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Cytokine Polymorphisms in the Study of Helicobacter pylori Related Gastric Pathology

A Thesis submitted to the University of Dublin, Trinity College

for the degree of

Doctor of Philosophy (Ph.D.)

By

Gwen Murphy

Supervisor

Professor CA O'Morain

Dept. Clinical Medicine

University of Dublin

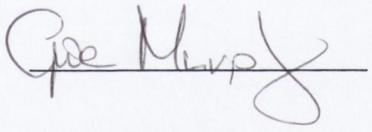
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Gwen Murphy

TABLE OF CONTENTS

1. GENERAL INTRODUCTION 1

1.1 *HELICOBACTER PYLORI* 2

1.1.1 Epidemiology and transmission of infection 3

1.2 *HELICOBACTER PYLORI* INFECTION AND GASTRIC PATHOLOGY .. 5

1.2.1 Classification of H. pylori related gastric pathology 6

1.2.2 Intestinal metaplasia and Gastric cancer 13

1.3 GASTRIC CANCER 16

1.3.1 Classification of Gastric cancer 20

1.3.2 Gastric cancer and Correas cascade of gastric carcinogenesis 22

1.3.3 Gastric cancer and Helicobacter pylori 26

1.4 DETERMINING PATHOGENICITY OF *HELICOBACTER PYLORI* INFECTION 28

1.4.1 Pathogenicity: Bacterial factors 29

1.4.2 Pathogenicity: Environmental factors 32

1.4.3 Pathogenicity: Host factors 35

1.5 AIMS AND OBJECTIVES 41

1.5.1 General Objectives 41

1.5.2 Specific Objectives 42

2. GENERAL MATERIALS AND METHODS 44

2.1 SCREENING 45

2.1.1 Inclusion/Exclusion criteria 46

2.2 STUDY PATIENTS 49

2.2.1 'Normal' patient group 50

2.2.2 Gastritis study group 51

2.2.3 Intestinal metaplasia study group 52

2.2.4 Non-cardia gastric cancer study group 53

2.3 METHODS OF *HELICOBACTER PYLORI* DETECTION 54

2.4 SAMPLE COLLECTION 57

2.4.1 DNA extraction from blood samples 58

2.4.2 DNA extraction from formalin fixed paraffin embedded tissue *59*

2.5 TAQMAN® ASSAY DEVELOPMENT – ALLELIC DISCRIMINATION 62

2.5.1 Introduction to Taqman® technology *63*

2.5.2 Assay Optimisation *65*

2.6 ATROPHY AND INTESTINAL METAPLASIA TYPING 69

2.6.1 ELISA Assays for Pepsinogen I & II *69*

3. PATIENT COHORT 71

3.1 INTRODUCTION 72

3.1.1 Specific Aims and Objectives *74*

3.2 PATIENTS AND METHODS 75

3.2.1 Summary of methods *75*

3.2.2 Statistics *76*

3.3 RESULTS 77

3.3.1 Disease and H. pylori status *77*

3.3.2 Histology *80*

3.3.3 Helicobacter pylori status *85*

3.3.4 Medical history *86*

3.4 DISCUSSION 87

4. SERUM PEPSINOGEN AND PATIENT CLASSIFICATION.. 89

4.1 INTRODUCTION 90

4.1.2 Specific Aims and Objectives *92*

4.2 PATIENTS AND METHODS 93

4.2.1 Summary of methods *93*

4.2.2 Statistics *94*

4.3 RESULTS 95

4.3.1 Serum Pepsinogen levels *95*

4.3.2 Serum Pepsinogen levels and Helicobacter pylori infection *97*

4.3.3 Serum Pepsinogen levels across patient groups *100*

4.3.4 Serum Pepsinogen levels and AST *104*

4.4 DISCUSSION 106

5. POLYMORPHISMS IN INFLAMMATORY MEDIATORS .. 108

5.1 INTRODUCTION 109

5.1.1 Inflammation and carcinogenesis 109

5.1.2 Single nucleotide polymorphisms 109

5.1.3 Polymorphisms in 'inflammatory' genes 111

5.1.4 Hypothesis and aims 116

5.2 PATIENTS AND METHODS 118

5.2.1 Summary of Methods 118

5.2.2 Specifics of Taqman Assay Design 119

5.2.3 Statistics 123

5.3 RESULTS 124

5.3.1 Primary Analysis 124

5.3.2 Secondary Analysis 133

5.4 DISCUSSION 141

6. GENERAL DISCUSSION 145

APPENDIX 1: CASE RECORD FORM 150

APPENDIX 2: *H. PYLORI* IGG ELISA 152

APPENDIX 3: TAQMAN ASSAYS 154

APPENDIX 4: CHEMICALS AND EQUIPMENT 158

APPENDIX 5: SNP SEQUENCES 161

APPENDIX 6: PRIMER & PROBE SEQUENCES 171

REFERENCES 175

LIST OF TABLES

TABLE 1.1: THE UPDATED SYDNEY SYSTEM 13
TABLE 1.2: NATIONAL CANCER REGISTRY OF IRELAND..... 19
TABLE 1.3: LAURÉN CLASSIFICATION 21
TABLE 2.1: PATIENT DATA AND CRITERIA FOR INCLUSION..... 49
TABLE 2.2: FINAL CLASSIFICATION OF *H. PYLORI* STATUS 56
TABLE 2.3 LAYOUT OF 96-WELL PLATE FOR PROBE OPTIMATION..... 66
TABLE 2.4 PRIMER OPTIMISATION 67
TABLE 2.5: PRIMER OPTIMISATION, COCKTAIL 68
TABLE 3.1: PATIENT DEMOGRAPHICS..... 79
TABLE 3.2: TUMOUR TYPE AND HISTOLOGICAL GRADE..... 84
TABLE 3.3: CLASSIFICATION OF *H. PYLORI* STATUS 85
TABLE 5.1: CONTROL GENOTYPE AND ALLELE FREQUENCIES 125
TABLE 5.2: IL-1B AND IL-1RN GENOTYPE FREQUENCIES 127
TABLE 5.3: IL-10 GENOTYPE FREQUENCIES 128
TABLE 5.4: TNF AND TLR4 GENOTYPE FREQUENCIES 129
TABLE 5.5: IL-10 HAPLOTYPE FREQUENCIES 130
TABLE 5.6: ODD RATIOS FOR CARRIAGE OF INDIVIDUAL ALLELES ACROSS ALL PATIENT GROUPS. 134

LIST OF FIGURES

FIGURE 1.1: GROSS ANATOMY OF THE STOMACH 8
FIGURE 1.2: THE UPDATED SYDNEY SYSTEM 10
FIGURE 1.3: AGE-STANDARDISED INCIDENCE RATES BY HEALTH-BOARD AREAS. 18
FIGURE 1.4: CORREAS CASCADE 23
FIGURE 2.1: OUTLINE OF STUDY DESIGN..... 48
FIGURE 2.2: DNA EXTRACTED FROM FORMALIN FIXED PARAFFIN EMBEDDED TISSUE 61
FIGURE 2.3: OUTLINE OF TAQMAN ALLELIC DISCRIMINATION ASSAY.....64
FIGURE 2.4: OUTLINE OF PEPSINOGEN ASSAYS USED IN THE DIAGNOSIS OF ATROPHY/INTESTINAL METAPLASIA 70
FIGURE 3.1: MEDIAN SCORES FOR INFLAMMATION IN PATIENTS 82
FIGURE 4.1: SERUM PG I AND PG II LEVELS IN MALE AND FEMALE PATIENTS..... 96
FIGURE 4.2: SERUM PG I AND PG II LEVELS IN *H. PYLORI* INFECTED AND UNINFECTED GROUPS. 98
FIGURE 4.3: SERUM PG I AND PG II LEVELS IN ALL PATIENTS. 99
FIGURE 4.4: CORRELATION BETWEEN PG I/PG II RATIOS AND GRADES OF INFLAMMATION IN THE *H.PYLORI* POSITIVE PATIENTS. 102
FIGURE 4.5: SERUM PG I AND PG II.LEVELS AND AST 105
FIGURE 5.1: GRAPHICAL REPRESENTATION OF TAQMAN ALLELIC DISCRIMINATION ASSAYS 121
FIGURE 5.2: GRAPHICAL REPRESENTATION OF TAQMAN ALLELIC DISCRIMINATION ASSAYS. 122
FIGURE 5.3: AGE & SERUM PGI AND PGII IN DIFFERENT IL-1B –511 GENOTYPE GROUPS OF *H. PYLORI* NEGATIVE PATIENTS.....137
FIGURE 5.4: AGE & SERUM PGI AND PGII IN DIFFERENT IL-1B –511 GENOTYPE GROUPS OF *H. PYLORI* POSITIVE INTESTINAL METAPLASIA PATIENTS..... 139

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SUMMARY

Persistent local inflammation is now accepted as a risk factor in the development of a number of cancers. Non-cardia gastric cancer is thought to develop after a long lead-time whereby persistent inflammation, caused by *Helicobacter pylori* (*H. pylori)* colonisation of the gastric mucosa progresses to atrophic gastritis, intestinal metaplasia, dysplasia and carcinoma.

Though a relatively uncommon cancer in Western society, mortality figures for those with gastric cancer remain high though incidence rates are falling year on year. Most cases of gastric cancer present at an advanced stage and prognosis for these cases remains dismal. Early detection and diagnosis are inherent in combating poor survival rates.

The classical epidemiological approach to causality postulates that both cancerous and pre-cancerous outcomes depend on the interaction of three factors: the infecting agent, the host and the environment. Recent investigations have increasingly considered the role of host/human genetics in *H. pylori* associated gastro-duodenal disease.

This study aimed to recruit a patient population including patients with gastric cancer as well as 'pre-cancerous' patients to form a novel *H. pylori* related disease database. A panel of single nucleotide polymorphisms (SNPs) in a number of inflammatory mediators was designed. These SNPs are believed to modulate immune response with either high or low production of the encoded protein dependent on expression or absence of the various polymorphic alleles.

DNA from 96 *H. pylori* uninfected controls, 91 patients with chronic gastritis, 63 patients with intestinal metaplasia and 59

patients with non-cardia gastric cancer was typed for polymorphisms at the IL-1B-31, -511 and +3954 and IL-1RN+2018 loci, the TNF-238 and -308 loci, the IL-10-592, -819 and -1082 loci and the TLR4+896 and +936 loci. Genotyping was performed following the development of Taqman® allelic discrimination assays. All alleles at all loci were in Hardy-Weinberg equilibrium and agreed with previously reported allelic frequencies.

Allelic frequency across the patient groups did not vary. Age and gender adjusted regression analysis, however, identified significant risk of gastric cancer development to be associated with carriage of the TLR4+896G allele (odds ratio 4.9, 95% confidence interval, 1.11, 21.79) and independently with homozygosity for the IL-10 ATA/ATA haplotype (OR, 5.93, 95% CI, 0.99, 35.4). The polymorphisms were not associated with atrophy, as measured by serum pepsinogen I and II levels.

This study reports that polymorphisms in TLR4 and IL-10 genes are associated with increased risk of gastric cancer development, possibly through modulation of the initial immune response to *H. pylori* infection. Further study with this patient cohort may well develop the clinical significance of these findings. Genetic profiling may identify patients at risk early on in the carcinogenic cascade and allow targeted surveillance and improved prognosis.

ABBREVIATIONS

AST	Anti-secretory therapy
bp	basepair
CagA	Cytotoxin associated antigen
CI	Confidence interval
CRF	Case Record Form
H. pylori	*Helicobacter pylori*
HRP	Horseradish peroxidase
IARC	International Agency for Cancer Research
IgG	Immunoglobulin G
IL-10	Interleukin 10
IL-1B	Gene encoding IL-1Beta
IL-1Beta	Interleukin 1 Beta
IL-1ra	Interleukin 1 receptor antagonist
IL-1RN	Gene encoding IL-1ra
IL-6	Interleukin 6
IL-8	Interleukin 8
IM	Intestinal metaplasia
Kb	Kilobase
LPS	Lipopolysacchardide
OR	Odds ratio
PAI	(cag) pathogenicity island
PPI	Proton pump inhibitor
SNP	Single nucleotide polymorphism
TNF(A)	Tumour Necrosis Factor
VacA	Vacuolating cytotoxin A

PUBLICATIONS FROM THIS THESIS

Abstracts

Murphy G, Thornton J, McManus R, Crotty P, Buckley M, O'Connor H, O'Morain CA

Toll-like receptor 4 polymorphism not associated with *H.pylori* related intestinal metaplasia. Eur Cytokine Netw 2003 Sept; 14S: A339

Murphy G, Thornton J, McManus R, Crotty P, Buckley M, O'Connor H, O'Morain CA

IL-1B polymorphisms not associated with *Helicobacter pylori* positive gastritis and intestinal metaplasia. Ir J Med Sci 2003 June

Murphy G, Thornton J, McManus R, Crotty P, Buckley M, O'Connor H, O'Morain CA

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IL-1B polymorphisms are not associated with *Helicobacter pylori* positive intestinal metaplasia. Gastroenterology 2003 Apr; 124(4): A404

Murphy G, O'Sullivan M, Hall W, O'Morain CA

Expression of Transforming Growth Factor β1 receptor type II on human gastric mast cells. Gut 2001 Nov; 49(3): A1885

Ryan BM, Murphy G, O'Morain CA

Host cytokine responses to *Helicobacter pylori*: an important determinant of clinical outcome. Ir J Med Sci. 2001 Apr-Jun;170(2):90-1.

Original Papers

Polymorphisms in TLR4 and IL-10 genes are associated with increased risk of non-cardia gastric cancer.

Murphy G, McManus R, Swan N, Ryan B, O'Morain CA.

Host factors: *H. pylori* and Gastric Cancer, An Update

Murphy G, O'Morain CA.

TLR4 polymorphisms in *H. pylori* related gastric disease

Murphy G, McManus R, Swan N, O'Connor H, O'Morain CA.

To the mother of all the chicks and the father of all 'Dad Facts'.....

"Reading a scientific article isn't the same as reading a detective story. We want to know from the start that the butler did it."

O.D. Ratnoff

Chapter 1

General Introduction

1.1 *Helicobacter pylori*

> In 1892, an Italian pathologist Giulio Bizzozero first described helicobacters as spiral shaped organisms colonising the stomach of dogs[2]. Almost a hundred years later, a letter in *The Lancet* first described the isolation of this same bacteria from patients with duodenal ulcers and gastric ulcers[3]. In a prophetic tone, the letter concluded that the bacteria '*may have a part to play in poorly understood, gastritis associated diseases*'.

Helicobacter pylori (*H. pylori*), is a curved or spiral shaped, smooth walled, gram-negative rod. Typically, it measures between 2.5 and 4 μm in length.

The organism's remarkable motility is chiefly conferred on it by the presence of between 2 and 6 unipolar flagella. These flagella allow the bacteria to pass rapidly through the acidic lumen of the stomach and penetrate to mucous layer, thus protected from further contact with the low pH environment of the stomach.

Adherence to the epithelial layer is essential in the initial colonisation of the stomach by the bacteria. The production of enzymes by *H. pylori* in particular, urease, further increases the likelihood of successful colonisation[4, 5].

1.1.1 Epidemiology and transmission of infection

Generally acquired in childhood, the infection rarely resolves spontaneously and may persist life-long with concurrent gastric pathology.

Transmission of the bacteria is not entirely understood. The leading hypothesis is that *H. pylori* is transmitted from person-to-person. Whether transmission is by gastro-oral, oral-oral or faecal-oral is a matter of debate[6-8]. It is therefore not surprising that infection rates are linked to socio-economic conditions, in particular, those of early childhood. Infection is understood to pass from person to person between those in close contact such as within families or community homes[9, 10]. Increased risk has been linked to sharing beds in childhood and related to crowded or cramped living quarters [11].

The prevalence of infection, in Western society, has decreased significantly in recent decades [12, 13]. It is unlikely that the advent of frequent use of antibiotics alone is responsible for the demise of the bacterial infection. More probable is the significant increase in the number of barriers to the spread of infection, which again can be related to increasing modernisation or improved socio-economic status. Parsonnet summarised this in 'Factors associated with the disappearance of *Helicobacter pylori* in the West'[14]:

> *"...numerous barriers to faecal-oral spread have been imposed over the 20th century including: access within homes to piped water for toilets and for washing, universal availability of soap, decreased family size and household crowding with improved*

childhood survival, and disappearance of night soil use for fertilization."

This is thought to explain the disparity in infection rates across varying age groups - approximately 20% of the population under the age of 40 years and 50% of those over the age of 60 years are believed to harbour *H. pylori* infection. This is referred to as a cohort effect whereby the current older generation contracted the infection as infants or young children when *H. pylori* infection was more prevalent [15].

1.2 *Helicobacter pylori* infection and gastric pathology

> *"H. pylori, each year, kills more than a million people and causes considerable discomfort to tens of millions."*
>
> *Adrian Lee, 2000*[16]

H. pylori infection generally begins in the antrum causing an antral gastritis. If left untreated, the opportunistic infection may spread, colonising the acid-producing corpus region, resulting in a pan-gastritis.

1.2.1 Classification of H. pylori related gastric pathology

In 1990, a working party at the World Congress Gastroenterology in Sydney established guidelines for both Pathologists and Gastroenterologists in the classification and grading of gastritis[17]. The 'Sydney System' of gastritis classification was reappraised 4 years later and re-named the 'Updated Sydney System'[18]. These are the currently accepted guidelines in sampling and classification of gastric pathology, particularly that related to *H. pylori* infection.

The system comprises of guidelines for endoscopic procedure and sample preparation, as well as for histopathological diagnosis.

Sydney System[18] Endoscopic guidelines and sample preparation

a) *Biopsy sites:* At least five gastric biopsy specimens to be taken, including two from the pyloric antrum (2 to 3 cm from the pylorus), one from the distal lesser curvature, the other from the distal greater curvature, two from the corpus (or body) about 8 cm from the cardia (or antrum cardiacum - one from the lesser and the other from the greater curvature) and one from the incisura angularis. (Figure 1.1)

b) *Samples:* Biopsy samples from each area should be separately identifiable.

c) *Communication:* Information on the patients' endoscopic findings, clinical history and biopsy sites to be transmitted to the pathologist for accurate clinicopathologic correlation in gastritis.

d) *H. pylori*: Specific staining for *H. pylori* should be carried out prior to declaring an inflamed biopsy specimen negative.

e) *Intestinal metaplasia*: An AB/PAS stain will facilitate the recognition of intestinal metaplasia.

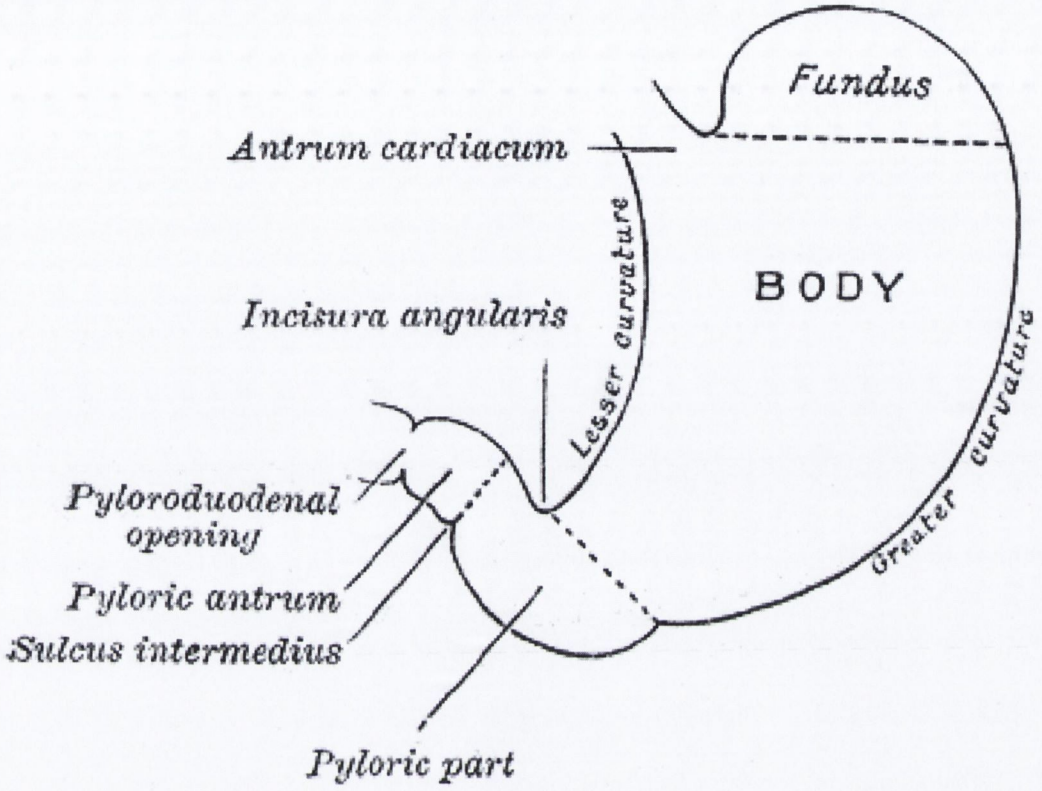


Figure 1.1: Gross anatomy of the stomach[19]

Gastritis

H. pylori related gastritis is a heterogeneous condition. Gastric inflammation may be patchy (focal), or extensive and associated with atrophy. In the initial stages of *H. pylori* related gastritis an inflammatory cell infiltrate is observed in the superficial half of the mucosa. This chronic superficial gastritis may progress to a more extensive gastritis where gastric inflammation spreads deeply to involve the full thickness of the mucosa. This chronic gastritis can, in turn, cause atrophy of the gastric glands (chronic atrophic gastritis). In cases of gastric atrophy, gastric inflammation itself often resolves though the gastric glands are destroyed[20].

The Updated Sydney System included a visual analogue scale (Figure 1.2) to aid in the grading of gastritis, intestinal metaplasia and atrophy. Chronic active gastritis is indicated by a 'significant' infiltrate of mononuclear cells and neutrophils. The presence of plasma cells, normally sparse or absent in the gastric mucosa are also an indication of chronic gastritis.

The term 'activity', in the context of the System is related to "the presence of neutrophil polymorphs in a background of chronic inflammation". Reactive oxygen species and proteases, derived from neutrophils cause tissue damage. As such, 'activity' is linked to tissue damage.

Figure 1.2: Visual Analogue Scale: The Updated Sydney System[18] (Slide courtesy of Professor Mike Dixon)

Intestinal metaplasia

> *"Natura abhorret vacuum"*
>
> (~ Nature dislikes a vacuum)
>
> R.M Genta, 2000[21]

Intestinal metaplasia (IM) is defined as the replacement of the gastric mucosa with mucosa which, morphologically and histochemically, resembles that of the large, or small intestine. IM can frequently be found in the wake of atrophic gastritis where the gastric glands have been destroyed and intestinal-type epithelium fills this void, it should be stressed, however, that atrophy and IM may exist alongside one another as well as independently.

