rathinavelu S, Kao JY, Todisco A, Del Valle J, Weinstock JV, Low MJ and JL Merchant. Treatment of Helicobacter gastritis with IL-4 requires somatostatin. Proc Natl Acad Sci U S A. 2003; 100(22): 12944-9.

Zavros Y and JL Merchant. Modulating the cytokine response to treat Helicobacter gastritis. Biochem Pharmacol. 2005; 69(3): 365-71.

Zheng HT, Peng ZH, Li S and L He. Loss of heterozygosity analyzed by single nucleotide polymorphism array in cancer. World J Gastroenterol. 2005; 11(43): 6740-4.

Zheng PY and NL Jones. Helicobacter pylori strains expressing the vacuolating cytotoxin interrupt phagosome maturation in macrophages by recruiting and retaining TACO (coronin 1) protein. Cell Microbiol. 2003; 5(1): 25-40.

Zheng W and RA Flavell. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell. 1997; 89(4): 587-96.

Zhou H, Du MQ and VM Dixit. Constitutive NF-kappaB activation by the t(11;18)(q21;q21) product in MALT lymphoma is linked to deregulated ubiquitin ligase activity. Cancer Cell, 2005; 7: 425-431.

Zhou W, Yamazaki S, Yamakawa A, Ohtani M, Ito Y, Keida Y, Higashi H, Hatakeyama M, Si J, Azuma T. The diversity of vacA and cagA genes of Helicobacter pylori in East Asia. FEMS Immunol Med Microbiol. 2004; 40(1): 81-7.

Zhou Y, Ye H, Martin-Subero JI, Hamoudi R, Lu YJ, Wang R, Siebert R, Shipley J, Isaacson PG, Dogan A and MQ Du. Distinct comparative genomic hybridisation profiles in gastric mucosa-associated lymphoid tissue lymphomas with and without t(11;18)(q21;q21). Br J Haematol. 2006; 133(1): 35-42.

Zucca E, Bertoni F, Roggero E, Bosshard G, Cazzaniga G, Pedrinis E, Biondi A, Cavalli F. Molecular analysis of the progression from Helicobacter pylori-associated chronic gastritis to mucosa-associated lymphoid-tissue lymphoma of the stomach. N Engl J Med. 1998; 338(12): 804-10.