On the basis of morphological and histochemical changes, IM is categorised as either complete or incomplete.

> *Type I (complete IM):* Complete, or Type I, intestinal metaplasia is defined as where *"the gastric mucosa changes to the normal small bowel epithelium, characterised by fully developed goblet cells and enterocytes with a brush border. In advanced cases, the contour of the mucosa changes with the development of villi and crypts. Paneth cells may be present in the base of the crypts."*[20] Goblet cells stain positive for sialomucins[18].
>
> *Type II (incomplete IM):* Incomplete, Type II intestinal metaplasia is classified by *"the absence of recognisable absorptive cells. The epithelium consists of a mixture of intestinal-type goblet cells and columnar mucus secreting cells,*

morphologically resembling those of the normal gastric epithelium."[20] Goblet cells, positive for sialomucin, are interspersed with gastric-type cells, positive for either neutral mucins or sialomucins[18].

Type III (incomplete IM): Incomplete, Type III intestinal metaplasia is characterized by *"tortuous and branched crypts lined by tall columnar cells containing either sialomucins or sulphomucins."*[18]

It should be noted that these variants rarely exist as a pure entity and mixtures of the various types may be found even within the same gastric pit.

Diagnosis and description of IM and atrophy are included in the Updated Sydney System (Figure 1.2 and Table 1.1).

Generally IM first appears at the junction between the antrum and the corpus as small foci of metaplasia[22]. As the metaplasia progresses, these foci fuse to form extensive metaplasia. Distal extension of the metaplasia may involve the entire antrum and proximal extension the length of the proximal lesser curve. Progression can also extend to the oxyntic mucosa on the anterior and posterior walls of the stomach as finger-like processes following the mucosal folds.

As IM begins focally, it is common to find *H. pylori* in surrounding gastric tissue where it has initiated chronic gastritis or atrophic gastritis, which in turn, led to the development of IM. As *H. pylori* adheres specifically to the gastric epithelium it is not commonly found *in situ* in areas of IM.

Classification of *Helicobacter pylori* related gastritis

a) Presence or absence of:

   - *H. pylori*
   - Chronic inflammation
   - Neutrophil polymorph activity
   - Glandular atrophy
   - Intestinal metaplasia

   should be recorded in all cases of gastritis.

b) Variables can be graded on a mild, moderate or marked scale (see Visual Analogue Scale, Figure 1.2).

c) Pattern of chronic gastritis should be assessed and categorised in all cases where both antrum and corpus biopsies are submitted. Where a two-grade difference between sites is found, these cases should be distinguished as antral predominant or corpus predominant.

d) Atrophy or intestinal metaplasia, where present, should be categorised as either multifocal or diffuse.

e) The pattern of inflammation and atrophy should be used in classifying the gastritis and as an indicator of its aetiology.

Table 1.1: The Updated Sydney System recommendations: Classification of *Helicobacter pylori* related gastritis

Compiled from Dixon et al. 1996[18]

1.2.2 Intestinal metaplasia and Gastric cancer

IM in the stomach was noted as early as the 19th century. In 1955 Morson noted, from his own studies, that cancer often arose in this IM tissue[23].

Numerous experimental studies have shown that exposure of gastric tissue to mutagens and/or carcinogens results not only in development of IM alongside gastric carcinoma. Rat studies have shown IM to develop following administration of N-methyl-N'-nitro-N-nitroguanidine in drinking water[24, 25] and following exposure to X-radiation[26].

The strength of epidemiological evidence is often questioned in studies investigating the association between IM and gastric cancer. Understandably, an association is difficult to illustrate in patients – the nature of endoscopic procedure means that only small areas of the stomach are biopsied so that the true extent of IM may be missed due to 'biopsy error'. This complicates studies in that follow-up biopsies could demonstrate a progression from atrophic gastritis to IM and IM to gastric cancer, or identify areas of metaplasia missed by biopsy in previous endoscopic procedures.

Spread of IM to the oxyntic area of the stomach (the acid producing area) compromises acid secretion leading to hypochlorhydria (diminished gastric secretion of hydrochloric acid). Hypochlorhydria is, in itself, a risk factor for the development of gastric cancer[27]. This increase in the pH of the gastric juice allows the accumulation of nitrous compounds, which can induce the development of carcinoma either in the IM tissue or in the residual gastric tissue.

Finally, Stemmermanns 'Status Report'[28] on IM concludes that *"gastric cancers usually develop in the distal stomach, usually in association with antral gastritis and intestinal metaplasia"*.

Importantly, the incomplete type of intestinal metaplasia is more commonly associated with an increased risk of malignant transformation than is the complete type.

Reversibility of IM following eradication therapy is a thorny issue for reasons of biopsy error. It would seem that there maybe a 'point of no return', ie., an alteration in the physiology and morphology of the gastric mucosa after which the process in 'irreversible' and removing the factor which may have begun the process (such as *H. pylori*) will have no effect. Professor Mike Dixon has further expanded this theory outlining two principal pathways leading to gastric mucosal atrophy[29] – either destruction of the stem cell compartment and/or glands *or* selective destruction of specialized epithelial cells with the preservation of the stem cell compartment. If the stem cells have been completely destroyed, regeneration is impossible, if not, removal of the 'injurious factor' may allow restoration of function with the regeneration of chief and parietal cells.

1.3 Gastric cancer

> *"In recent years it has become apparent that the most important single factor responsible for the development of non-cardia gastric cancer (by far the commonest type) is infection with the organism Helicobacter pylori."*
>
> Anthony Axon, 2002[30]

In June 1994, The International Agency for Cancer Research (IARC) concluded "infection with *Helicobacter pylori* is carcinogenic to humans (Group 1)"[31]. This distinction came exactly eleven years after the bacteria was first isolated from patients with gastritis and peptic ulceration[3] and was the result of a review of over 350 papers concerning the relationship between *H. pylori* and gastric cancer.

Gastric Cancer on the island of Ireland

The most recently published cancer statistics for the Republic of Ireland (Cancer in Ireland, 1994-2002[32]) report 450 new cases of stomach cancer in 1999, 171 in females, 279 in males. Annual mortality figures for 1999 reported 148 deaths in females, 204 in males, directly attributed to cancer of the stomach. Though mortality rates are decreasing (estimated annual percentage change of -3.3% for females and -3.9% for males), stomach cancer remains as a significant cause of morbidity with 695 reported episodes and an average length of hospital stay of 15 days.

A previous report by the National Cancer Registry in Ireland: 'All Ireland cancer statistics 1994-96'[1] detailed an average of 4 deaths for every 5 incident cases. The disease was found to principally

affect the elderly, with the national age-specific incidence rate peaking at or beyond 80 years of age. The mean age of diagnosis was 73 years for females and 70 years for males.

Age standardised incidence rates (as a percentage of All-Ireland rate) are illustrated in Figure 1.3 and number of deaths relating to stomach cancer (by gender and county) are outlined in Table 1.2.

Gastric Cancer rates in Ireland and in Europe

A recent paper by Levi *et al.*[33] reported *"steady and persisting falls"* in gastric cancer rates across Europe. The authors studied age-standardized mortality from gastric cancer in each of 25 individual European countries, as well as the in European Union (EU) as a whole, between 1950 and 1999. The authors also referred to a previous study which found, *"of the 80,000 fewer cancer deaths observed in the EU in 1997, as compared with 1988, 24,000 were due to the fall in gastric cancer rates."*[34]

According to the Levi study [33], Ireland followed the EU trend of declining rates of gastric cancer since 1950. A fall of 31.5% was reported in men between the ages of 35 and 64 years old with a similar decrease of 34.92% reported in females of the same age group.

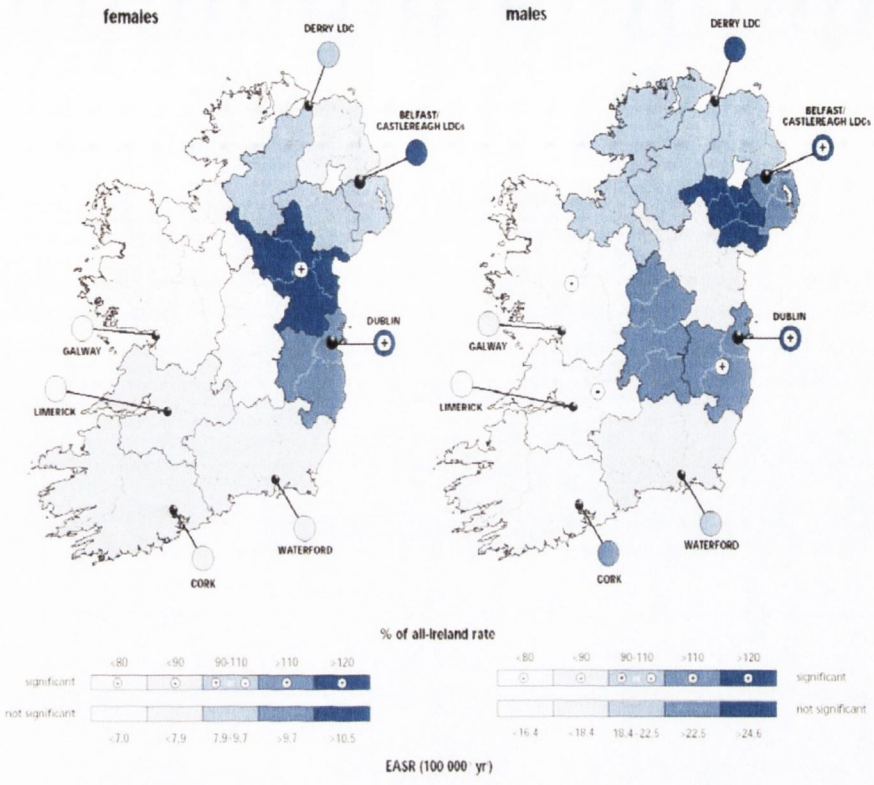


Figure 1.3: Age-standardised incidence rates (as % of All-Ireland rate) by health-board areas, 1994-96: malignant cancer of the stomach[1]. Darker shading indicates a percentage increase <u>above</u> the national average and paler shading regions of lower reported incidence, again relative to the national average.

Female deaths			Male deaths		
	All cancers	Stomach		All cancers	Stomach
Carlow	45	2	Carlow	43	2
Cavan	53	4	Cavan	79	5
Clare	74	4	Clare	100	5
Cork	417	15	Cork	469	24
Donegal	121	6	Donegal	163	11
Dublin	1021	50	Dublin	1068	66
Galway	157	8	Galway	209	12
Kerry	127	4	Kerry	156	8
Kildare	96	3	Kildare	108	7
Kilkenny	64	3	Kilkenny	76	6
Laois	49	1	Laois	64	3
Leitrim	29	2	Leitrim	46	2
Limerick	157	8	Limerick	175	7
Longford	32	2	Longford	39	2
Louth	86	5	Louth	92	7
Mayo	128	6	Mayo	168	9
Meath	80	3	Meath	93	5
Monaghan	40	3	Monaghan	55	4
Offaly	50	3	Offaly	64	4
Roscommon	55	2	Roscommon	67	3
Sligo	62	3	Sligo	79	5
Tipperary	142	6	Tipperary	159	8
Waterford	88	3	Waterford	110	6
Westmeath	57	3	Westmeath	74	6
Wexford	102	3	Wexford	117	5
Wicklow	88	3	Wicklow	100	7

Table 1.2: Number of cancer deaths and stomach cancer deaths by gender and by county, National Cancer Registry of Ireland: 1994-1999 averages[32].

1.3.1 Classification of Gastric cancer

Gastric adenocarcinoma is the most common form of malignant gastric tumour, accounting for about 90% of stomach cancers.

In 1965 Pekka Laurén published what he referred to as *'An Attempt at a Histo-Clinical Classification'* of gastric carcinoma[35]. He proposed this system against a background of *'considerable confusion in the histological terminology of gastric cancer'*. Laurén divided gastric adenocarcinoma into two histological sub-types, intestinal and diffuse type carcinoma.

Intestinal type gastric adenocarcinoma: Typically, the intestinal type is well differentiated, characterized by cohesive neoplastic cells that form discrete gland like structures (which frequently ulcerate), microscopically resembling colorectal carcinoma. Lauréns classification led to the observation that where IM is severe, the resulting cancer is generally intestinal[35].

Diffuse type gastric adenocarcinoma: The diffuse type is less well differentiated and is characterized by sheets of cells, with no gland formation. Sparse signet ring cells may be present, with or without mucin. Diffuse carcinomas often carry a poor prognosis.

Specific differences between the two types of carcinoma described by Laurén in terms of general and cellular structure, as well as secretion and mode of growth are outlined in Table 1.3.

Gastric carcinoma may further appear in a 'mixed' form, ie., a mixture of both classes, falling distinctly into neither category.

	Intestinal type gastric adenocarcinoma	*Diffuse type gastric adenocarcinoma*
General Structure	Distinct, large glandular lumina Often accompanied by papillary fold formation or solid components	Glandular lumina rarely seen Carcinoma cells scattered either as solitary cells or small clusters
Cellular Structure	Cells large, well defined Variable morphology Nuclei: large, variable shape, hyperchromatic	Cytoplasm indistinctly defined, fragile Cells of uniform shape Nuclei: smaller, smooth edged, only faintly hyperchromatic
Secretion	Few secreting cells Secretion, where present forms distinctly defined theca in cytoplasm	Secreting cells present Secretion evenly distributed in the cytoplasm
Mode of Growth	Spread to a clearly and often distinctly defined area Inflammatory cell infiltrate profuse, sometimes concentrated at the margin of spread of the tumour, polymorphonuclear	No well defined tumours Inflammatory cell infiltrate faint, sometimes very slight, mononuclear

Table 1.3: Compiled from Laurén, 1965[35]

1.3.2 Gastric cancer and Correas cascade of gastric carcinogenesis

> *"...Although H. pylori produces a number of toxins that may affect carcinogenesis, it would now seem that the important feature of infection is the induction of an on-going inflammatory reaction."*
>
> David Forman, 2000[36]

Pelayo Correa presented a pair of impressive cohort and cross-sectional studies in 1990[37]. These studies involved 1422 residents of Nariño, Columbia (an area of high incidence of gastric cancer) followed over an average of 5.1 years.

The author described a 'hierarchical distribution' of gastric lesions involving gland neck hyperplasia, atrophy, intestinal metaplasia and dysplasia and reported *"acute inflammation was not found to be a specific stage in the sequence, but rather a common finding in all stages of the precancerous spectrum"*.

These studies supported a model of gastric carcinogenesis proposed by Correa 2 years earlier, in 1988[38] (Figure 1.4), commonly referred to as 'Correas Cascade of Gastric Carcinogenesis'. It should be noted that this type of carcinogenic process applies to 'intestinal type' carcinoma as opposed to the 'diffuse' or 'infiltrative' gastric carcinoma.

The cascade outlines a process of carcinogenesis, which, far from being an, 'all or nothing' phenomenon, is a gradual transition from initial gastritis to atrophic gastritis, intestinal metaplasia, dysplasia and carcinoma.

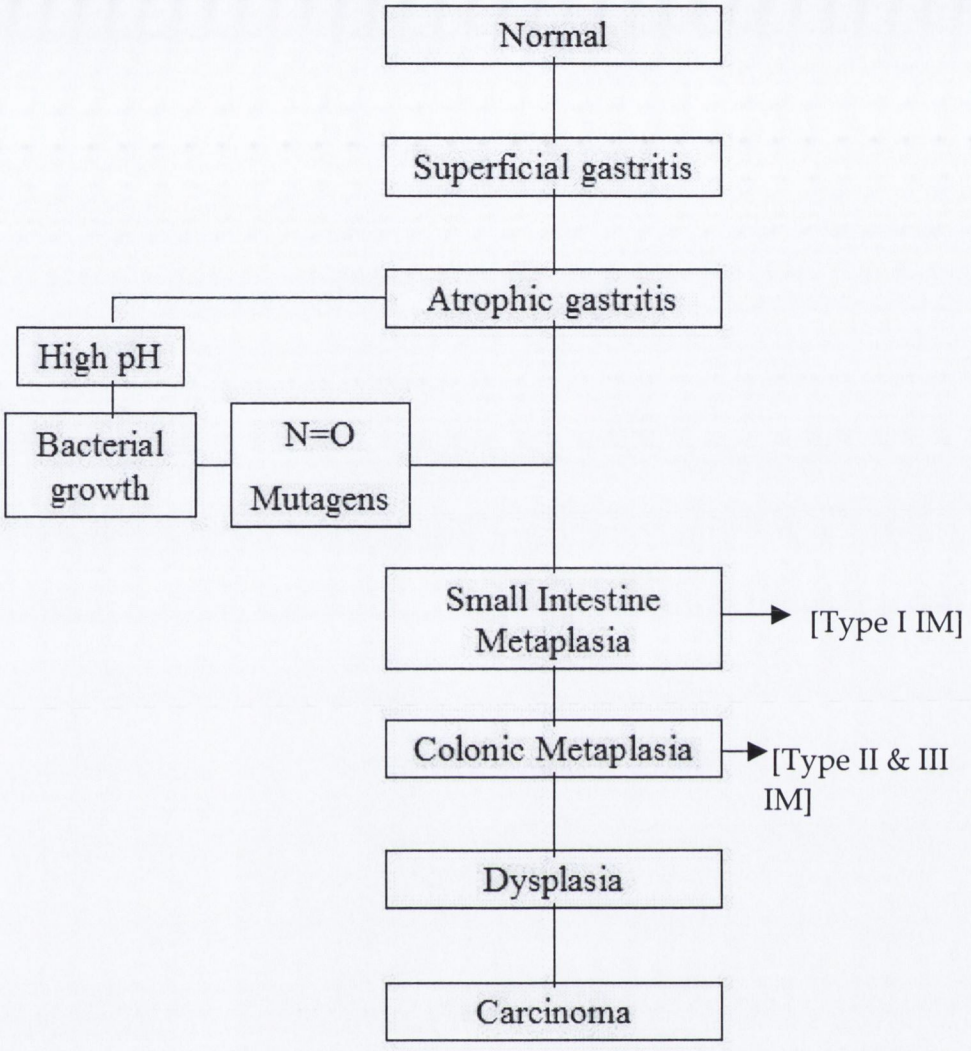


Figure 1.4: Correas cascade, Pelayo Correa, 1992[27]

The early stages of the cascade do not change the normal phenotype of the epithelial cells but are associated with cell loss and subsequent cell regeneration – normal cell cycle events, exaggerated in the presence of chronic inflammation induced by *H. pylori*.

Correa suggests after the step of atrophy *"forces involved in the process apparently require the presence of genotoxic agents, since at this stage hereditary cellular changes are implied which should represent changes in the structure or function of nuclear DNA."* He suggests that these 'genotoxic agents' are nitrites produced by bacterial reduction of dietary nitrate. Contributors to the process include irritants such as salted foods, use of aspirin and genetic susceptibility as outlined in studies investigating gastric cancer in migrants from high risk areas of gastric cancer and early blood group studies [39, 40] (further detailed in 1.4.3, Pathogenicity: Host Factors).

Another dimension: parallel pathways

With the classification of *H. pylori* as a Class I carcinogen, IARC stressed the importance of studying the dynamics of *H. pylori* infection within the stomach, given that non-atrophic gastritis predominantly involves the antrum and multi-focal atrophic gastritis compromises both the antrum and the corpus. Thus, the site of infection and the initial inflammation influences the subsequent gastric pathology.

Furthermore, in patients with duodenal ulceration gastritis is generally non-atrophic and antral-predominant *H. pylori* gastritis with normal or increased acid secretion[41, 42]. In patients with non-cardia gastric cancer (or gastric ulcers) gastritis is more widely

spread and acid secretion is reduced sometimes to the point of achlorhydria[41, 43].

IARC also cite a number of studies investigating the effects of long-term acid-secretory therapy (omeprazole) on *H. pylori* gastritis and the site of gastritis. One such study followed 91 patients on long-term omeprazole therapy and found that while 1% of these patients had atrophic gastritis on commencing therapy 25% developed atrophic gastritis on follow-up[44].

These observations are of fundamental significance to the study of *H. pylori* related disease and emphasise the complexity of the *H. pylori* associated pathologic process. The success of colonisation, site of colonisation and the inflammatory response to colonisation are key stages in gastric disease pathogenesis.

1.3.3 Gastric cancer and Helicobacter pylori

> *"Although the ultimate mechanism of carcinogenesis remains unknown, it seems clear that infection with H. pylori is the major driving force in the process."*
>
> Pelayo Correa, 2004[45]

Since the World Health Organisation classification of *H. pylori* as a class I carcinogen in 1994, numerous studies have added weight to the association between the bacteria and gastric carcinogenesis. The most impressive of these studies is that of the Uemura group in Japan[46]. This study, was a long-term prospective follow-up study and probably the most powerful to date. The Japanese cohort consisted of 1526 patients with duodenal ulcers, gastric ulcers, gastric hyperplasia or non-ulcer dyspepsia on enrolment. 1246 patients were identified as being infected with *H. pylori,* while the remaining 280 patients were shown to be uninfected. The mean follow-up time was 7.8 years. Of those patients positive for *H. pylori*, 3% developed gastric cancer while none of those in the uninfected group went on to develop cancer. Of the 3% that developed gastric cancer, 58% (n=21) were in patients who had presented with functional dyspepsia, underlining the importance of *H. pylori* infection in risk of cancer development, even with normal endoscopic investigation.

A recently published study reported the findings of a 5 year prospective randomised control study based in the Yantai county of the Shandong province in China (where gastric cancer incidence is amongst the highest in China)[47]. 587 *H. pylori* infected

subjects were randomised to receive either eradication therapy (omeprazole, amoxicillin and clarithromycin) or placebo. Five years after receiving treatment the *progression* of gastric IM was retarded in the group that had received eradication therapy. Those who had received eradication therapy had a significantly lower risk of IM progression than those who received placebo (OR: 0.63, 95% CI, 0.43-0.93) and in the cases where eradication therapy proved successful the risk was further reduced (OR: 0.48, 95% CI, 0.32-0.74). Rates of gastric cancer, however, were not statistically different in those who had received *H. pylori* eradication relative to those who had been treated with placebo. Another study from the Fujian Province in China (another area associated with a 'high risk' of gastric cancer development)[48] found that the incidence of gastric cancer to be similar between participants receiving *H. pylori* eradication treatment and those receiving placebo during a period of 7.5 years.

Negative findings (such as those outlined above), even after a relatively long follow-up time (7.5 years), are not surprising given the long-lead time in development of gastric cancer. An accurate assessment of the effect of *H. pylori* eradication treatment on incidence rates of gastric cancer would require tens of thousands of subjects followed for a number of decades. For this reason, intermediate end-points, such as intestinal metaplasia are useful in the study of gastric carcinogenesis.

1.4 Determining pathogenicity of *Helicobacter pylori* infection

H. pylori infection is almost endemic. It can be assumed that only a few people are not exposed to infection yet only a proportion of those exposed develop *H. pylori* infection and far less develop clinically significant disease. The reason for this disparity is multi-factorial, involving interaction between the bacteria, environmental factors and the human host.

1.4.1 Pathogenicity: Bacterial factors

Helicobacter pylorus is a particularly heterogeneous and highly adaptable bacterium. Virulence can vary greatly from strain to strain and strains vary greatly with geographical location. A number of bacterial virulence factors have been associated with particular gastric pathologies and severity of pathology.

Cytotoxin associated antigen (CagA) and the *cag* associated pathogenicity island (*cag*-PAI)

One of the first virulence factors identified in *H. pylori* was a protein, CagA, encoded by the *cag*A gene. This gene is present in between 60% and 70% of *H. pylori* strains in developed countries. Upon binding of the bacteria to the gastric epithelial cells, CagA is translocated into the membrane of the host gastric cells via a type IV secretion system[49], essentially, a molecular syringe. The function of the bacterial protein once inside the host cell is not known, though CagA is tyrosine phosphorylated on translocation by a host-cell kinase within the epithelial cell [50].

The *cag*A gene is the terminal open reading frame of a 40 Kilobase segment of the *H. pylori* genome, referred to as the *cag*-associated pathogenicity island (*cag*-PAI). Infection with *cag*-PAI positive strains results in an increased expression of IL-8 by gastric epithelial cell lines [51,52] via a nuclear factor κB (NF-κB) pathway[53,54].

Clinical observation has tended to associate these strains with a more severe disease phenotype, though these associations vary greatly. Peek *et al.*[55], reported that gastric biopsies from the antrum of patients with peptic ulcers, who were infected with *cag* positive *H. pylori* strains, had a greater degree of inflammation

and more often expressed inflammatory cytokines Interleukin-1β (IL-1β) and Interleukin-8 (IL-8) than those infected with *cag* negative strains or those without *H. pylori* infection.

The vacuolating cytotoxin A (VacA)

The vacuolating cytotoxin gene A (*vacA*) is a locus, located outside the *cag* PAI, which has been associated with ulcer disease and gastric adenocarcinoma. Approximately 50% of *H. pylori* isolates are thought to express *vacA*. VacA forms pores in lysosomal membranes, increasing anion permeability and generating vacuoles [56, 57]. It is also hypothesised that VacA may increase paracellular permeability to small molecules [58].

Two divergent regions in the *vacA* gene have been described, one in the peptide coding region (*s1a, s1b, s1c* and *s2* allelic types), the other mid-region (*m1, m2a* and *m2b* allelic types. A study published in 2002 by Figueiredo *et al.*[59], found infection with *vacAs1-*, *vacAm1-*, and *cagA*-positive strains of *H. pylori* was associated with an increased risk for gastric carcinoma, with odds ratios of 17 (95% CI, 7.8, 38), 6.7 (95% CI, 3.6, 12), and 15 (95% CI, 7.4, 29), respectively. 'Mosaic' *s1m1* strains have been shown to be more toxic than *s1m2* strains and associated with more severe gastritis, atrophy and intestinal metaplasia[60].

Numerous other bacterial factors have now been identified which may influence both success of bacterial infection and course or severity of gastric disease. These include the *babA2*, *iceA* and *napA* genes[61-63].

Factors apart from virulence factors *per se*, will influence the success of *H. pylori* colonisation and therefore affect subsequent

gastric pathology. These factors include the bacterial lipopolysaccharide, bacterial urease and indeed motility.

1.4.2 Pathogenicity: Environmental factors

A number of environmental factors associated with altered risk of gastric cancer have been described by epidemiological studies. These factors may act as inhibitors (e.g. antioxidants) or enhancers (gastric irritants) of the inflammatory process.

Diet

Gastric cancer incidence and mortality vary between and within countries, in migrant populations and their offspring, in differing socio-economic groups and over time. It is plausible that dietary habits may be aetiologically important in the development of atrophic gastritis and gastric cancer. As such, the role of diet has been widely investigated with inconclusive results.

Fruit and Vegetables: A protective association has been acknowledged, almost unanimously, with fresh fruit and vegetables[64-66]. Micronutrients, vitamins C (ascorbate) and E (α-tocopherol), carotenoids (β-carotene) and selenium, have most consistently been attributed with protective roles [67,68].

Widespread growth of electric refrigeration over the last century has been associated with the general decline in gastric cancer rates across the EU[69].

Salt, Nitrite and Nitrate: A high intake of salt has also been associated with increased gastric cancer risk [70-72]. La Vecchia *et al.*[73] reported a two- to threefold increased gastric cancer risk associated with a high intake of salt, nitrites and methionine.

A number of *N*-nitroso compounds have been shown to be carcinogenic in animal models. In the human stomach, these compounds can be formed from dietary nitrite (usually from preserved meat) or nitrate (usually from vegetables or drinking

water). Studies have consistently reported a negative association between dietary nitrate intake and gastric cancer. Case-control studies on the effects of nitrite intake have, in general, reported trends towards increased risk of gastric cancer in high nitrite consumers (non-significant), relative to low nitrite consumers [74, 75]. Studying one food group or micronutrient in isolation can lead to incomplete results, as demonstrated by Buiatti *et al.*[74], where a diet high in nitrite did *not* confer increased risk of gastric cancer where that diet was also high in antioxidants from fruit and vegetables.

It would seem that a specific risk has not been associated, consistently, with a specific food group or micronutrient. It is clear, however, that a diet high in fruit and vegetables may protect against gastric cancer[76], as well as a variety of other diseases.

The issue of diet is tied to socio-economic conditions. Studies in the UK and Europe have shown that maintaining a healthy diet can be expensive [77-79]. As such, nutrition, particularly early in life, is defined by socio-economics governing the variety and types of foods to which children are exposed. A Swedish study investigated the effects of unfavourable socio-economic conditions early in life, with development of gastric cancer subsequently. The study reported short height or a large number of siblings to be positively associated with subsequent cancer risk[80].

Smoking

The relationship between gastric cancer and smoking has been extensively studied, yet to date evidence of an association is

lacking. Most studies have reported a weak to moderate association.[39, 81-84]

It is important environmental factors involved in the development of gastric disease and gastric cancer, are not studied in isolation. It is more likely that lifestyle, as a whole, including dietary habits, socio-economics and social conditions, determines risk.

1.4.3 Pathogenicity: Host factors

> *"Napoleon Bonaparte's brother, two sisters, father and possibly father's sister died of stomach cancer. When Napoleon himself died...his official cause of death, supported by post-mortem examination, was perforated malignant gastric ulcer."*
>
> *-Julie Parsonnet[85](cites[86])*

As early as 1988, 5 years after Warren and Marshalls description of *H. pylori* and suggestion of its possible involvement in gastric disease, the possibility of a role for human genetics in the development of *H. pylori* related disease was being addressed. Pelayo Correa observed:

> *"It is entirely possible that the genetic makeup of the subject and the intensity or frequency of the individual components of environmental insults determine the pattern of phenotype changes."* [38]

One population study[39] (alluded to in 1.3.2, Gastric cancer and Correas cascade of gastric carcinogenesis) investigated native and migrant Japanese and noted a reduction in risk for development of gastric cancer (intestinal type) on emigration to Hawaii. Migration prompted a delay of approximately 15 years in the start of the age-specific incidence curve, after this the incidence curve of the Japanese-Hawaiians mirrors the curve of the native Japanese.

In 2000, Brenner *et al.*[87] concluded that, *"familial aggregation of stomach cancer may be explained at least partly by familial clustering of*

H. pylori infection." This study identified a higher prevalence of *H. pylori* infection among subjects with a parental history of gastric cancer (69%), than those with no such history (44%). The higher prevalence of *H. pylori* infection among first-degree relatives of patients with gastric cancer was explored and further strengthened in an independent, though concurrent study by El-Omar *et al.*[88]. El-Omars group studied a predominantly Scottish cohort and reiterated the findings of Brenner *et al.* The Scottish cohort consisted of 100 first-degree relatives of patients with non-cardia gastric cancer (n=40) and 100 healthy subjects with no family history of gastric cancer. The group assessed *H. pylori* status, gastric histology and gastric acid secretion. The study reported the prevalence of hypochlorhydria was significantly higher in the cancer relatives compared to the healthy controls (27% *versus* 3%). The presence of atrophy was also recorded and differed significantly between the groups: 34% in cancer relatives and 5% in controls. These differences in gastric histology and acid secretion were almost entirely confined to *H. pylori* infected individuals.

Together, the studies of El-Omar *et al.* and Brenner *et al.*, suggested that not only was the prevalence of *H. pylori* infection higher in first-degree relatives of gastric cancer patients, they also displayed significant changes in gastric histology and function.

It seemed that early stages of Correas cascade were present in relatives of gastric cancer patients. Understanding the mechanisms whereby these conditions presented themselves seemed an important line of enquiry in the search for better understanding gastric carcinogenesis.

Long-term proton pump inhibitor therapy and gastritis

Benzimidazole was developed in 1979, later becoming the drug omeprazole. Omeprazole is a potent proton pump inhibitor (PPI) and acts by inhibiting the enzyme H^+K^+ adenosine triphosphatase (ATPase) on the parietal cell to prevent acid secretion.

With the subsequent recognition of a role for *H. pylori* in gastric disease studies investigating the effect of omeprazole treatment on *H. pylori* gastritis began. While treatment with acid-suppressing omeprazole led to a decrease in bacterial load in the antrum, corpus gastritis appeared to worsen[89-91]. Acid suppression treatment appeared to have the affect of altering the pattern of gastritis from a typical, *H. pylori* antral predominant gastritis, to pan-gastritis incorporating the body of the stomach. Severity/degree of gastritis seemed also to be influenced by PPI use. Studies on the effect of maintenance doses of omeprazole on both *H. pylori* infected and uninfected individuals, have reported development of atrophic gastritis to be more frequent in individuals infected with the bacteria, relative to those without [44, 92].

This altered pattern of *H. pylori* infection is central to the parallel pathways of *H. pylori* related gastric disease outlined by the International Agency for Research on Cancer (alluded to in 1.3.2). Here the pattern of gastritis induced by infection can be either antrum or corpus predominant and accordingly can be associated with development of duodenal ulcer (in the former) or achlorhydria and gastric cancer, in the latter. El-Omar hypothesised that something inherent to infected subjects could determine the pattern of their gastritis and so determine the course of their disease.

Interleukin 1β

In 2000, the first study to tie acid suppression and inflammation, or development of gastritis, specifically to human genetics was published[93]. This came with the acknowledgement of Interleukin 1β (IL-1β) as a highly potent inhibitor of gastric acid secretion as well as a potent pro-inflammatory agent.

The proposal of a role for IL-1β in determining the severity of gastric disease in patients infected with *H. pylori* married Correas cascade of carcinogenesis through inflammation, to reports of increased incidence of atrophy and hypochlorhydria in relatives of gastric cancer patients.

Not surprisingly, IL-1β is upregulated in the presence of *H. pylori* along with other pro-inflammatory cytokines including IL-6, IL-8 and tumour necrosis factor α (TNF-α)[94]. The genes coding for IL-1β, IL-1α and the receptor antagonist, IL-1ra (IL-1B, IL-1A and IL-1RN, respectively), are located together on chromosome 2q, spanning a region of approximately 430-kilobases. A number of single base transitions, or SNPs (single nucleotide polymorphisms, further described in 5.1.2), have been identified within these genes.

El-Omars group hypothesised, based on preliminary data, that these SNPs influenced IL-1β production and sought *"to determine whether these polymorphisms are important with respect to the different outcomes of H. pylori infection..."* C-T base transitions at positions -31, -511 and +3954, within IL-1B (the gene coding for IL-1β), as well as an 86 base pair repeat in the IL-1RN gene (encoding the IL-1 receptor antagonist) were investigated. The group reported the carriage of the C allele at position -31 and allele 2(IL-1RN*2), of the 5 possible allelic variations in IL-1RN, were associated with

increased risk of developing a hypochlorhydric response to *H. pylori* and further developing gastric cancer. The investigation included a study into the possible effect of the polymorphisms with use of electrophoretic mobility shift assay (EMSA) technique. Briefly, this study demonstrated that LPS stimulation induced a fivefold increased in DNA binding on the IL-1B –31C oligonucleotide or essentially, that carriage of the C allele was associated with enhanced IL-1β production. IL-1RN*2 had already been associated with enhanced IL-1β production *in vitro.* The effects reported for the IL-1RN*2 genotype, as well as those illustrated in the EMSA work for the IL-1B –31C+ genotypes were assumed to lead to a high producing IL-1β phenotype. The effects of this 'high' IL-1β production were hypothesised to aid a pro-inflammatory response, designed to clear *H. pylori* infection, while concurrently resulting in acid suppression in the stomach, thus allowing the spread of infection and development of a pangastritis fed by pro-inflammatory cytokines. The group hypothesised that this acid suppression and gastritis might then progress to destruction of parietal cells and irreversible hypochlorhydria. It was estimated that up to 38% of gastric cancer were caused by the combination of the IL-1B –31C and IL-1RN*2 genotypes in the host.

Recent work has identified SNPs in other cytokines, including TNFα and IL-10, also believed to alter cytokine production levels [95]. These pro-inflammatory genotypes of TNFα and IL-10 have each been associated with more than doubling the risk of development of non-cardia gastric cancer[96]. Where these genotypes were found together found with previously identified 'risk' genotypes in IL-1B gene and IL-1RN gene, the associated

risk was found to be cumulative dependent on how many 'risk' genotypes were carried.

The El-Omar paper of 2000[93], was followed by a flurry of investigations into the IL-1B polymorphisms and a number of other inflammatory mediators and association with gastric and other cancers. Studies reporting associations with these pro-inflammatory genotypes have been enthusiastically greeted. They appear to identify specific factors accounting for part of the hereditary nature of gastric cancer. They also fit with Correas hypothesis of a cascade leading from initial infection and prolonged inflammatory response to the development of atrophy and neoplasia. The association, however, is by no means conclusive and some studies have reported no such association for these polymorphisms[97-103].

1.5 Aims and Objectives

1.5.1 General Objectives:

To investigate the frequency of a number of single nucleotide polymorphisms in a variety of genes involved in immune system modulation across a spectrum of *Helicobacter pylori* related gastric disease. Specifically groups under investigation will be:

(i) Patients with *Helicobacter pylori* related gastritis

(ii) Patients with *Helicobacter pylori* related intestinal metaplasia (including patients where gastritis and metaplasia coexist)

(iii) Patients with non-cardia gastric cancer

Results will be presented relative to a population with histologically proven 'normal' gastric mucosa and no evidence of *Helicobacter pylori* infection.

1.5.2 Specific Objectives:

The investigation of patients' inflammatory genetics is dependent on patient recruitment as well as the design and production of a number of allelic discrimination assays. As such the specific objectives of the study are as follows:

(i) The prospective recruitment of the following patient groups:

1. Patients with normal gastric mucosal histology and no evidence of *H. pylori* infection.
2. Patients with histologically proven *H. pylori* related chronic gastritis.
3. Patients with histologically proven intestinal metaplasia that have been shown to have evidence of current or recent *H. pylori* infection.

(ii) The retrospective recruitment of:

4. Patients with histologically proven non-cardia gastric adenocarcinoma.

(iii) Design of a patient database to include relevant patient background information including:

1. Medical/surgical history
2. Family history of gastrointestinal disease
3. Primary symptom complaint
4. Duration of current disease
5. Medication history
6. Previous/current laboratory medicine reports

(iv) Further use of serological enzyme linked immunosorbent assays in the classification of *H. pylori* infection and gastric mucosal atrophy status.

(v) To develop Taqman® allelic discrimination assays for the investigation of the following single nucleotide polymorphisms:

- IL-1B-31, -511 and +3954
- IL-1RN +2018
- TNF(A) -238 and -308
- IL-10 -1082, -819 and -592
- TLR4 +896 and +936

(vi) To investigate the frequency of these polymorphisms across the patient groups listed above and to further investigate the effect of confounding variables (age, gender, gastric mucosal atrophy status etc.) on any relative risk conferred by the above polymorphisms.

Chapter 2

General Materials and Methods

2.1 Screening

The study involved both prospective and retrospective patient recruitment at the Department of Gastroenterology, Adelaide and Meath Hospital, incorporating the National Childrens Hospital (AMNCH), Tallaght, Dublin 24 and the Department of Pathology, St. James's Hospital, Dublin 8 (in the case of those patients treated at the former Adelaide and Meath Hospitals, Dublin)

Ethics

The study was approved by the Joint Research Ethics Committee of the Federated Dublin Voluntary Hospitals and St James's Hospital, following submission of a detailed study protocol. Informed consent was obtained from all patients prospectively recruited at AMNCH.

Non-cardia gastric cancer patients were collected retrospectively from the archives of the Adelaide and Meath Hospitals. As these patients were deceased the study protocol followed the Royal College of Pathologists guidelines: *'Transitional guidelines to facilitate changes in procedures for handling 'surplus' and archival material from human biological samples'*[104].

2.1.1 Inclusion/Exclusion criteria

The foundation of the study was rigorous patient classification. A detailed Case Record Form (CRF) was completed for each patient screened (see Appendix I), detailing each patient's medical history, primary complaint at presentation, medication history, and family and social history.

Diagnosis

The endoscopists report for each patient was noted. This report was used in conjunction with that of the histopathologist in determining patient's diagnosis.

Only patients with gastric mucosal biopsy specimens and therefore histopathological diagnosis, from both antral and corpal gastric sites, were included in the study.

Concurrent disease

A detailed medical history for each patient was a condition of inclusion in the study. Patients with a concomitant inflammatory condition or disease previously associated with altered inflammatory response or cytokine levels were excluded from the study.

For this reason exclusion criteria were listed as follows:

- Inflammatory bowel disease
- Coeliac disease
- Rheumatoid arthritis
- Diabetes
- Renal disease/transplant
- Primary cancer other than gastric cancer
- Liver disease
- Hepatitis

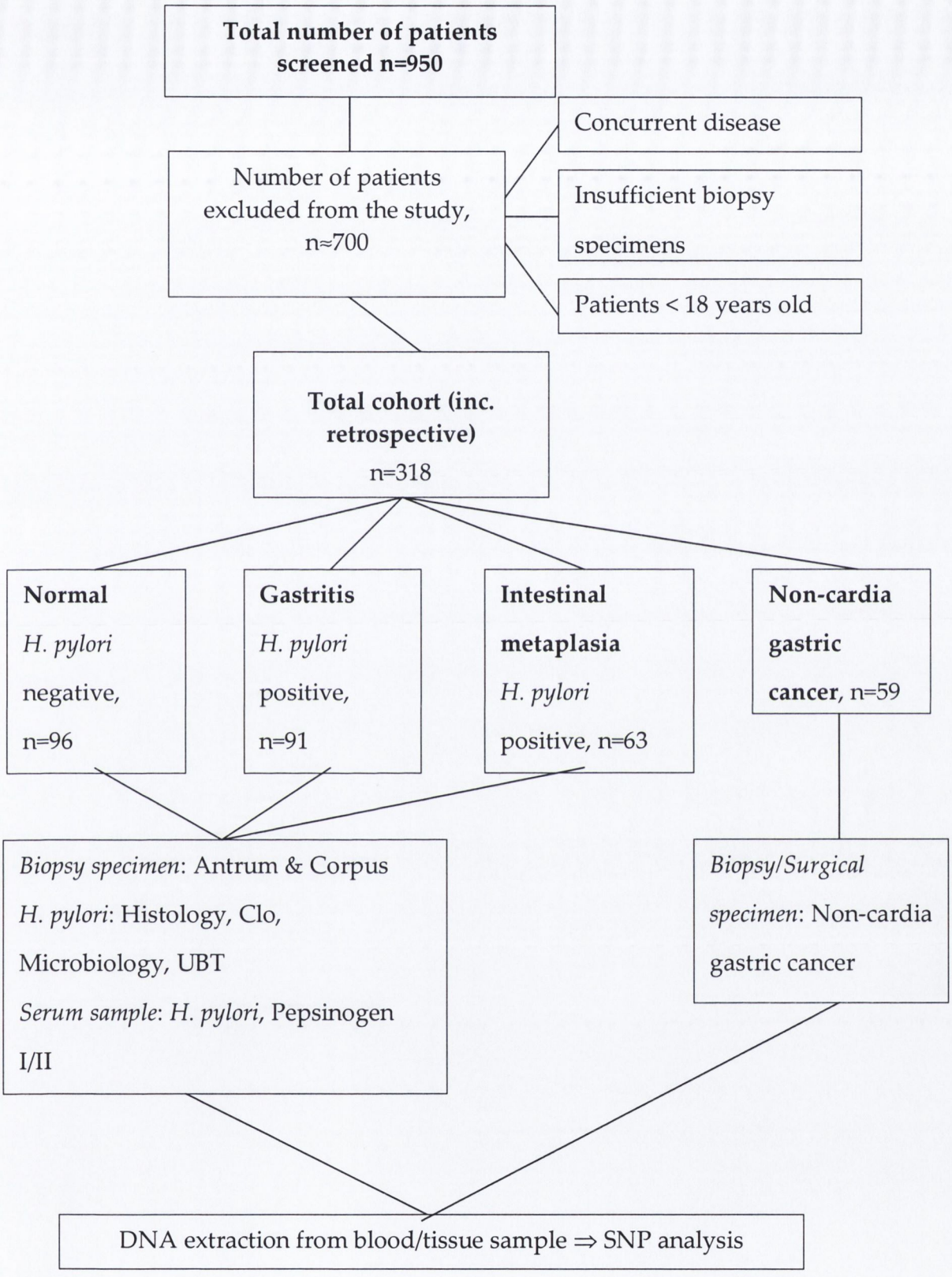


Figure 2.1: Outline of study design

2.2 Study Patients

Patients were classified so that each group was as homogenous as possible.

Study protocol and patient classification are illustrated in Figure 2.1.

Briefly, patients were first screened for suitability for the study. Those patients deemed eligible for inclusion were then classified according to gastric histology, *H. pylori* status and medical history (outlined in Table 2.1).

Further classification determined gastric mucosal atrophy status using enzyme linked immunosorbent assays (ELISAs) for the presence of Pepsinogen I and II (detailed in Section 2.6).

	H. pylori infection	Gastric Biopsy specimen	Blood/Serum	Medical History
Normal		√	√	√
Gastritis	√	√	√	√
Intestinal metaplasia	√	√	√	√
Gastric Cancer		√		

Table 2.1: Patient data and criteria for inclusion

2.2.1 'Normal' patient group

***Definition**: The absence of significant gastric inflammation defined the normal study group.*

- **Histology**: Diagnosis was made on the basis of haematoxylin and eosin (H & E) stained gastric mucosal biopsy specimens from both the antrum and corpus as well as the endoscopist's report.
- ***H. pylori* status**: The normal group were diagnosed *H. pylori* uninfected. This diagnosis of *H. pylori* status was based on the results of both previous and present histology, culture, rapid urease and urea breath test results. *H. pylori* serology (or indeed specific histological staining of gastric mucosal biopsy tissue) is not performed routinely at AMNCH but all serum samples from patients included in the study were tested serologically for the presence of *H. pylori* antibodies.

  Where serology results suggested *H. pylori* infection, regardless of the results of the other tests, the patient was considered to have had a previous *H. pylori* infection and was therefore deemed to be *H. pylori* positive and excluded from the normal control group.

2.2.2 *Gastritis study group*

> ***Definition**: Gastritis was identified by a pathologist at AMNCH according to the Sydney classification criteria[18] using Haematoxylin & Eosin stained gastric mucosal biopsy tissue.*

- **Histology**: All gastritis study group patients displayed chronic gastritis.

  In addition, patients found to have gastric lesions, benign or malignant (gastric ulcer, adenoma, intestinal metaplasia etc.), were excluded from the gastritis group. It was intended that the gastritis group consist only of those with a *"chronic gastritis, which appears to result directly from infection with Helicobacter pylori"*.

  Patients were identified as having mild, moderate or marked degrees of gastritis.

- ***H. pylori* status:** Each patient's current *H. pylori* status was determined according to standard diagnostic tests as outlined already (2.2.1, *H. pylori* Status). In addition to these routine tests, serology was carried out to investigate the possibility of previous *H. pylori* infection. Patients were included in the study where evidence was found to indicate either current or previous (according to serology) infection with *H. pylori*.

2.2.3 *Intestinal metaplasia study group*

***Definition**: Intestinal metaplasia was also identified by a pathologist at AMNCH, again using Haematoxylin & Eosin stained gastric mucosal biopsy tissue.*

- **Histology**: Both those showing extensive metaplasia throughout the stomach and those with focal metaplasia at specific gastric sites were included in the group.

  In general, IM was found against a backdrop of chronic gastritis. Any further gastric lesion/abnormality identified either by endoscopy or histopathologically meant exclusion from the group.
- ***H. pylori* status:** It has been observed that *H. pylori* may often be absent in patients with intestinal metaplasia, due to the progressive change in the milieu of the gastric mucosa. Serology was again carried out on all IM patients to assess the possibility of previous *H. pylori* infection.

2.2.4 Non-cardia gastric cancer study group

Patients with primary non-cardia gastric cancer were identified *retrospectively* from hospital records (using the laboratory medicine database at St James's Hospital, Dublin) as having previously attended Professor Colm O'Morain at either the Adelaide or Meath hospitals between 1986 and 1997.

- **Histology:** Slides of either gastric mucosal biopsy tissue taken at the time of endoscopy, or surgical tissue (generally taken during post-mortem) were selected from the archives at St James's Hospital, Dublin. The slides were then reviewed by a consultant pathologist (Dr. Niall Swan) at AMNCH, and the cancer typed, in so far as possible, according to Lauréns classification (Classification of Gastric Cancer, 1.3.1: Table 1.3). Where slides were no longer available, the original tissue blocks were sought, and fresh slides made and stained (GM).
- ***H. pylori* status:** In general, the *H. pylori* status of the non-cardia gastric cancer group was unknown. Investigations for *H. pylori* in these patients, as detailed in the patient records, were found to be rare. As these patients were deceased serological investigation of *H. pylori* status was impossible.

2.3 Methods of *Helicobacter pylori* detection

Determination of *H. pylori* status was intensive, and involved four main techniques as outlined below. Criteria for diagnosis using techniques described below are outlined in Table 2.2.

Histology

Histological determination of *H. pylori* infection was based on gastric mucosal biopsy tissue taken at the time of endoscopy. Where *H. pylori* was observed (by a pathologist) in H & E stained gastric mucosal biopsy tissue sections, results were expressed semi-quantitatively according to the Updated Sydney System [18].

Rapid Urease Test (Clo™)

Further use of urease, inherent to *H. pylori,* is made with the rapid urease test. On hydrolysis of urea the bacteria releases carbon dioxide and ammonia. The rapid urease test is an agar based pH indicator which changes colour with the increase in pH caused by the generation of ammonia. Clo™ tests are performed on all patients undergoing upper endoscopy.

Culture

Gastric mucosal biopsy specimen for bacterial culture are not always taken during endoscopy at AMNCH and where they are, culture is not always possible.

Serology

Serological detection of *H. pylori* IgGs is not performed routinely at AMNCH. Testing for possible previous, as well as current *H. pylori* infection, was an integral part of the study; as such, commercially available *enzyme linked immunosorbent assay* (ELISA)

kits were used to test the serum of all included patients for the presence of *H. pylori* IgGs (Biohit Plc., Helsinki, Finland, Appendix II).

As the cancer cohort was collected retrospectively from the archives, no serum samples were available for these patients and serology testing to determine *H. pylori* status of the gastric cancer patients was not possible.

Criteria for *H. pylori* diagnosis
• Where ELISA based serum testing for *H. pylori* IgG proved positive, in the absence of a positive result in any of the other tests, the patient was understood to have/have had *H. pylori* infection.
• Where serology was negative or borderline and the results of at least two other tests were positive, the patient was understood to be *H. pylori* positive.
• In order to be classified as *H. pylori* negative, the patient in question must have had serological testing for *H. pylori* IgGs, and this testing must have proven negative.

Table 2.2: Criteria used in the study for final classification of *H. pylori* status

2.4 Sample collection

A full blood sample was drawn into a BD Vacutainer™ (Preanalytical Systems, UK) tube, aliquoted, made anonymous with allocation of a study number and stored at -80°C. These samples were later used for extraction of DNA.

Serum samples were drawn into a Vacuette® (Z serum clot activator, Greiner bio-one, UK) tube and spun at 2000g for 8 minutes. Serum was then drawn off, aliquoted, and stored at -80°C. These samples were later used for *H. pylori* and Pepsinogen I and II serological assays.

2.4.1 DNA extraction from blood samples

DNA was extracted from whole blood samples using a QIAamp® DNA Mini Kit (Qiagen®, UK).

Briefly, 200µl whole blood was added to 20µl Protease, with 200µl lysis buffer (Buffer AL, Qiagen®, UK), and the mixture was incubated at 56°C for 10 minutes. 200µl ethanol was added and the mixture vortexed and added to a QIAamp Spin Column. The tube was then centrifuged at 8000 rpm for 1 minute, the filtrate was removed and 500µl Buffer AW1 (Qiagen®, UK) was added. The tube was spun again at 8000 rpm for 1 minute and the filtrate removed. 500µl of a second buffer (Buffer AW2, Qiagen®, UK) was added to the column and the tube centrifuged at 14000 rpm for 3 minutes. An additional step was included in the protocol at this stage, where the filtrate was removed and the tube spun again at 14000 rpm for 1 minute to remove residual buffer and then the filtrate was again discarded. Elution buffer (200µl Buffer AE, Qiagen®, UK) was added and the tube was incubated at room temperature for 5 minutes before being spun at 8000 rpm for 1 minute. The eluted DNA was then stored at -20°C.

2.4.2 *DNA extraction from formalin fixed paraffin embedded tissue*

A number of different techniques were investigated for optimal DNA extraction from formalin fixed paraffin embedded tissue. The technique that gave the best yields, and was used throughout the project, is outlined below.

DNA from cancer patients included in the study was extracted from archival gastric mucosal biopsy specimens and surgical specimens (gastrectomy or post-mortem tissue).

Three alternative methods of DNA extraction from formalin fixed, paraffin embedded (FFPE) tissue were investigated. The most successful method used a 'DNA Isolation Kit for Mouse Tail Tissue' (Gentra®, USA). Briefly, 10 x 0.5µm sections of FFPE tissue were cut on a microtome (Leica, Germany) and placed in 1.5 ml microcentrifuge tubes to which 300µl Xylene (Scarlau, Spain) was added. The tubes were then centrifuged at full speed (14000 rpm) for 3 minutes and the Xylene was then discarded. This procedure was repeated twice more. 300µl Ethanol (Aldrich, USA) was then added to the tissue, the tubes were vortexed for 5 minutes at room temperature, spun at 14000 rpm for 3 minutes, and the ethanol discarded. This procedure was then repeated. Cell lysis solution (300µl, Gentra®, USA) was then added to the tissue and the contents of the tube thoroughly mixed. 1.5µl Proteinase K (20mg/ml, Gentra®, USA) was added to the tissue, and each tube was inverted 25 times and left to incubate at 55°C for 24 hours. After 24 hours, a further 1.5µl of Proteinase K was added and the tubes returned to incubate at 55°C for a further 24 hours, after which the process was

repeated. The supernatant (containing the DNA) in each tube was then poured off to a 1.5ml microcentrifuge tube containing 300µl Isopropanol (Sigma-Aldrich, Germany) and 0.5µl Glycogen (20mg/ml, Roche Diagnostics Germany). The tubes were then inverted 50 times and centrifuged at 14,000 rpm for 5 minutes. Following centrifugation the supernatant was poured onto clean absorbent paper, 300µl 70% Ethanol was added and the tubes inverted. The tubes were then centrifuged again at 14,000 rpm for 1 minute and the ethanol poured off. Each tube was then left open to air dry at room temperature for 10-15 minutes. 20µl DNA Hydration Solution (Gentra®, USA) was then added and the DNA rehydrated by incubating at 65°C for 1 hour (the tubes were tapped intermittently to disperse the DNA). DNA was then stored at -20°C.

DNA 'quality' was assessed post-extraction with the use of 1% agarose gels (Figure 2.2), where extracted DNA was examined relative to a standard DNA ladder (Hind III ladder, Sigma Aldrich, UK).

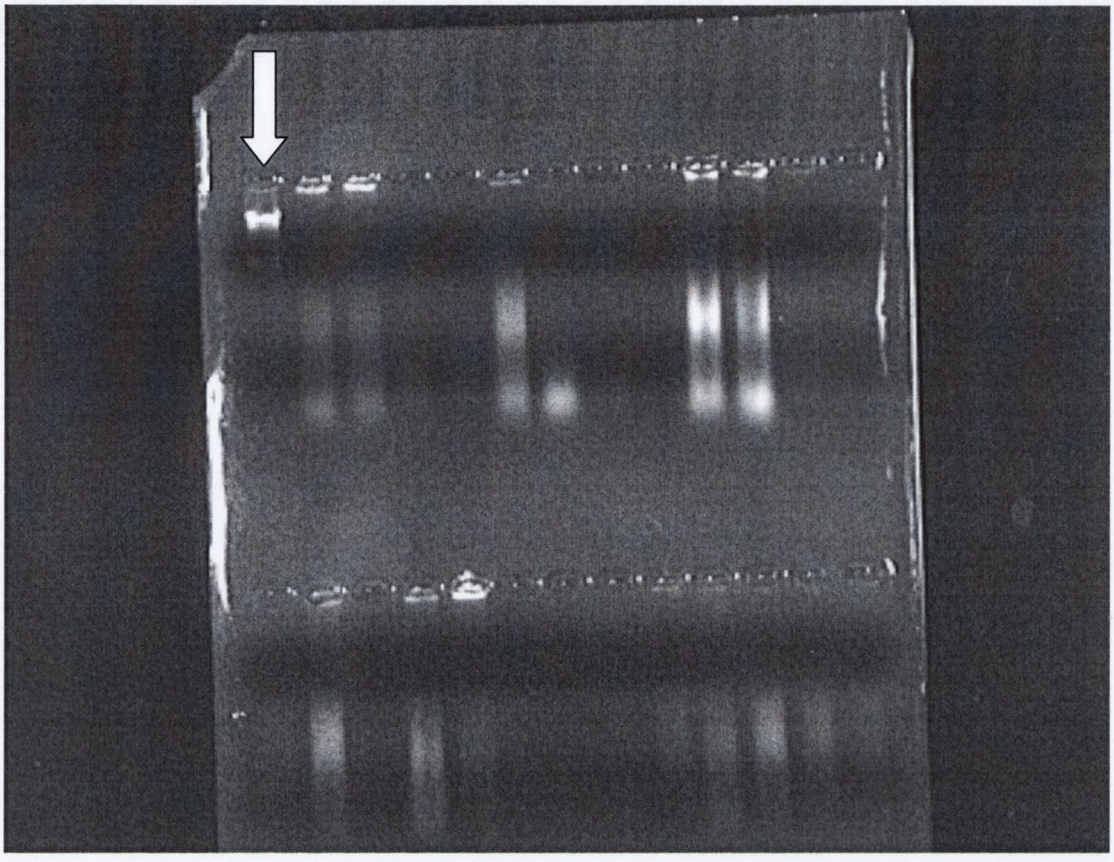


Figure 2.2: DNA extracted from formalin fixed paraffin embedded tissue visualised on a 1% agarose gel. DNA ladder shown was run in the first lane – top left hand side.

2.5 Taqman® Assay Development – Allelic Discrimination

Genotyping of the IL-1B -31, -511, +3954, TLR4 +896 and TNF -238 and -308 was performed using Taqman® technology at the Department of Molecular Medicine, Sir Patrick Dunns Laboratory, St. James's Hospital, Dublin.

2.5.1 Introduction to Taqman® technology

Taqman allelic discrimination assays combine PCR amplification and detection with 2 fluorogenic probes, each specific to each allele variant in a bi-allelic single nucleotide polymorphism (SNP; 5.1.2: Single nucleotide polymorphisms).

The assay is performed under competitive conditions where both probes are present in the same reaction tube. During the extension cycle of the PCR reaction, the DNA polymerase cuts the probe so that the quencher is released and the allele specific dye fluoresces (Figure 2.3).

This study used FAM and TET labelled fluorescent probes throughout.

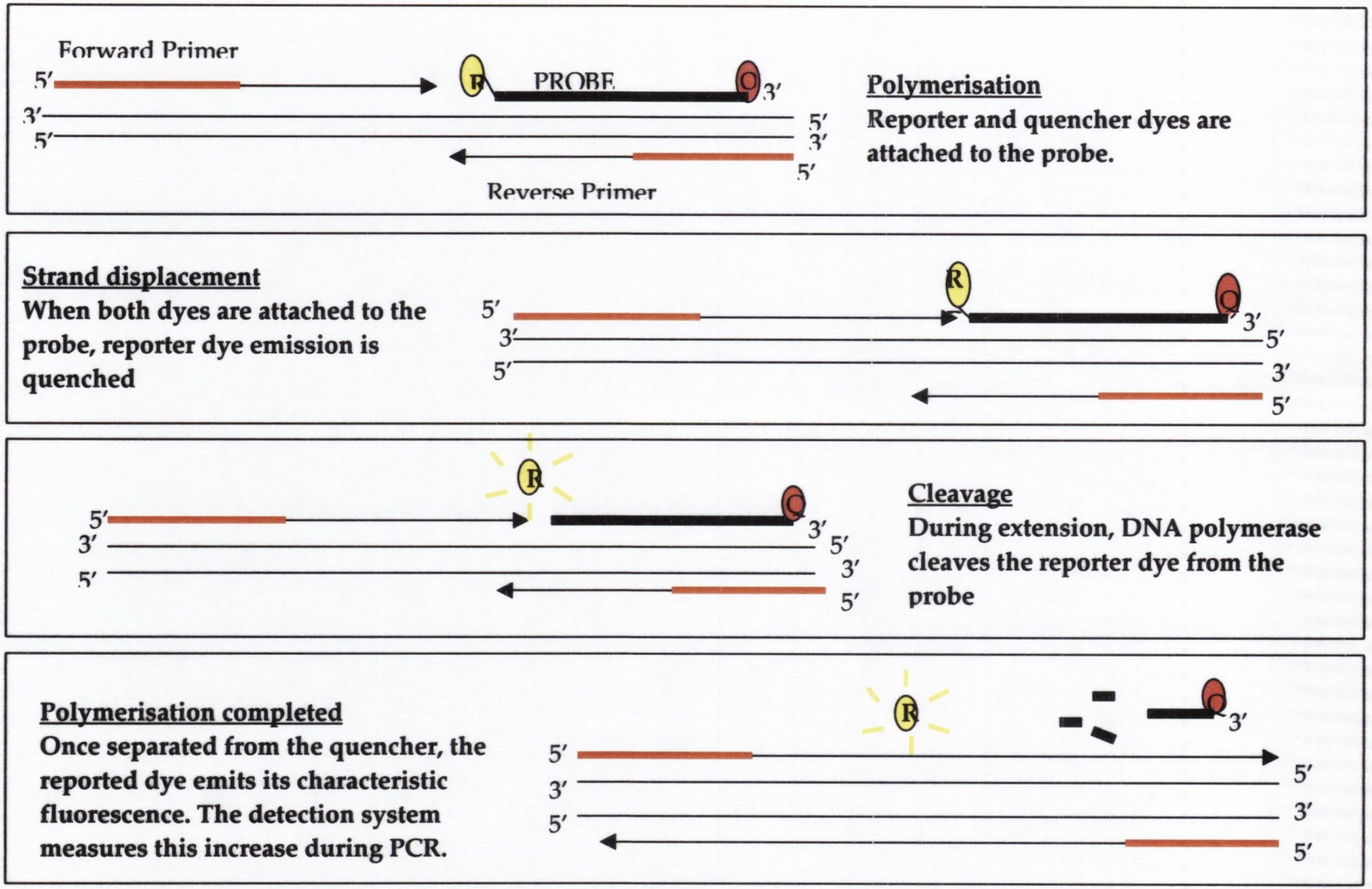


Figure 2.3: An outline of the stages of a Taqman® allelic discrimination assay. (Tamra=quencher in all assays)

2.5.2 Assay Optimisation

Assays were carried out on a 96-well reaction plate.

Each plate included 7 'No Template Controls' (NTCs) and at least 1 'No Amplification Control' (NAC). In the case of the NTC, sterile deionised water was substituted for DNA template to ensure no amplification in the absence of sample DNA.

The NAC used 0.25M SDS (Sigma-Aldrich, Germany) substituted for DNA template to check contamination in the components of the reaction cocktail.

Assay optimisation consists of two parts –probe and primer optimisation. Once the working concentrations of probes and primers have been ascertained the next step is the optimisation of thermal cycling conditions.

Probe Optimisation

The aim of this part of the protocol is to ascertain the probe concentrations for the assay, essentially the probe concentration where the fluorescence of each probe is approximately equal.

As it is the fluorescence of the probes alone that is under investigation, no thermal cycling is required at this stage and NTCs and NACs are not included in the plate.

Fluorescent probes (SGS, Sweden) were reconstituted to give a final concentration of 5µM. Once reconstituted the probes were aliquoted and stored, in darkness, at -20°C.

During the optimisation, the probe labelled, TET is kept at a concentration of 200 nM, while the concentration of the probe labelled FAM ranges from 50 to 200 nM.

The layout of the optimisation plate is shown below (Table 2.3).

Wells	Universal PCR Mastermix 2X (µl)	5µM FAM Probe (µl)	5µM TET Probe (µl)	Deionized Water (µl)	Total Volume per Well (µl)	Final FAM Probe Conc. (nM)	Final TET Probe Conc. (nM)
A1,2,3	6.25	0.125	0.5	5.625	12.5	50	50
A3,4,5	6.25	0.25	0.5	5.5	12.5	50	100
A6,7,8	6.25	0.375	0.5	5.375	12.5	50	150
A9,10,11	6.25	0.5	0.5	5.25	12.5	50	200

Table 2.3 Layout of 96-well plate for probe optimisation

Primer Optimisation

Primers (MWG Biotech, UK) were reconstituted in sterile water and adjusted to a working concentration of 10µM, aliquoted and stored at -20°C.

The aim of the optimisation is to determine the minimum primer concentration that gives the maximum R_n.

Unlike the probe optimisation step, both forward and reverse primer concentrations are altered (Table 2.4), so that each concentration of forward primer is investigated with the full range of reverse primer concentrations, and *vice versa*.

	Forward primer (nM)		
Reverse primer (nM)	50	300	900
50	50/50	50/300	50/900
300	300/50	300/300	300/900
900	900/50	900/300	900/900

Table 2.4: Forward and reverse primer concentration combinations for optimisation

Unlike probe optimisation, primer optimisation includes thermal cycling. 2µl of template (sample DNA) is added to each 10.5µl reaction and each reaction is run in multiples of 4 (ie., across 4 wells). 4 NTCs (2µl water) and 4 NACs (2µl 0.25M SDS) are also included in the plate.

In the case of IL-1B –511, a 96-well plate was set-up as illustrated in Table 2.5. Wells A1 to C12 are run with sample DNA, the remaining 8 wells (D1 – D8) are the NTCs and NACs.

Wells	Universal Mastermix (µl)	5µM FAM	5µM TET	Deionised Water (µl)	10µM Forward Primer (µl)	10µM Reverse Primer (µl)	Total volume per well (µl)
A1, 2, 3, 4	25	0.9	2	13.6	0.25	0.25	42
A5, 6, 7, 8	25	0.9	2	12.35	0.25	1.5	42
A9, 10, 11, 12	25	0.9	2	9.35	0.25	4.5	42
B1, 2, 3, 4	25	0.9	2	12.35	1.5	0.25	42
B5, 6, 7, 8	25	0.9	2	11.1	1.5	1.5	42
B9, 10, 11, 12	25	0.9	2	8.1	1.5	4.5	42
C1, 2, 3, 4	25	0.9	2	9.35	4.5	0.25	42
C5, 6, 7, 8	25	0.9	2	8.1	4.5	1.5	42
C9, 10, 11, 12	25	0.9	2	5.1	4.5	4.5	42
D1, 2, 3, 4	25	0.9	2	5.1	4.5	4.5	42
D5, 6, 7, 8	25	0.9	2	5.1	4.5	4.5	42

Table 2.5: Primer optimisation, cocktail recipe for each combination. Reactions run in multiples of 4.

Thermal cycling conditions

Cycling conditions varied for each assay, as detailed in further chapters.

2.6 Atrophy and Intestinal metaplasia typing

Atrophy was identified serologically using well-validated, commercially available ELISA kits for Pepsinogen I and Pepsinogen II (Biohit, Helsinki, Finland). It is reported that the values obtained in pepsinogen assays can predict the presence of intestinal metaplasia[105].

2.6.1 ELISA Assays for Pepsinogen I & II

Serum samples were defrosted and diluted in diluent buffer in line with the assay protocols (1 in 10 for Pepsinogen I, 1 in 5 for Pepsinogen II). All reactions were run in duplicate.

The same basic procedure was carried out for both assays with some differences in incubation time or temperature (Figure 2.4).

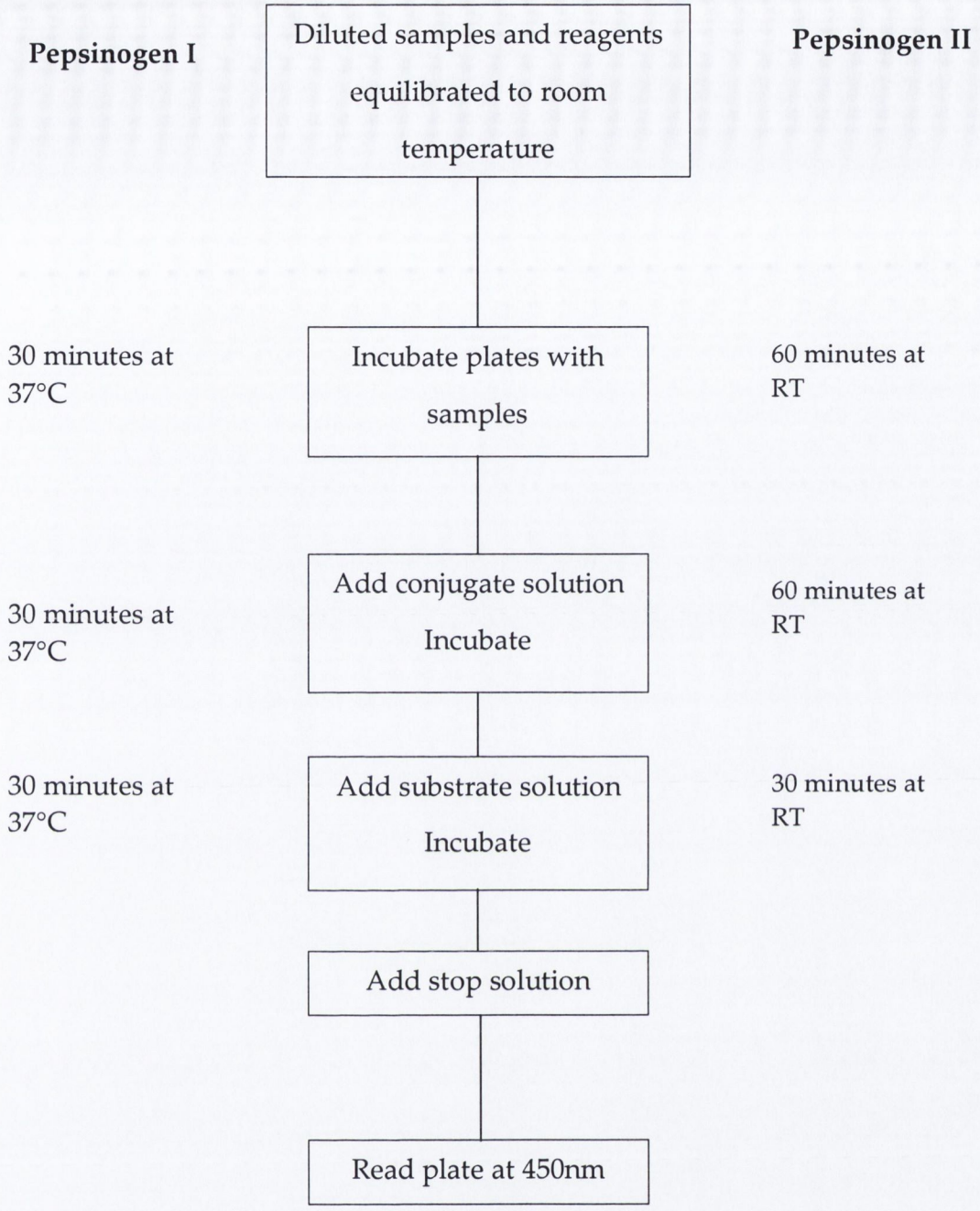


Figure 2.4: Outline of Pepsinogen assays used in the diagnosis of atrophy/intestinal metaplasia

Chapter 3

Patient Cohort

3.1 Introduction

"A very small fraction of infected subjects develop gastric cancer... This points to a major scientific challenge: what mechanisms lead to cancer in certain subjects and what mechanisms prevent the development of cancer in other chronically infected subjects. This conundrum leads us to the search for answers in the events that precede cancer development, namely the precancerous process."

Pelayo Correa, 2003[106]

The long lead-time of gastric cancer development allows certain windows of opportunity in the study of carcinogenesis. *H. pylori* infection, the single most important factor responsible for the development of gastric cancer, affects more than 50% of the world's population[107]. It seems reasonable to assume, given the risk of exposure, that a majority of people are exposed to *H. pylori*, yet only a relative minority present clinically significant symptoms. Bacterial factors are undoubtedly important - the virulence of the bacterial strain will influence the success of colonisation. Host genetics also play a part (as outlined in 1.4.3: Pathogenicity: Host factors). Increasing evidence suggests Correas cascade to be a viable model for the development of intestinal type non-cardia gastric cancer. The cascade suggests two particular points at which host genetics may influence increase of gastric cancer – firstly, whether the host

immune system will allow successful colonisation of *H. pylori* and secondly, whether the immune system is genetically predisposed towards 'overreacting', allowing an exaggerated chronic inflammatory response to *H. pylori* infection. This inflammation will in itself play a part in genetic and epigenetic changes involved in the molecular alterations underlying gastric carcinogenesis. Any genetic factors suspected of being involved in gastric carcinogenesis must therefore be investigated not only in the context of case (cancer)/control studies, but also across a wider spectrum of benign and pre-malignant gastric disease.

3.1.1 Specific Aims and Objectives

To our knowledge, there is no existing Irish cohort of patients with *H. pylori* associated gastric cancer and pre-malignant conditions such as intestinal metaplasia, and chronic gastritis.

The aim of this study was to build a cohort of patients with *H. pylori* related gastritis and intestinal metaplasia as well as non-cardia gastric cancer patients and normal controls. As such, it was necessary to:

I.) Retrospectively collect a non-cardia gastric cancer cohort (prospective collection would be unfeasible given the relatively low annual incidence of gastric cancer).

II.) Prospectively collect and classify patient groups including normal, *H. pylori* uninfected controls and patients with *H. pylori* associated gastritis and intestinal metaplasia.

3.2 Patients and Methods

3.2.1 Summary of methods

The process of screening and classifying screened patients is outlined in general materials and methods (2.1: Screening – 2.3: Methods of *Helicobacter pylori* detection). In summary:

- Patients attending endoscopy day clinic were recruited to the study (2.1: Screening).
- Demographic data, clinical details and symptom profiles were documented (2.2: Study Patients).
- Patient groups within the cohort were defined according to histopathology reports and *H. pylori* testing (2.2-2.3).
- Gastric cancer patients were collected retrospectively from the archives (2.2.4: Non-cardia gastric cancer study group). Slides, of either gastric mucosal biopsy, or surgical tissue, were collected from the archives. Where no slides were available, the original tissue blocks were sought, and new slides cut and stained. These slides were then reviewed by a consultant pathologist (Dr. Niall Swan) and the cancer typed.
- Serological *H. pylori* IgG status was tested, using an ELISA test based on an enzyme immunoassay technique. This consisted of a partially purified *H. pylori* bacterial antigen adsorbed on a microplate and a detection antibody labelled with horseradish peroxidase (HRP).

3.2.2 Statistics

Descriptive data was expressed as 'means ± standard deviation' or 'median' (interquartile range). Data was recorded in SPSS (Version 10.0 SPSS Inc.) and in JMP (JMP IN, Version 4.0.4, SAS Institute Inc.). Statistical analysis was performed using both SPSS and JMP packages, as appropriate.

3.3 Results

In all, 950 patients were screened for inclusion in the study. The final cohort included 250 patients who had been prospectively included in the study, as well as 68 patients recruited retrospectively through the archives.

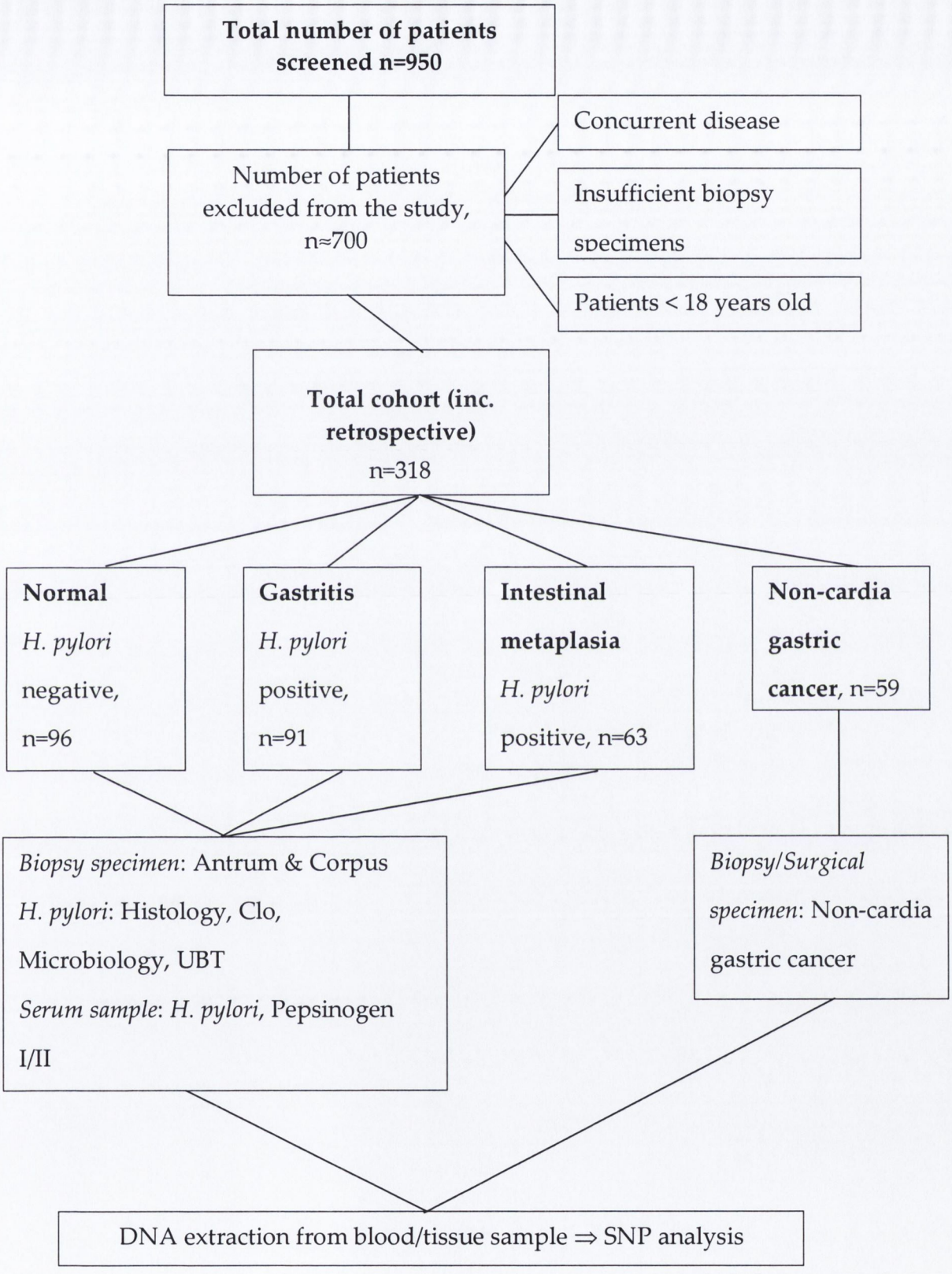
3.3.1 Disease and H. pylori status

The process of inclusion in the study is outlined in Figure 2.1 (Chapter 2). The final patient cohort consisted of:

Collected prospectively
- Histologically normal, *H. pylori* negative controls, n=96
- Chronic gastritis, *H. pylori* positive, n=91
- Intestinal metaplasia, *H. pylori* positive, n=63

Collected retrospectively
- Non-cardia gastric cancer, n=68

Patient demographics are further described in Table 3.1.



Repeat of Figure 2.1 (Chapter 2): Outline of final cohort

	Normal	Gastritis	IM	Gastric cancer
Number	96	91	63	68
Description	Normal histology, *H. pylori* negative	Chronic gastritis, *H. pylori* positive	Intestinal metaplasia, *H. pylori* positive	Non-cardia gastric cancer
Sex F:M	11:8	6:4	11:10	7:6
Age yr. Mean ± sd (range)	44 ± 17 (18-82)	47± 16 (18-90)	57± 13* (26-84)	68± 12* (38-92)
Primary complaint	Epigastric pain – 38%	Epigastric pain – 34%	Epigastric pain – 35%	N/A
Acid-secretory therapy	Yes: 29%	Yes: 34%	Yes: 29%	N/A

*$p < 0.05$: IM and gastric cancer significantly older than normal and gastritis groups

Table 3.1: Patient demographics

3.3.2 Histology

Gastritis

75 of the gastritis group (82%) were found to have *H. pylori* related pangastritis on endoscopy and subsequent gastric mucosal biopsy specimen examination. 10 patients showed gastritis confined to the antrum, 4 presented with corpus gastritis and 2 with gastritis of the incisura. All were defined by a histopathologist as having chronic gastritis. Assessing the severity or grade of gastritis (as determined by a histopathologist), the majority of patients with gastritis were found to have moderate gastritis. Median gastritis grade at each site, for all patients, is shown in Figure 3.1.

Intestinal metaplasia

Gastritis was also reported in patients with intestinal metaplasia. 46 (73%) of the patients with intestinal metaplasia were found to have a pangastritis, 14 (22%) had antral predominant gastritis and 1 patient had a gastritis confined to the corpus. 2 patients with intestinal metaplasia were reported as not displaying any significant inflammation.

Site and extent of metaplasia varied across the group. The majority of the group were reported to display a focal metaplasia (n=54) with 9 patients reported to show an extensive metaplasia across the stomach. Just over half of the group (n=33) displayed focal IM of the antrum. Focal IM was confined to the corpus in 4 patients and the incisura in 6. 11 patients showed focal metaplasia at more than 2 sites. The majority of patients with

intestinal metaplasia were shown to have a moderate degree of gastritis (Figure 3.1).

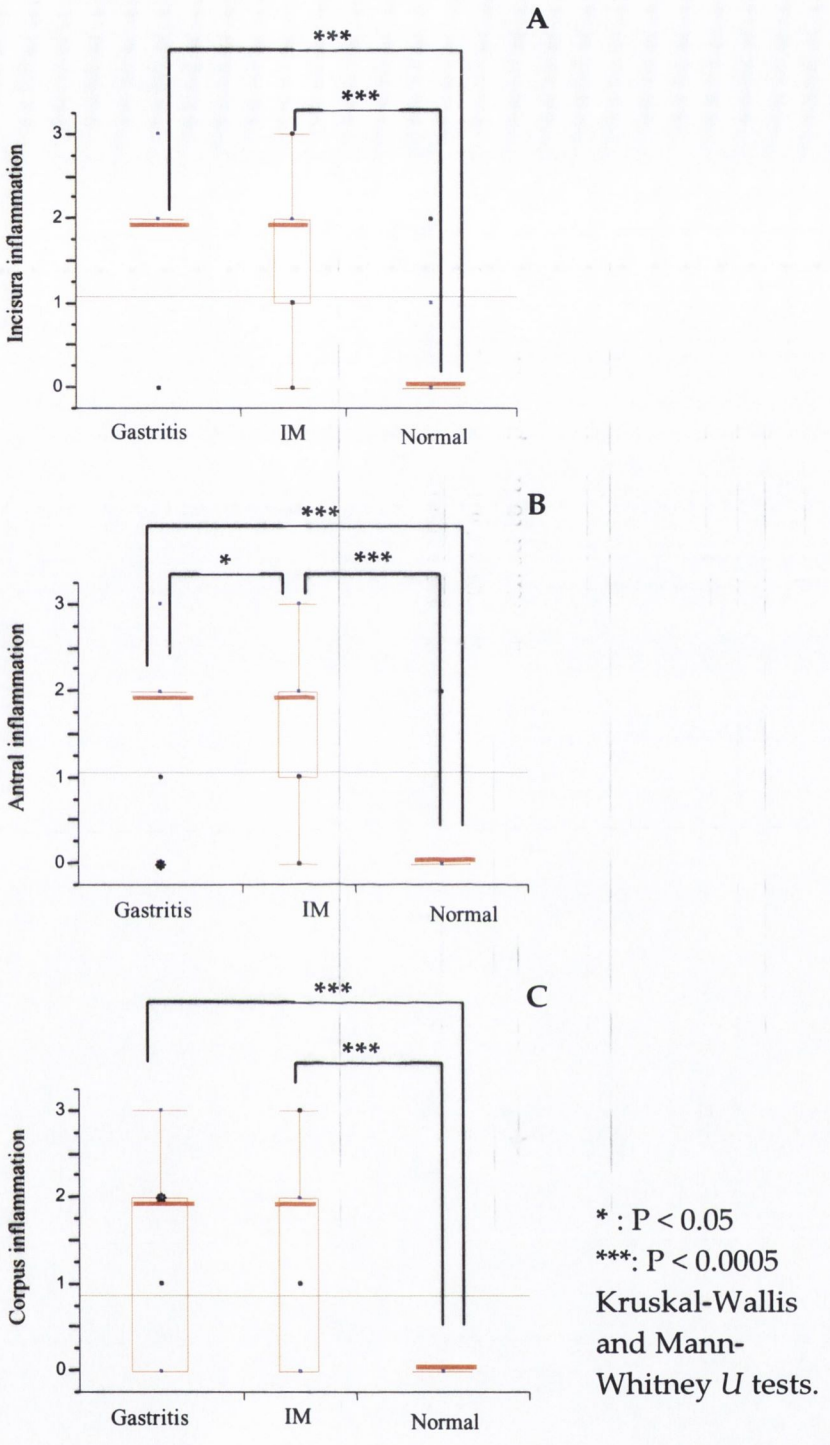


Figure 3.1: Median scores for (A) incisura, (B) antral and (C) corpus inflammation in *H. pylori* positive gastritis and IM patients and *H. pylori* negative controls. Data are expressed as median (bold double lines) with interquartile ranges (box: 25-75%, bars 10-90%).

Non-cardia gastric cancer

68 patients were identified through the archives as having attended the Gastroenterology Departments of the Adelaide or Meath Hospitals, and having been under the care of Professor O'Morain in the period between 1986 and 1997.

Full patient records were unavailable for these patients.

In all, 9 of the 68 patients tracked through the archives were excluded from the study. 1 patient was identified as having a neuro-endocrine tumour and 1 patient had a gastric lymphoma. In 4 cases, where the archives recorded a patient as having gastric cancer, no tumour was identified on the slides. These patients were also excluded from the study. In 3 cases no archival tissue could be found.

Typing of the available gastric mucosal biopsy or surgical tissue revealed a majority of the cancer patients had an intestinal type gastric cancer (n=29), 18 patients had a diffuse type cancer and 12 were of mixed cancer type.

Tumour grade and types are summarised in Table 3.2.

Tumour type	Tumour grade		
	G1	G2	G3
Intestinal	6	15	8
Diffuse	0	0	18
Mixed	0	5	7

Table 3.2: Tumour grade and histological type across 59 gastric cancer patients included in the final analysis.

3.3.3 Helicobacter pylori status

Accurate diagnosis of *H. pylori* status is a controversial point in many studies. For this reason we assessed the presence (or absence) of *H. pylori* using a variety of methods. The criteria used in the study for final classification of *H. pylori* status have already been outlined (Table 2.2). Test results for the prospective cohort are outlined below (Table 3.3).

	Normal		**Gastritis**		**IM**	
***H. pylori* status**	+	-	+	-	+	-
Rapid Urease test (Clo™)	3	79	63	20	31	26
Microbiology	1	76	33	38	18	28
Histology	1	93	68	20	47	11
Serology (BioHit)	0	96	58	26	37	21

Table 3.3: Classification of *H. pylori* status across patient cohort

3.3.4 Medical history

Full and detailed patient reports were only available for those patients collected prospectively.

Most patients were symptomatic on presentation to the day ward at the Gastroenterology Department, AMNCH, with the exception of 4 patients who were being screened due to family history of gastrointestinal cancer.

Patients reported a myriad of complaints. Epigastric pain was the single most frequent complaint overall, 88 (35%) of the prospectively collected patients (n=250) reported chest pain or epigastric pain as their primary complaint. This complaint was distributed evenly across the patient groups with 37.5% (35/96) of the control patients with normal histology, 34% (31/91) of gastritis patients and 35% (22/63) of IM patients reporting epigastric pain as their primary complaint.

28% (25/88) of patients reporting chest pain had no previous medical history to report.

Medication

Any medication use reported by the patient was noted. The number of patients reporting regular use of anti-secretory therapy (AST; proton pump inhibitors or Histamine H_2 receptor antagonists) was marginally higher in the gastritis group than the normal or IM group (34% in the gastritis group *versus* 29% in both the IM and normal group).

3.4 Discussion

We recruited over 950 patients to the study, over 300 of whom form the cohort for this study. This is the first such cohort, that we know of, in Ireland. A number of general observations can be made.

The IM and gastric cancer groups are significantly older than both the gastritis and control patient groups. This reflects the nature and progression of *H. pylori* infection. Severity of gastric pathology is related to duration of infection, therefore, it stands to reason that the groups with the most significant pathology are older than those at an early stage of infection and disease. Age as a confounding factor, however, must be considered in all subsequent studies of the population.

Extensive metaplasia is quite rare in the cohort (9/63), the majority of patients having a focal pattern of intestinal metaplasia. These focal areas arose on a background of pangastritis, supporting the model of IM developing as isolated foci, gradually fusing to form larger areas of metaplasia[22]. This IM cohort can, therefore, be said to be at a relatively early stage in the IM-atrophy-dysplasia-neoplasia cascade.

An equal proportion of each patient group reported usage of acid-secretory therapy (AST), either proton-pump inhibitors or H_2 antagonists. This showed no gender bias. It may also imply that the group described histologically as normal are symptomatic and may have functional dyspepsia. A third of the cohort were presenting with their first episode of gastric symptoms, reporting no previous medical history. Though all

gastritis patients were found to have histologically chronic gastritis, they may have been reporting significant symptoms for the first time (at a tertiary referral centre).

One of the benefits of prospective patient recruitment is that it allows an intensive process of testing for *H. pylori* infection status. This is a controversial issue and frequent caveat in cohort studies investigating the effect of infection on risk of cancer development[108, 109]. Our study tested for both present and previous infection, decreasing the likelihood of false-positive results, which may lead to underestimation of cancer risk.

Prospective recruitment of gastric cancer patients was impossible, due to the relatively low incidence of gastric cancer in Ireland. The National Cancer Registry of Ireland report an average annual incidence of 148 cases of stomach cancer in Dublin[32] (1994-1999 averages). The difficulties in defining, retrospectively, a cohort of non-cardia gastric cancer patients begin with a relatively low incidence of disease, and are compounded by difficulty in obtaining tissue for classification of cancer and the lack, or absence, of detailed patient charts. Though these are important issues in interpreting results of later genetic work, this is a significant group of patients and an important group to study.

This study resulted in the formation of a well-defined patient population for use in further studies of patient genetics and serum atrophy markers.

Chapter 4

Serum Pepsinogen and Patient Classification

4.1 Introduction

The need for intermediate end-points in the study of gastric cancer has already been outlined. The issue of which groups can be defined as 'pre-cancerous' is a controversial one.

Atrophic gastritis is characterised by significant loss of gastric glands and has been identified as a significant risk factor for gastric adenocarcinoma [27, 110-112]. Diagnosis of atrophy is a veritable minefield. In the words of Dr Genta: *"a diagnosis so loaded with significant prognostic implications should not be made lightly but, surprisingly, the histopathological criteria for atrophic gastritis have been and remain vague."*[21]

In terms of histopathological classification, the issue of biopsy error is compounded by the inflammatory response in the tissue obscuring the view of the gastric glands (- or lack there of) so that is virtually impossible to distinguish *invisibility* from *loss*. The Histopathology department at the Adelaide and Meath Hospital do not routinely report atrophy in their analysis of gastric mucosal biopsy tissue.

With a significant population of chronic gastritis and intestinal metasplasia patients included in the study, it was important to address the question of atrophic gastritis in these patients. Given the myriad of difficulties in histological diagnosis of atrophy increasing efforts are being made to develop serological based assays for the diagnosis of atrophic gastritis.

Pepsinogen I (PG I), is a precursor enzyme of pepsin and is synthesized by the chief cells and neck cells of the gastric corpus. The majority of PG I is secreted directly into the gastric lumen

but a small amount can be found in the blood. Serum levels of PG I reliably correlate with the number of chief cells in the gastric corpus mucosa. Pepsinogen II (PG II) is produced by the chief cells and mucous neck cells of the gastric mucosa, in pyloric glands in the antrum and Brunner's glands in the proximal duodenum.

Severe corpus inflammation and atrophy are associated with a marked reduction in the pepsinogen level (corresponding to the 'loss' of chief cells) and the PG I/PG II ratio and both have been used as surrogate markers of atrophy[113-115].

Commercially available ELISA kits for the measurement of serum levels of pepsinogen I and pepsinogen II (Pepsinogen I & II ELISA kits, Biohit Plc, Helsinki, Finland - part of the GastroPanel® test of Biohit Plc, Helsinki, Finland) were chosen, in an attempt to further classify our patient population as atrophic- or non-atrophic- gastritis patients.

4.1.2 Specific Aims and Objectives

- To identify a possible subpopulation of patients with atrophic gastritis amongst the intestinal metaplasia and chronic gastritis group through the use of serologically based ELISA assays whether our patient population included patients with atrophic gastritis
- To determine the serum levels of pepsinogen I and pepsinogen I/II ratio across the normal, gastritis (*H. pylori* positive) and intestinal metaplasia (*H. pylori* positive) groups and compare these to previously published levels in similar patient groups.
- To investigate the influence of possible confounding factors on serum pepsinogen levels.

4.2 Patients and Methods

4.2.1 Summary of methods

The process of screening and classifying screened patients is outlined in General Materials and Methods (2.1-2.3, as before). In summary:

- Patient groups within the cohort were defined according to histopathology reports and *H. pylori* testing (2.2-2.3).

- Atrophy was identified in the prospective cohort (2.6: Atrophy Typing) with the use of sandwich immunoassay technique with either a PG I or PG II specific capture antibody (monoclonal) absorbed on a microplate and a detection antibody labelled with horseradish peroxidase (HRP). The sensitivity and specificity of the commercially available PG I and PG II assays were 92% and 90%, respectively.

4.2.2 Statistics

Spearman's rank correlation coefficient (ρ) and Pearson's correlation coefficient (R) were calculated for the association of serum PG levels and age using SPSS software (Version 10.0 SPSS Inc.). Spearmans correlation was also used in investigating the relationship between grade or severity, of gastritis and serum PG levels.

Differences in serum PGs levels among patients groups were examined using the Kruskal-Wallis test, followed by Mann-Whitney U and Student's t test (JMP IN, Version 4.0.4, SAS Institute Inc.).

Regression analysis was performed using JMP (JMP IN, Version 4.0.4, SAS Institute Inc.). Odds ratios are reported with 95% confidence intervals.

4.3 Results

4.3.1 Serum Pepsinogen levels

Serum Pepsinogen and age

There were significant correlations between age at diagnosis and PG I (ρ = 0.17, $P < 0.05$, weak correlation) and PG II levels (ρ= 0.3013, $P < 0.0001$). An inverse correlation was found between PG I/PG II and age at diagnosis (ρ= -0.2583, $P < 0.0001$), in that with increasing age patients' serum PG I/PG II ratio declined steadily.

Serum Pepsinogen and gender

Gender differences in general, were confined to PG I levels with a significant difference (P=0.006) observed where the median for females was 90.49 µg/l (range, 68.4-133 µg/l) and 115.8 µg/l for males (range, 82.9-114.6 µg/l, Figure 4.1).

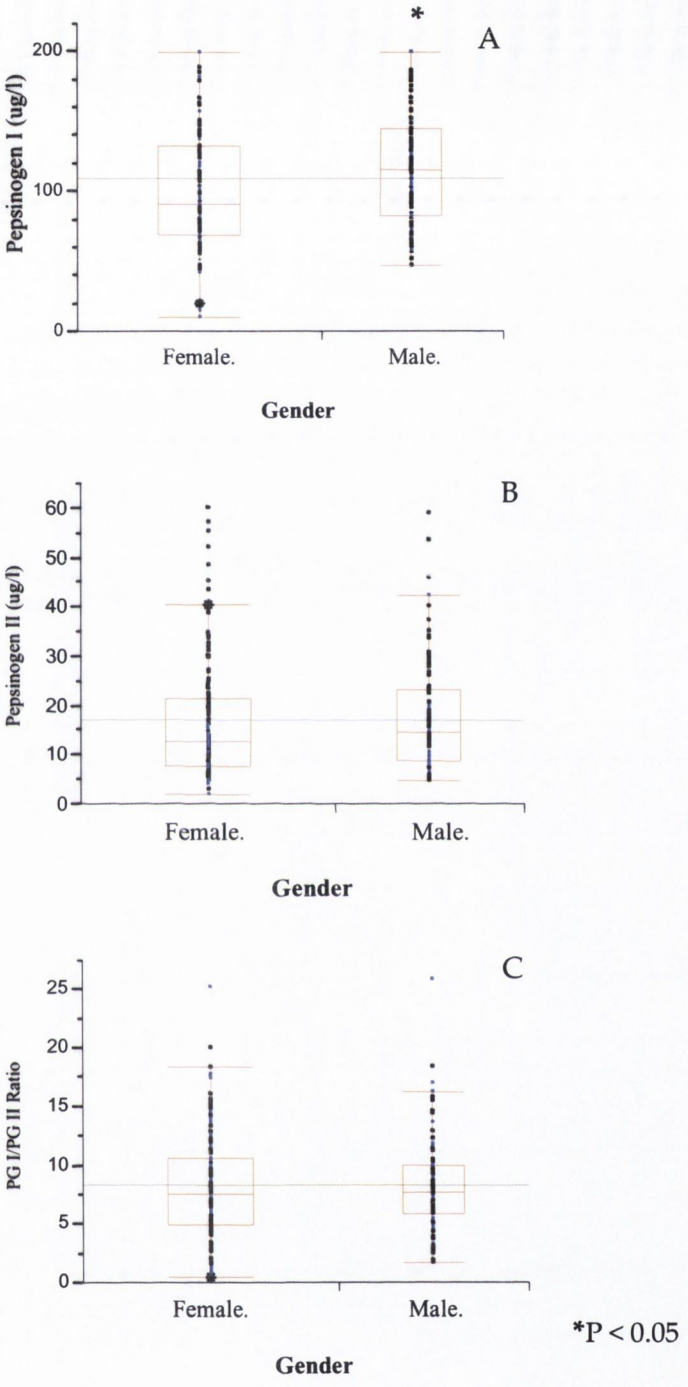


Figure 4.1: Serum PG I (A) and PG II (B) levels, as well as PG I/PG II ratios in male and female patients. Data are expressed as medians with interquartile ranges (box: 25-75%; bars: 10%-90%). Statistical significance was assessed by Student's t test.

4.3.2 Serum Pepsinogen levels and Helicobacter pylori infection

In general, significant differences were observed in PG I and PG II levels (and PG I/PG II ratios) between groups infected with *H. pylori* and those without infection (Figure 4.2). The median serum level of PG I in normal, *H. pylori* uninfected, controls was lower, at 92 µg/l (range, 68.6-122 µg/l) than that of the *H. pylori* infected group (112 µg/l; range, 79.1-147.4 µg/l). This difference was mirrored in serum PG II levels. The median serum PG I/PG II ratio was lowest in the *H. pylori* infected group at 7.1 (range, 4.9-9.2) compared to 9.5 (range, 6.3-12.3) in the uninfected, normal controls.

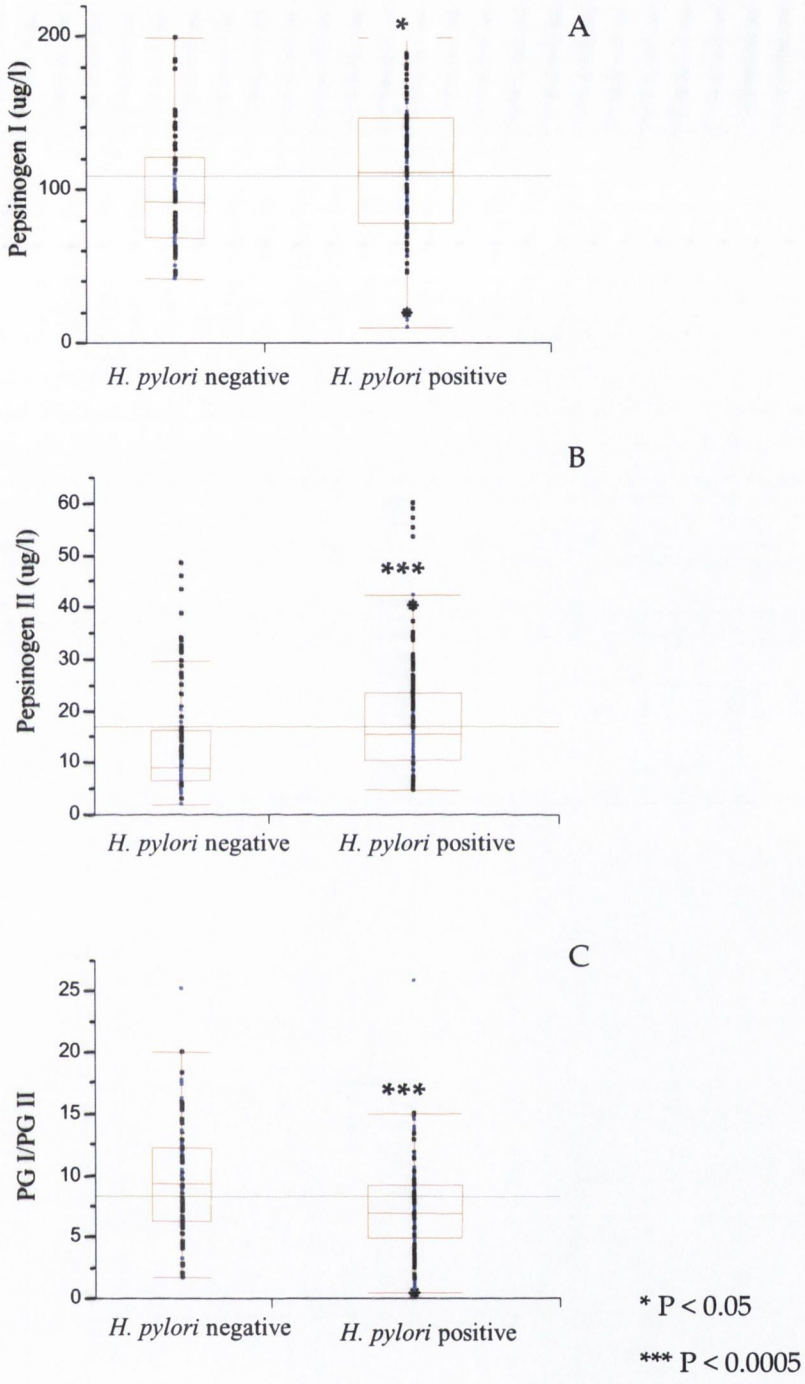


Figure 4.2: Serum PG I (A) and PG II (B) levels, as well as PG I/PG II ratios in *H. pylori* infected and uninfected groups. Data are expressed as medians with interquartile ranges (box: 25-75%; bars: 10%-90%). Statistical significance was assessed by Student's t test.

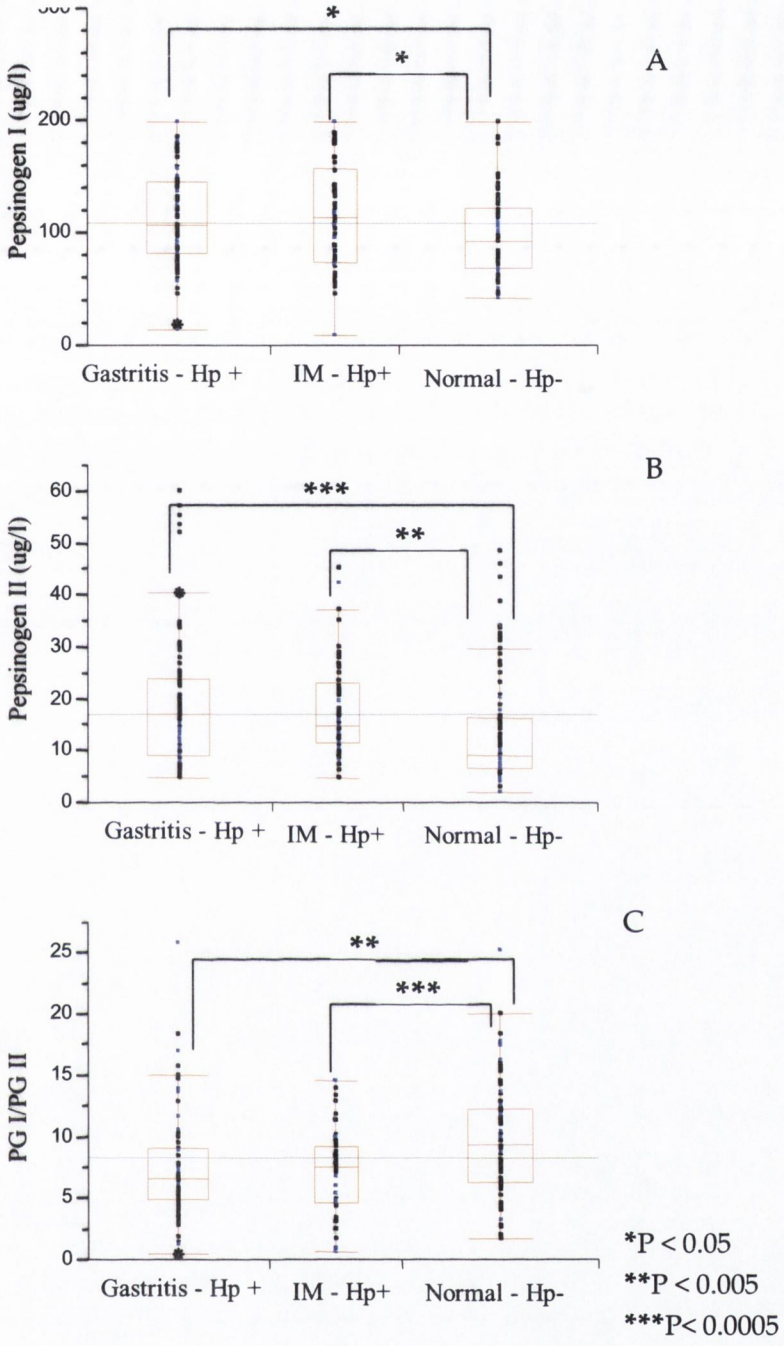


Figure 4.3: Serum PG I (A) and PG II (B) levels, as well as PG I/PG II ratios in *H. pylori* positive gastritis and intestinal metaplasia patients and *H. pylori* negative controls. Data expressed as before. Statistical significance was assessed by Student's t test.

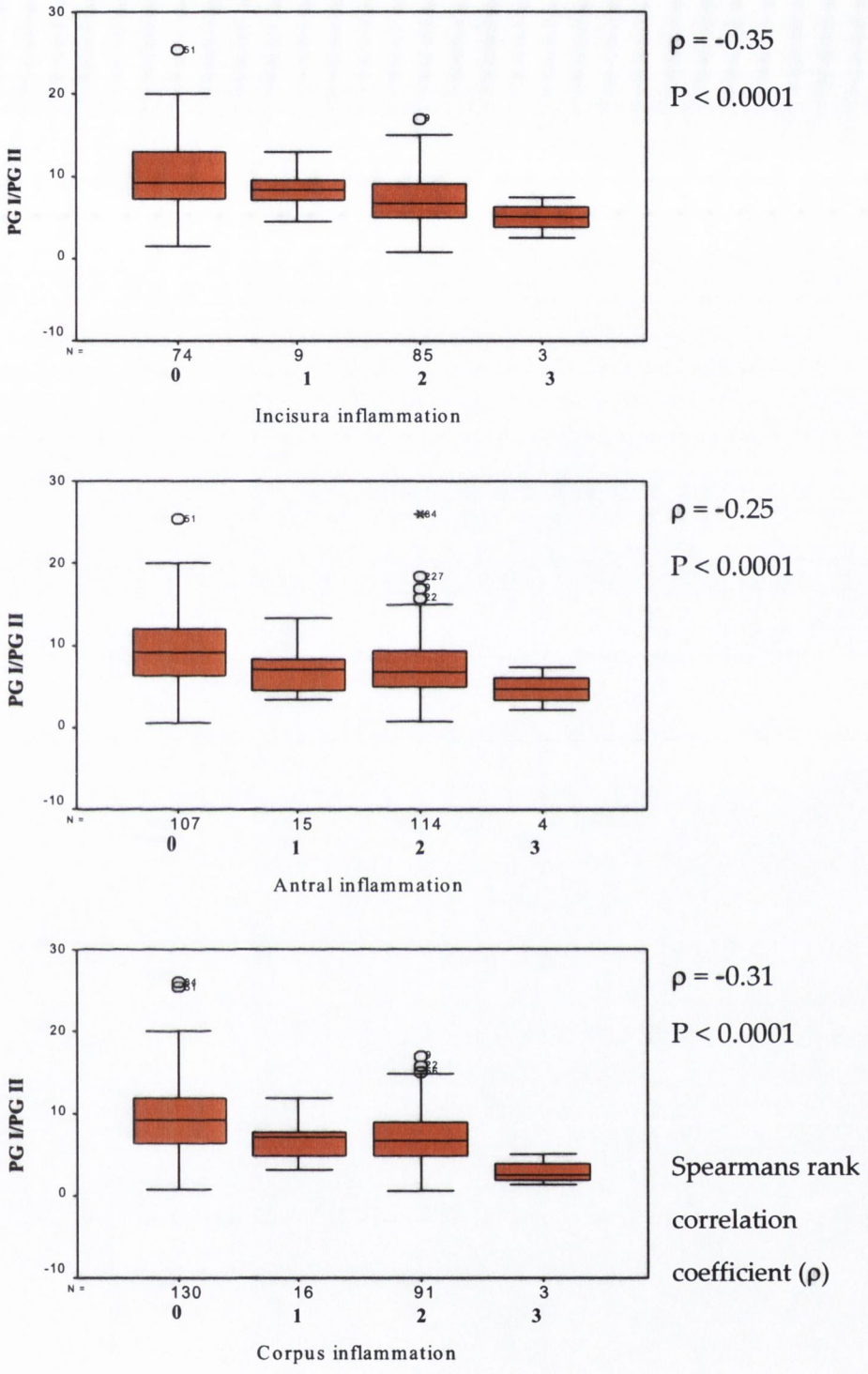


Figure 4.4: Correlation between PG I/PG II ratios and grades of antral and corpus inflammation in the *H.pylori* positive patients. Boxes represent medians and interquartile ranges.

Low serum PG I

Serum PG I is generally considered 'low' when it falls below 25 μg/l[116-120]. 5 patients of the 150 *H. pylori* positive patients were found to have serum PG I levels below 25 μg/l. Of these patients two were in the chronic gastritis patient group and 3 had focal intestinal metaplasia. Furthermore, none of those patients classified histopathologically as having 'extensive intestinal metaplasia' were considered to have 'low' serum PG I. The median serum PG I level for these patients was 73.8 μg/l (range, 66.5-106.8).

PG I/PG II Ratio < 3

A PG I/PG II ratio of below 3 is regarded as an indication of atrophic gastritis[121]. The number of patients with a PG I/PG II ratio below 3 was similar across patient groups – 6 patients with chronic gastritis, 7 with intestinal metaplasia (5 with focal IM, 2 with extensive IM) and 5 patients with histologically normal gastric mucosa were found to have a PG I/PG II ratio below 3.

4.3.4 Serum Pepsinogen levels and anti-secretory therapy

The effect of acid-secretory therapy on serum levels of Pepsinogen I and II and the Pepsinogen I/Pepsinogen II ratio was investigated. In general (ie., across the entire population), acid-secretory therapy was found to have a significant effect on serum PG I levels, with median PG I levels being significantly higher in those patients on therapy compared to those not taking AST (118.73 µg/l; range, 80-162.8 µg/l *versus* 95.3 µg/l; range, 71.1-129.2 µg/l, Figure 4.5). No significant difference was noted in the median PG II levels or PG I/PG II ratios. The lack of significance between PG I/PG II ratios, though the PG I association with AST was significant is probably due to the dilutary effect of PG II levels not being significantly different and therefore the ratio remains unaffected.

The effects of AST were then studied in each patient group, normal, gastritis and intestinal metaplasia. Any effect of AST on PG levels was confined to the intestinal metaplasia group where the median PG I/PG II ratio was significantly lower in patients reporting no AST usage (7.1; range, 4.6-8.6) compared to IM patients on AST (8.5; range, 7.5-10).

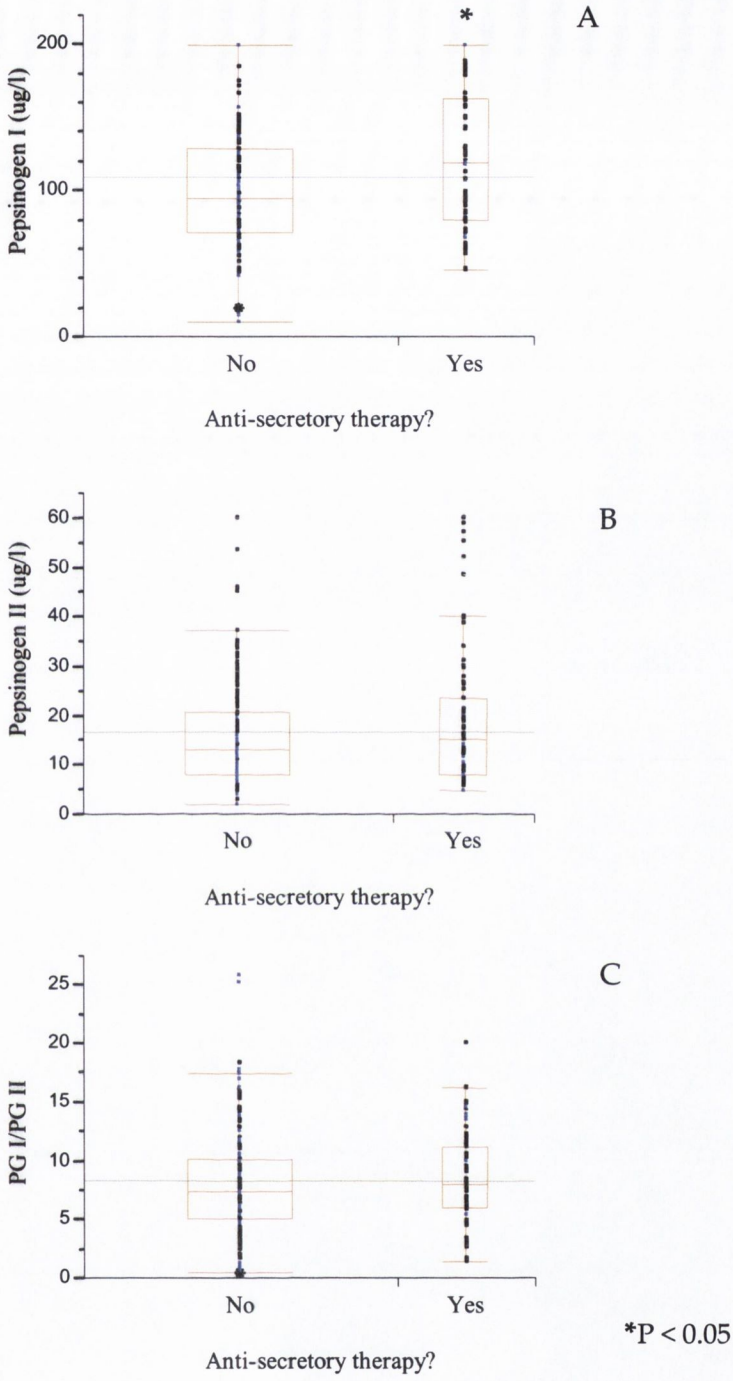


Figure 4.5: Serum PG I (A) and PG II (B) levels, as well as PG I/PG II ratios (C) in patients on anti-secretory therapy versus patients on no such therapy. Data expressed as medians with interquartile ranges. Statistical significance was assessed by Student's t test.

4.4 Discussion

The study aimed to investigate serum pepsinogen levels across the prospective patient cohort of *H. pylori* associated gastritis and intestinal metasplasia relative to normal controls.

In general serum PG I levels and PG I/PG II ratios reported in this study are higher than those generally reported in these patient groups[105, 122-125].

The median PG I/PG II ratio was highest in patients with no significant inflammation and no *H. pylori* infection where those with inflammation and concurrent *H. pylori* infection were significantly lower. Looking across patient groups median PG I/PG II ratios were highest in the normal control group followed by the IM group and the gastritis group had the lowest PG I/PG II ratio.

Numerous studies have reported increased levels of serum PG I in the presence of *H. pylori* infection and associated inflammation[113, 114], some studies have further shown a decrease in PG I levels on eradication of *H. pylori* [126-127]. Therefore, increased serum PG I levels in the gastritis group of the current study is not surprising. What is atypical, is that the median PG I level of the IM patient group was not significantly different to that of the gastritis group. Similarly the PG I/PG II ratio was lowest in the gastritis group rather than the IM group. It is likely that the IM patient group PG levels are shadowing those of the gastritis group due to the presence of *H. pylori* infection and the focal nature of metaplasia. Should the surface area of intestinal metaplasia increase and/or *H. pylori* infection disappear it can be

assumed, serum PG levels would fall significantly below those of the gastritis patient group.

Anti-secretory therapy seemed to have little to no effect on serum PG levels. Though PG I levels in those on AST were higher than those not on AST the PG I/PG II ratio did not reflect this difference in that PG ratios between those on AST and those who were not were similar.

In identifying patients with atrophy and intestinal metaplasia serologically, the results of this study are disappointing. Suggested diagnostic cut-off points of 25 μg/l for PG I levels and PG I/PG II ratio of less than 3 proved unhelpful, in this patient population. Only 2 of the 9 patients with histopathologically diagnosed 'extensive' metaplasia were found to have PGI/PG II ratio below 3. While failure to detect focal IM serologically might be expected, it is confusing that extensive changes could not be diagnosed serologically.

Given the absence of diagnostic identification of extensive intestinal metaplasia and atrophy, the usefulness of serum pepsinogen assays, in the context of this study, was questionable.

Chapter 5

Polymorphisms in Inflammatory Mediators

5.1 Introduction

5.1.1 Inflammation and carcinogenesis

Correas cascade presents a plausible progression from chronic *H. pylori* infection to development of pre-cancerous changes of the gastric mucosa and neoplasia. It can be assumed that any factor mediating the inflammatory process could influence both success of initial bacterial colonisation and subsequent gastric pathology. In such a highly regulated arrangement as the inflammatory system, the question remains: which factors might be of primary importance and how might these factors be influenced?

5.1.2 Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) make up about 90% of all naturally occurring variation in the human genome[128].

SNPs can be simply defined as *"single base pair positions in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals in some population(s), wherein the least frequent allele has an abundance of 1% or greater."*

Bi-allelic (or di-allelic) SNPs are by far the most common type of SNP, tri-allelic and tetra-allelic are possible but rare.

Bi-allelic SNPs

Bi-allelic SNPs comprise four distinct types: one transition C⇔T (G⇔A, pyrimidine for pyrimidine or purine for purine) and three transversions C⇔A (G⇔T), C⇔G (G⇔C) and T⇔A (A⇔T, pyrimidine is exchanged from purine and *vice versa*). The distribution of the four varieties is not equal, with approximately 2/3 of all SNPs being of the C⇔T (G⇔A) variety.

Coding and non-coding SNPs

The frequency of single base differences in genomic DNA is thought to be about once in every 1000 base pairs [129-131]. The effect of the SNP can be estimated according to its position in the gene. Those in the coding region of the gene may alter the resultant protein if the single base change alters the amino acid code.

In general, mutations in the non-coding DNA would not be expected to have a phenotypic effect unless they occur in the DNA sequences involved in gene regulation in the promoter region. Polymorphisms occurring in the promoter regions upstream of genes can affect the process of transcription. Transcription requires the co-ordinated action of multiple regulatory proteins through both protein-DNA and protein-protein interactions[132]. A single base pair variation in the DNA sequence of the region may alter the affinities of existing protein-DNA interactions or, indeed, recruit new proteins to bind to the DNA, altering the specificity and kinetics of the transcriptional process[133-135].

5.1.3 Polymorphisms in 'inflammatory' genes

A number of SNPs have been identified in genes encoding for inflammatory mediators.

Interleukin-1β and Interleukin-1ra

The pro-inflammatory cytokine Interleukin 1β (IL-1β) is emerging as a key mediator in the pathophysiology of host-environment interaction.

IL-1β has been shown to be upregulated in the presence of *H. pylori*[94]. As part of the inflammatory cascade IL-1β induces expression of many other genes including other pro-inflammatory cytokines such as tumour necrosis factor α (TNFα), IL-2, IL-6, IL-12 and cyclooxygenase 2 (COX-2). IL-1 is also a highly potent inhibitor of gastric acid secretion.

The most widely studied SNPs, in the field of *H. pylori* related gastric pathology, are those of the IL-1B gene, encoding the inflammatory mediator and acid-suppressor, IL-1β and a microsatellite repeat in IL-1RN encoding the endogenous receptor antagonist (IL-1ra) to IL-1β. (These studies have been briefly outlined in 1.4.3 Pathogenicity: Host factors).

The IL-1B and IL-1RN genes are located on chromosome 2q within a 430-kilobase region. Three di-allelic polymorphisms have been identified in the IL-1B gene[95]. All represent C-T base transitions. Two of the SNPs are located in the promoter region of the gene at -511 and -31 with the third SNP located downstream, in the coding region of the gene, at position +3954. The promoter region SNPs are in complete linkage disequilibrium (the -511T allele is always found with the -31C allele and likewise for the opposite alleles)[93].

These polymorphisms are thought to contribute to a high-producing IL-1β phenotype. The -511T allele, has been associated with slightly, but not significantly, increased IL-1β secretion from peripheral blood mononuclear cells[136].

The IL-1RN gene, encoding IL-1 receptor antagonist has a penta-allelic 86-bp variable number tandem repeat (VNTR) and Allele 2 (IL-1RN*2) of the 5 variants has been associated with enhanced IL-1β secretion[136-138]. This was supported in a recent study which also found that IL-1β mRNA levels, in the gastric mucosa were highest when IL-1B-511T and IL-1RN*2 were expressed together[97].

It has further been demonstrated that a SNP at position +2018 (a T/C transition) of the IL-1RN gene can be used as a marker for the presence of IL-1RN*2, in that allele 2 of the SNP (+2018C) is always found with allele 2 of the VNTR[139].

Tumour Necrosis Factor

Tumour necrosis factor α (TNFα), another pro-inflammatory cytokine, is also an inhibitor of gastric acid secretion, though not as potent as IL-1β. It is also upregulated in response to *H. pylori* infection and has a significant role in the pro-inflammatory cascade.

The TNF gene (or TNF-A) gene coding for TNFα is polymorphic at positions -308 and –238 in the promoter region of the gene. Furthermore, these polymorphisms (G/A transitions) have been shown to be associated with the outcome of several infections which have potential to chronically infect the host including hepatitis B infection, malaria and leishmaniasis[140] as well as Epstein-Barr virus associated gastric cancer [141]. The TNF -308A allele has been significantly related to infection with the *H. pylori* cagA subtype in Korean patients with gastric disease[142] as well as doubling the risk of non-cardia gastric cancer[96].

The function of these polymorphisms remains controversial with evidence for and against a subsequent effect on transcriptional activation/production of TNF upon expression of the variant alleles[97, 143-146].

Interleukin-10

The extent of the host inflammatory response to infection with *H. pylori* represents a balance of both pro- and anti-inflammatory agents. It is hypothesised that anti-inflammatory cytokines, such as Interleukin 10 (IL-10) play a significant role in the initial host response to bacterial infection and therefore, pathophysiology of subsequent disease.

Three bi-allelic polymorphisms have been described in the promoter region of the IL-10 gene at positions -1082 (G/A), -819 (C/T) and -592 (C/A). These polymorphisms were found to be in strong linkage disequilibrium and segregate predominantly as 3 (of a possible 8) haplotypes: GCC, ACC and ATA[147]. These haplotypes have been associated with altered IL-10 production: haplotype GCC has been associated with high, and ATA with low, IL-10 production capacity from in vitro stimulated peripheral blood mononuclear cells [147, 148]. The low producing (and therefore pro-inflammatory haplotype) has been previously associated with more than doubling the risk of non-cardia gastric cancer [96].

Toll-like Receptor-4 and success of *Helicobacter pylori* colonisation

Toll is a *Drosophila* gene, which plays an important role in combating the invasion of pathogens. Homologues of the *Drosophila* Toll have been identified in mammals and are now commonly referred to as the Toll-like receptor (TLR) family. The TLRs contain a cytoplasmic portion, which shows a high similarity to that of the IL-1 receptor family. This homologous region is termed the Toll/IL-1R (TIR) domain. Signalling pathways via the TLR family are also similar to those pathways via the IL-1 receptor family.

Common co-segregating missense mutations (Asp299Gly and Thr399Ile) have been identified which alter the extracellular domain of the receptor. Both polymorphisms have been associated, in humans, with a blunted response to inhaled lipopolysaccharide[149]. Given this association TLR4 could be involved in the initial host response to *H. pylori* and therefore, success of colonization. TLR4 mRNA and protein have been found in human gastric mucosal cells[150] and exposure to *H. pylori* LPS stimulated distinct TLR4 signalling. This suggests that TLR4-mediated intracellular signalling may play an important role in initial *H. pylori*-epithelial interactions, leading to the initiation of an inflammatory response by the gastric mucosa. As yet the TLR4 polymorphisms have not been studied in relation to *H. pylori* colonisation or related benign or malignant disease outcome in humans.

5.1.4 Hypothesis and aims

Our general hypothesis centred on the possible pro-inflammatory effect of this profile of polymorphisms. Together, these polymorphisms may form a pro- or anti- inflammatory profile and that this, in turn, controls:

- an individuals' response to *H. pylori* infection and could therefore determine success of *H. pylori* colonisation

- an individuals' cytokine polymorphism profile may determine the extent of the chronic inflammatory reaction to colonisation and therefore be influential in the inflammatory cascade and eventual development of non-cardia gastric cancer.

Given the long lead-time to development of gastric cancer it is logical to investigate these polymorphisms in as many pre-cancerous stages as possible – i.e. chronic gastritis and intestinal metaplasia.

Specific Aims and Objectives

I.) To develop Taqman allelic discrimination assays for the investigation of the following polymorphisms:

- IL-1B -31, -511, +3954 and IL-1RN+2018
- TNF -238 and -308
- IL-10 -1082, -819 and -592
- TLR4 +896 and +936

II.) To investigate these polymorphisms across a cohort of gastric cancer patients, patients with *H. pylori* positive intestinal metaplasia, patients with *H. pylori* positive gastritis as well as a *H. pylori* uninfected control population with no gastric pathology.

III.) To further investigate the effect of confounding variables (age, gender, serum pepsinogen levels) on any relative risk conferred by a 'pro-inflammatory' polymorphism profile.

5.2 Patients and Methods

5.2.1 Summary of Methods

Methods involved in patient recruitment are detailed in general materials and methods (Sections 2.1 – 2.3) and further outlined in Section 3.2.1.

Processes involved in DNA extraction from tissue and allelic discrimination assays are outlined in detail in 'Sample collection' (2.4) and 'Taqman assay development' (2.5). In summary:

- Histologically normal, *H. pylori* uninfected patients (n=96), patients with *H. pylori* associated gastritis (n=91) and intestinal metaplasia (n=63) were recruited prospectively (Sections 2.1-2.3)
- Non-cardia gastric cancer patients (n=59) were collected retrospectively through the archives of the Adelaide and Meath hospitals at St. James's Hospital, Dublin.
- DNA was obtained from blood samples, in the prospective cohort (Section 2.3.1) or from formalin fixed, paraffin embedded tissue in the case of the retrospective cohort (Section 2.3.2)
- Allelic discrimination assays were designed for identification of SNPs across patient groups (Section 2.5)

5.2.2 Specifics of Taqman Assay Design

Genotyping of the IL-1B -31, -511, +3954, TLR4 +896 , TNF -238 and -308 assays was carried out using an ABI Prism 720 at the Department of Molecular Medicine, Sir Patrick Dunns Laboratory, St. James's Hospital, Dublin. Further typing of the TLR4+936, IL-1RN+2018, IL-10-592, -819, -1082 and HH2R –1018, was performed by KBiosciences (Hertfordshire, EN11 OEX, UK; www.kbioscience.co.uk).

Design of primers and probes

Primers and probes were designed using sequences from online databases, Genbank[151] and SNP500Cancer[152]. Sequences used in the design of all assays are listed in Appendix VI. Primers and probes for in-house assays were designed using PrimerExpress®, Software (v2.0, Applied Biosystems, UK).

Assay development

Probe and primer optimisation was carried out for all inhouse designed assays (as outlined in 2.5.2 Assay Optimisation). This was followed by optimisation of the thermal cycling conditions for each assay.

Each plate for each assay was set up on a Perkin Elmer 9600 PCR machine under the following condtions:

IL-1B-31, -511, +3954 Assays

50°C – 2mins

95°C – 10mins

95°C for 15s }

62°C for 1 min } X 40 cycles

15°C Holding temperature

TNF-238 and -308 Assays

50°C – 2mins

95°C – 10mins

95°C for 15s }

58°C for 1 min } X 40 cycles

15°C Holding temperature

TLR4+896 Assay

50°C – 2mins

95°C – 10mins

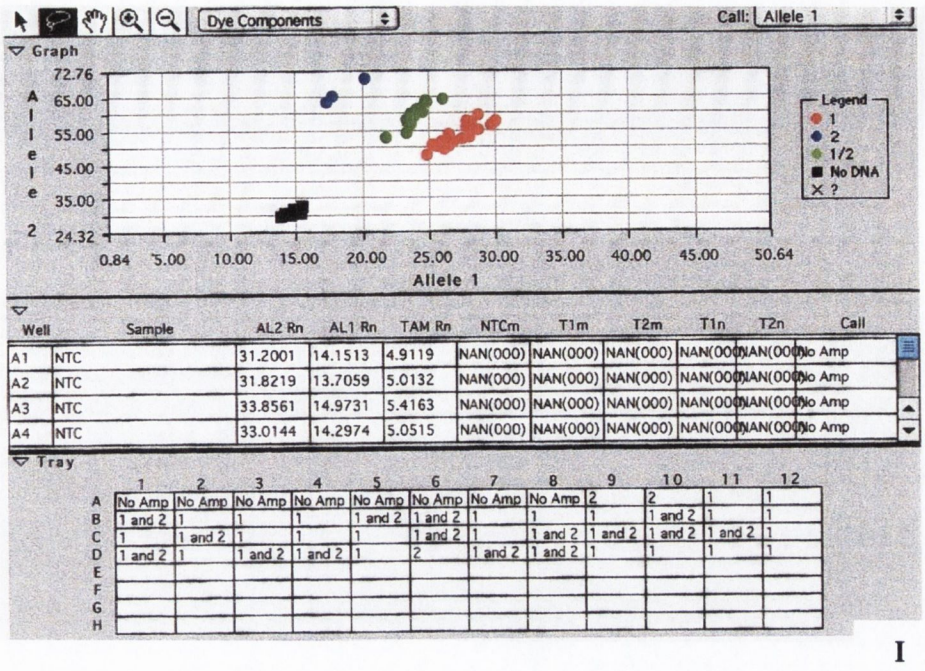
95°C for 15s }

62°C for 1 min } X 40 cycles

15°C Holding temperature

Specifics of assay cocktails are detailed in Appendix III.

Figures 5.1 and 5.2 illustrate the graphic produced by the ABI software after successful PCR amplification of the regions of interest. More common alleles are generally designated 'allele 1' and appear along the X axis, where the less common allele is referred to as allele 2 and appears along the 'Y' axis of the graphic.

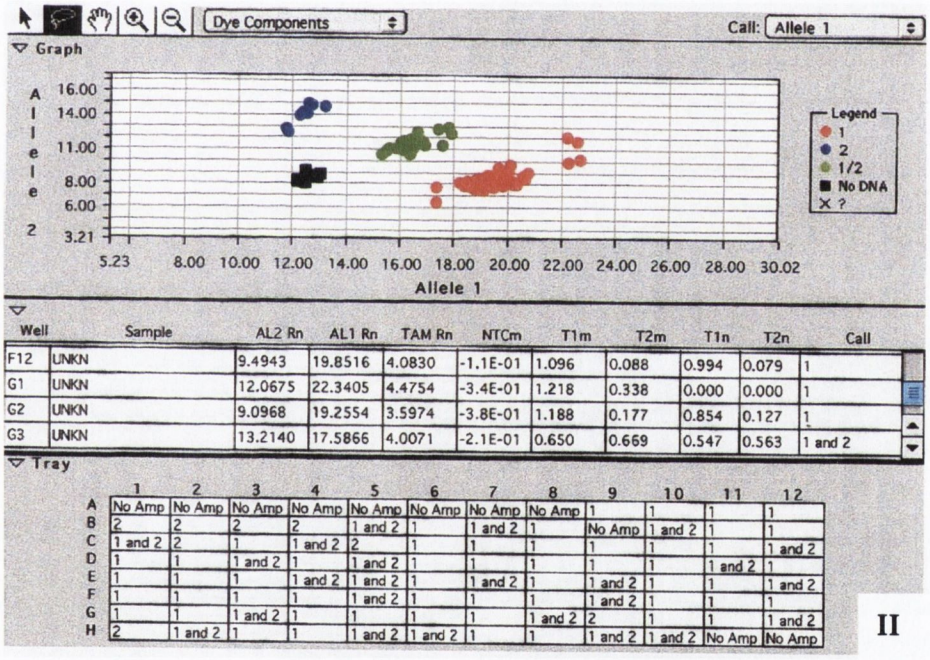


Well	Sample	AL2 Rn	AL1 Rn	TAM Rn	NTCm	T1m	T2m	T1n	T2n	Call
A1	NTC	31.2001	14.1513	4.9119	NAN(000)	NAN(000)	NAN(000)	NAN(000)	NAN(000)	No Amp
A2	NTC	31.8219	13.7059	5.0132	NAN(000)	NAN(000)	NAN(000)	NAN(000)	NAN(000)	No Amp
A3	NTC	33.8561	14.9731	5.4163	NAN(000)	NAN(000)	NAN(000)	NAN(000)	NAN(000)	No Amp
A4	NTC	33.0144	14.2974	5.0515	NAN(000)	NAN(000)	NAN(000)	NAN(000)	NAN(000)	No Amp

Tray

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	2	2	1	1
B	1 and 2	1	1	1	1 and 2	1 and 2	1	1	1	1 and 2	1	1
C	1	1 and 2	1	1	1	1 and 2	1	1 and 2	1 and 2	1 and 2	1 and 2	1
D	1 and 2	1	1 and 2	1 and 2	1	2	1 and 2	1 and 2	1	1	1	1
E												
F												
G												
H												

I



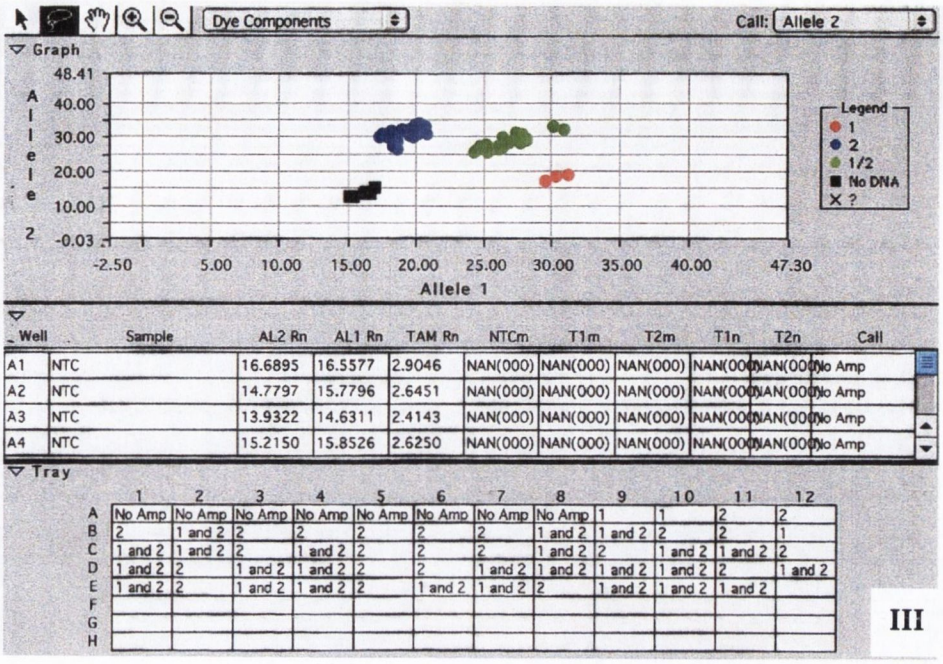
Well	Sample	AL2 Rn	AL1 Rn	TAM Rn	NTCm	T1m	T2m	T1n	T2n	Call
F12	UNKN	9.4943	19.8516	4.0830	-1.1E-01	1.096	0.088	0.994	0.079	1
G1	UNKN	12.0675	22.3405	4.4754	-3.4E-01	1.218	0.338	0.000	0.000	1
G2	UNKN	9.0968	19.2554	3.5974	-3.8E-01	1.188	0.177	0.854	0.127	1
G3	UNKN	13.2140	17.5866	4.0071	-2.1E-01	0.650	0.669	0.547	0.563	1 and 2

Tray

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	1	1	1	1
B	2	2	2	2	1 and 2	1	1 and 2	1	No Amp	1 and 2	1	1
C	1 and 2	2	1	1 and 2	2	1	1	1	1	1	1	1 and 2
D	1	1	1 and 2	1	1 and 2	1	1	1	1	1	1 and 2	1
E	1	1	1	1 and 2	1 and 2	1	1 and 2	1	1 and 2	1	1	1 and 2
F	1	1	1	1	1 and 2	1	1	1	1 and 2	1	1	1
G	1	1	1 and 2	1	1	1	1	1 and 2	2	1	1	1 and 2
H	2	1 and 2	1	1	1 and 2	1 and 2	1	1	1 and 2	1 and 2	No Amp	No Amp

II

Figure 5.1: Graphical representation of Taqman allelic discrimination assays for IL-1+3954 (I) and TNF-308 (II). 96 well plates were read using PE Applied Biosystems SDS 7200 System.

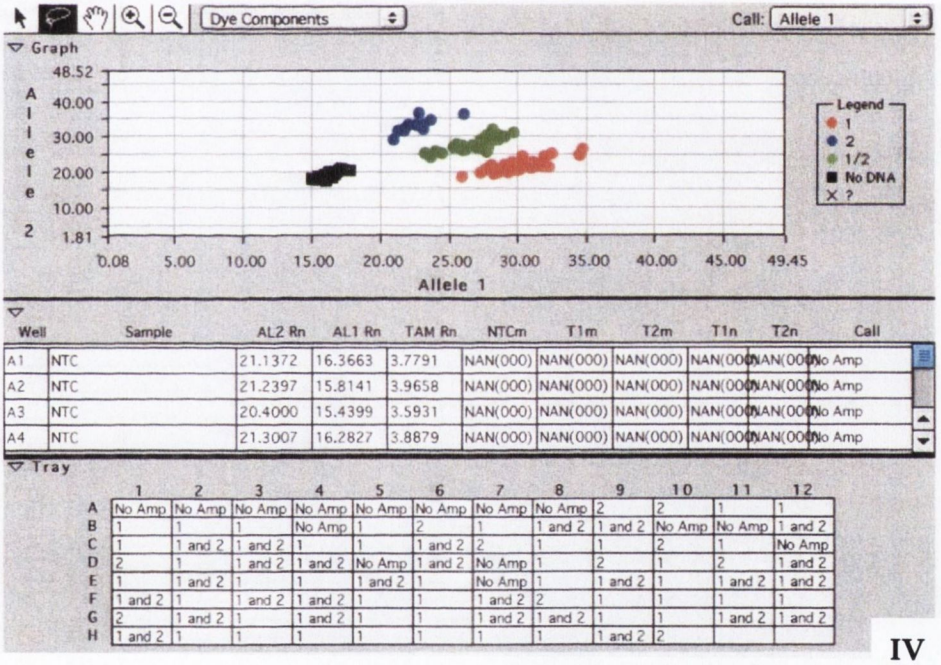


Well	Sample	AL2 Rn	AL1 Rn	TAM Rn	NTCm	T1m	T2m	T1n	T2n	Call
A1	NTC	16.6895	16.5577	2.9046	NAN(000)	NAN(000)	NAN(000)	NAN(000)	NAN(000)	No Amp
A2	NTC	14.7797	15.7796	2.6451	NAN(000)	NAN(000)	NAN(000)	NAN(000)	NAN(000)	No Amp
A3	NTC	13.9322	14.6311	2.4143	NAN(000)	NAN(000)	NAN(000)	NAN(000)	NAN(000)	No Amp
A4	NTC	15.2150	15.8526	2.6250	NAN(000)	NAN(000)	NAN(000)	NAN(000)	NAN(000)	No Amp

Tray

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	1	1	2	2
B	2	1 and 2	2	2	2	2	2	1 and 2	1 and 2	2	2	1
C	1 and 2	1 and 2	2	1 and 2	2	2	2	1 and 2	2	1 and 2	1 and 2	2
D	1 and 2	2	1 and 2	1 and 2	2	2	1 and 2	1 and 2	1 and 2	1 and 2	2	1 and 2
E	1 and 2	2	1 and 2	1 and 2	2	1 and 2	1 and 2	2	1 and 2	1 and 2	1 and 2	
F												
G												
H												

III



Well	Sample	AL2 Rn	AL1 Rn	TAM Rn	NTCm	T1m	T2m	T1n	T2n	Call
A1	NTC	21.1372	16.3663	3.7791	NAN(000)	NAN(000)	NAN(000)	NAN(000)	NAN(000)	No Amp
A2	NTC	21.2397	15.8141	3.9658	NAN(000)	NAN(000)	NAN(000)	NAN(000)	NAN(000)	No Amp
A3	NTC	20.4000	15.4399	3.5931	NAN(000)	NAN(000)	NAN(000)	NAN(000)	NAN(000)	No Amp
A4	NTC	21.3007	16.2827	3.8879	NAN(000)	NAN(000)	NAN(000)	NAN(000)	NAN(000)	No Amp

Tray

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	2	2	1	1
B	1	1	1	No Amp	1	2	1	1 and 2	1 and 2	No Amp	No Amp	1 and 2
C	1	1 and 2	1 and 2	1	1	1 and 2	2	1	1	2	1	No Amp
D	2	1	1 and 2	1 and 2	No Amp	1 and 2	No Amp	1	2	1	2	1 and 2
E	1	1 and 2	1	1	1 and 2	1	No Amp	1	1 and 2	1	1 and 2	1 and 2
F	1 and 2	1	1 and 2	1 and 2	1	1	1 and 2	2	1	1	1	1
G	2	1 and 2	1	1 and 2	1	1	1 and 2	1 and 2	1	1	1 and 2	1 and 2
H	1 and 2	1	1	1	1	1	1	1	1 and 2	2		

IV

Figure 5.2: Graphical representation of Taqman allelic discrimination assays for IL-1-31 (III) and IL-1-511 (IV). 96 well plates were read using PE Applied Biosystems SDS 7200 System.

5.2.3 Statistics

Primary Analysis

Distribution of allele and genotype frequencies across patients groups was performed using JMP (JMP IN, Version 4.0.4, SAS Institute Inc.), SPSS (Version 10.0 SPSS Inc.) and Genepop packages (Genepop Online: http://wbiomed.curtin.edu.au/genepop/genepop_op2.html).

Testing for Hardy-Weinberg equilibrium was performed using Genepop. Pearsons χ^2 reported were calculated by SPSS and in agreement with Fisher's method χ^2 values reported by Genepop.

Haplotype analysis was performed using PHASE 2.1.1 (web-based version, PHASE software for haplotype estimation, Washington, USA[153, 154]).

Secondary Analysis

Logistic regression was performed using the JMP software package and STATA software (STATA v8.2, STATA Press). Odd ratios are reported with 95% confidence intervals. All odds ratios were adjusted for the effects of age using age both as a continuous model and as 10-year intervals. Adjustments for gender were made concurrently.

5.3 Results

5.3.1 Primary Analysis

Preliminary, or primary analysis considered allelic and genotypic frequency and distribution across the patient population. Its purpose was to identify any obvious bias in genotype/allele distribution and to check Hardy-Weinberg equilibrium for all alleles.

Control Allele and Genotype Frequencies

All genotype and allelic frequencies, as reported in Table 5.1, were in agreement with previous studies[96, 155-157] and with online databases such as Genbank[151]and SNP500Cancer[152].

All alleles at all loci were in Hardy-Weinberg equilibrium with nonsignificant χ^2 values. This implies that experimental technique was sound and results are robust.

Further more, power calculations were carried out and found that the sample size used gave the study 80-90% power to detect significance at the 0.05 level given a minor allele frequency of about 20% (Power & Sample Size Calculator, PS Program, v2.1.31). Power did not extend to the division of the cancer patient group into histological type (intestinal, diffuse, or mixed) for this reason it was not possible to investigate possible association between genotype and histological cancer type.

	Genotype frequency %			Allele frequency	
	1, 1	1, 2	2, 2	1	2
IL-1B -31 C/T	9 (T,T)	49 (C,T)	42 (C,C)	0.66 T	0.34 C
IL-1B -511 C/T	42 (C,C)	49 (C,T)	9 (T,T)	0.66 C	0.34 T
IL-1B +3954 C/T	56 (C,C)	40 (C,T)	4 (T,T)	0.76 C	0.24 T
IL-10 -1082 G/A	24 (A,A)	43 (A/G)	28 (G,G)	0.53 A	0.47 G
IL-10 -819 C/T	64 (C,C)	33 (C,T)	3 (T,T)	0.80 C	0.20 T
IL-10 -592 C/A	64 (C,C)	33 (C,A)	3 (A,A)	0.80 C	0.20 A
IL-1RN +2018 C/T	52 (T,T)	41 (C,T)	7 (C,C)	0.72 T	0.28 C
TLR4 +896 A/G	85 (A,A)	13 (A,G)	2 (G,G)	0.92 A	0.08 G
TLR4 +936 C/T	82 (C,C)	17 (C,T)	1 (T,T)	0.91 C	0.09 T
TNF -238 A/G	91 (G,G)	8 (A,G)	1 (G,G)	0.95 G	0.05 A
TNF -308 G/A	69 (G,G)	30 (A,G)	1 (A,A)	0.84 G	0.16 A

Table 5.1: Control genotype and allele frequencies for all single nucleotide polymorphisms (SNPs). SNPs at positions -31 and -511 in the Interleukin-1 (IL-1) promoter and SNPs were in complete linkage disequilibrium, as were SNPs at -592 and -819 in the Interleukin-10 (IL-10) promoter region.

Allele and genotype frequencies across groups

Preliminary χ^2 testing for differences in allele or genotype frequency across patient groups found no significant differences. χ^2 and p values are reported in Tables 5.2, 5.3 and 5.4.

Allele and genotype frequencies in combined population groups

Gastric disease groups were combined (gastritis, intestinal metaplasia and gastric cancer) and allele number, carriage of 'rare' alleles and homozygosity for 'rare genotypes' were investigated between normal controls and this composite 'gastric disease' group. No significant differences were observed between groups. An increase in TNF -308A homozygotes (TNF -308 A/A) in the gastric disease group was noted, though this was not significant (χ^2= 3.06, P = 0.08).

Groups were further clustered into 'benign' or '(pre-) malignant categories and number of risk alleles, carriage of 'risk' alleles and homozygosity for the 'risk' genotypes were investigated between the aggregate groups. No significant differences were observed.

Genotype	Controls (%)	Gastritis (%)	Intestinal metaplasia (%)	Gastric cancer (%)	Pearson χ^2 value (P value)
IL-1B-31					
T/T	40 (42)	46 (50)	32 (51)	18 (40)	
C/T	48 (50)	38 (42)	24 (38)	23 (51)	3.78
C/C	8 (8)	7 (8)	7 (11)	4 (9)	(P = 0.71)
IL-1B-511					
C/C	40 (42)	45 (49)	31 (49)	17 (39)	
C/T	47 (49)	39 (43)	24 (38)	23 (52)	3.87
T/T	9 (9)	7 (8)	8 (13)	4 (9)	(P = 0.69)
IL-1B +3954					
C/C	53 (55)	57 (63)	35 (56)	23 (51)	
C/T	39 (41)	29 (32)	24 (38)	19 (42)	2.65
T/T	4 (4)	5 (5)	4 (6)	3 (6)	(P = 0.85)
IL-1RN +2018					
T/T	47 (52)	51 (58)	36 (57)	19 (51)	
C/T	37 (41)	32 (36)	21 (33)	15 (41)	1.87
C/C	7 (7)	5 (6)	6 (10)	3 (8)	(P = 0.93)

Table 5.2: IL-1B -31, -511 and +3954 and IL-1RN +2018 genotype frequencies in *H. pylori* negative controls, *H. pylori* positive gastritis and intestinal metaplasia patients, and patients with non-cardia gastric cancer.

Genotype	Controls (%)	Gastritis (%)	Intestinal metaplasia (%)	Gastric cancer (%)	Pearson χ^2 value (P value)
IL-10-592					
C/C	58 (65)	48 (53)	36 (57)	27 (59)	
C/A	28 (32)	37 (41)	26 (41)	14 (30)	8.41
A/A	3 (3)	5 (6)	1 (2)	5 (11)	(P = 0.21)
IL-10-819					
C/C	59 (64)	48 (53)	36 (57)	27 (59)	
C/T	31 (33)	37 (41)	26 (41)	14 (30)	7.89
T/T	3 (3)	5 (6)	1 (2)	5 (11)	(P = 0.25)
IL-10-1082					
G/G	22 (24)	22 (24)	11 (18)	16 (35)	
G/A	43 (46)	45 (50)	37 (59)	17 (38)	7.04
A/A	28 (30)	23 (26)	14 (23)	12 (27)	(P = 0.32)

Table 5.3: IL-10 -592, -819 and -1082 genotype frequencies in *H. pylori* negative controls, *H. pylori* positive gastritis and intestinal metaplasia patients, and patients with non-cardia gastric cancer.

Genotype	Controls (%)	Gastritis (%)	Intestinal metaplasia (%)	Gastric cancer (%)	Pearson χ^2 value (P value)
TNF-238					
G/G	87 (91)	80 (88)	61 (97)	34 (83)	
G/A	8 (8)	11 (12)	2 (3)	7 (17)	8.46
A/A	1 (1)	0	0	0	(P = 0.21)
TNF-308					
G/G	66 (69)	52 (57)	42 (67)	30 (65)	
G/A	29 (30)	36 (40)	16 (25)	13 (28)	9.19
A/A	1 (1)	3 (3)	5 (8)	3 (7)	(P = 0.163)
TLR4+896					
A/A	82 (85)	77 (85)	53 (84)	20 (74)	
A/G	12 (13)	13 (14)	9 (14)	6 (22)	2.58
G/G	2 (2)	1 (1)	1 (2)	1 (4)	(P = 0.86)
TLR4+936					
C/C	75 (78)	75 (83)	52 (84)	36 (80)	
C/T	15 (16)	13 (14)	9 (14)	9 (20)	1.89
T/T	1 (1)	2 (3)	1 (2)	0	(P = 0.93)

Table 5.4: TNF -238 and -308 and TLR4 +896 and +936 genotype frequencies in *H. pylori* negative controls, *H. pylori* positive gastritis and intestinal metaplasia patients, and patients with non-cardia gastric cancer.

Haplotype analysis: Interleukin 10

Allelic data for each locus studied in the IL-10 gene was submitted to PHASE v 2.1.1 online haplotype analysis software.

Haplotype frequencies are reported below in Table 5.5 and degree of difference between groups are reported in Table 5.5: expanded.

Haplotype	*Normal*	*Gastritis*	*Intestinal metaplasia*	*Gastric cancer*
ACC	0.45	0.48	0.46	0.34
ATA*	**0.01**	**0.01**	**0.01**	**0.13**
GCC	0.35	0.26	0.32	0.41
GCA	0	0	0	0
GTA	0.19	0.25	0.21	0.12

Table 5.5: IL-10 haplotype frequencies across patient groups. Four IL-10 haplotypes were observed.

***IL-10 ATA haplotype is said to be the low producing haplotype while the GCC combination is said to be the high producing haplotype.**

Haplotype 1

ACC	*Normal*	*Gastritis*	*IM*	*Cancer*
Pearson χ^2 value	N/A	0.28	0.01	**4.38* (P < 0.05)**

Haplotype 2

ATA	*Normal*	*Gastritis*	*IM*	*Cancer*
Pearson χ^2 value	N/A	0.001	0.09	**18.35*** (P < 0.001)**

Haplotype 3

GCC	*Normal*	*Gastritis*	*IM*	*Cancer*
Pearson χ^2 value	N/A	3.39	0.28	1.57

Haplotype 5

GTA	*Normal*	*Gastritis*	*IM*	*Cancer*
Pearson χ^2 value	N/A	2.03	0.14	2.27

Table 5.5 – expanded: Degree of difference in IL-10 haplotype frequencies in disease groups relative to the normal controls, as measured by Pearsons' Chi Square test. Haplotype 4 (GCA) was not observed in this population and was omitted from analysis.

5.3.2 Secondary Analysis

Logistic regression

Logistic regression analysis was performed using JMP statistical package (detailed earlier).

There was no significant association between carrier status and age (means were compared using a two sample t-test). The proportions of carriers did not differ significantly between men and women (results based on a standard chi-square). As a result, standard odds ratios did not differ significantly from those adjusting for the effect of age and gender.

Age and gender adjusted odds ratios relating to carriage of the particular alleles and risk of disease are detailed in Table 5.6.

Carriage of the TLR4+896G allele was associated with a significantly increased risk of non-cardia gastric cancer with an age and gender adjusted odds ratio of 4.92 (95% CI: 1.11, 21.79).

SNP Locus	Odds ratio (95% confidence interval)			
	Gastritis (n=91)	Intestinal metaplasia (n=63)	Gastric cancer (n=39)	All gastric disease (n=193)
TLR 4 +896 G+	1.12 (0.49,2.52)	1.33 (0.49, 3.59)	**4.92 (1.11, 21.79)**	1.24 (0.61, 2.54)
TLR 4 +936 T+	0.97 (0.44, 2.11)	0.99 (0.38, 2.63)	2.32 (0.68, 7.91)	1.04 (0.52, 2.1)
TNF -238 A+	1.32 (0.52, 3.4)	0.26 (0.05, 1.34)	1.85 (0.51, 6.75)	1.08 (0.45, 2.56)
TNF -308 A+	1.65 (0.9, 3.02)	1.32 (0.62, 2.83)	1.89 (0.7, 5.15)	1.59 (0.92, 2.76)
IL-1B -31 C+	0.72 (0.4, 1.28)	0.83 (0.41, 1.67)	1.2 (0.48, 3.1)	0.82 (0.48, 1.37)
IL-1B -511 T+	0.75 (0.42, 1.34)	0.88 (0.43, 1.77)	1.27 (0.49, 3.23)	0.85 (0.51, 1.43)
IL-1B +3954 T+	0.74 (0.41, 1.34)	0.97 (0.47, 1.98)	1.9 (0.71, 5.13)	0.84 (0.5, 1.4)
IL-1RN +2018 C+	0.74 (0.41, 1.34)	0.81 (0.39, 1.67)	1.1 (0.39, 3.09)	0.79 (0.47,1.34)
IL-10 -592 A+	1.58 (0.86, 2.91)	1.42 (0.68, 2.94)	1.14 (0.45, 2.9)	1.37 (0.77, 2.34)
IL-10 -819 T+	1.47 (0.81, 2.68)	1.41 (0.68, 2.91)	1.14 (0.45, 2.9)	1.3 (0.76, 2.2)
IL-10 -1082 A+	0.92 (0.46, 1.84)	1.15 (0.46, 2.83)	0.52 (0.18, 1.5)	0.87 (0.47, 1.6)
IL-10 ATA/ATA	1.65 (0.38, 7.22)	0.9 (0.09, 9.63)	**5.93 (0.99, 35.4)**	1.55 (0.41, 5.87)

Table 5.6: Age and gender adjusted odd ratios (95% confidence intervals) for carriage of individual 'rare'/'risk' alleles across all patient groups.

Homozygosity for 'risk' alleles

A number of alleles were found to confer increased risk of gastric cancer (but not of gastritis or intestinal metaplasia) only when two copies of the risk allele were found at the gene locus.

Homozygotes for the low IL-10 ATA haplotype were found to be at significantly increased risk of developing cancer with an age and gender adjusted odds ratio of 5.93 (95% CI: 0.99, 35.4, P = 0.05). Homozygosity for IL-10-592A and IL-10-819T were associated with increased risk (OR 5.63, CI: 1, 36.3 and OR 5.76, CI: 1.03, 37.2, respectively, significance: $P < 0.05$) as individual SNPs additionally to the increased risk conferred by the IL-10 ATA haplotype, of which they form a part.

'Pro-inflammatory genetic profile'

Combinations of a number of cytokine polymorphisms, which have been reported previously to confer increased risk of disease, were also investigated [158]. These combinations included carriage of IL-1B-511T and homozygosity for IL-1RN*2 (≈ IL-1RN - 2018C/C) alone and in combination with carriage of TNF-A-308A and homozygosity for IL-10 haplotype ATA. None of genotype combinations were associated with significant increase in risk of developing gastric disease, in the present study.

Pro-inflammatory polymorphisms, inflammation and atrophy

None of the polymorphisms were associated with degree of inflammation in the gastritis or intestinal metaplasia groups.

The relationship between serum PG levels and carriage of all polymorphisms investigated was explored. Given the inverse correlation between serum PG levels and patient age, already identified (Serum Pepsinogen and age, 4.3.1) age was included as a confounder in these investigations on the affect of pro-inflammatory genotypes.

The relationship between carriage of the IL-1B-511 pro-inflammatory genotypes and serum PG levels in the normal and IM patient groups is shown in Figures 5.3 and 5.4. Though graphically striking the relationship between genotype and PG phenotype is not significant.

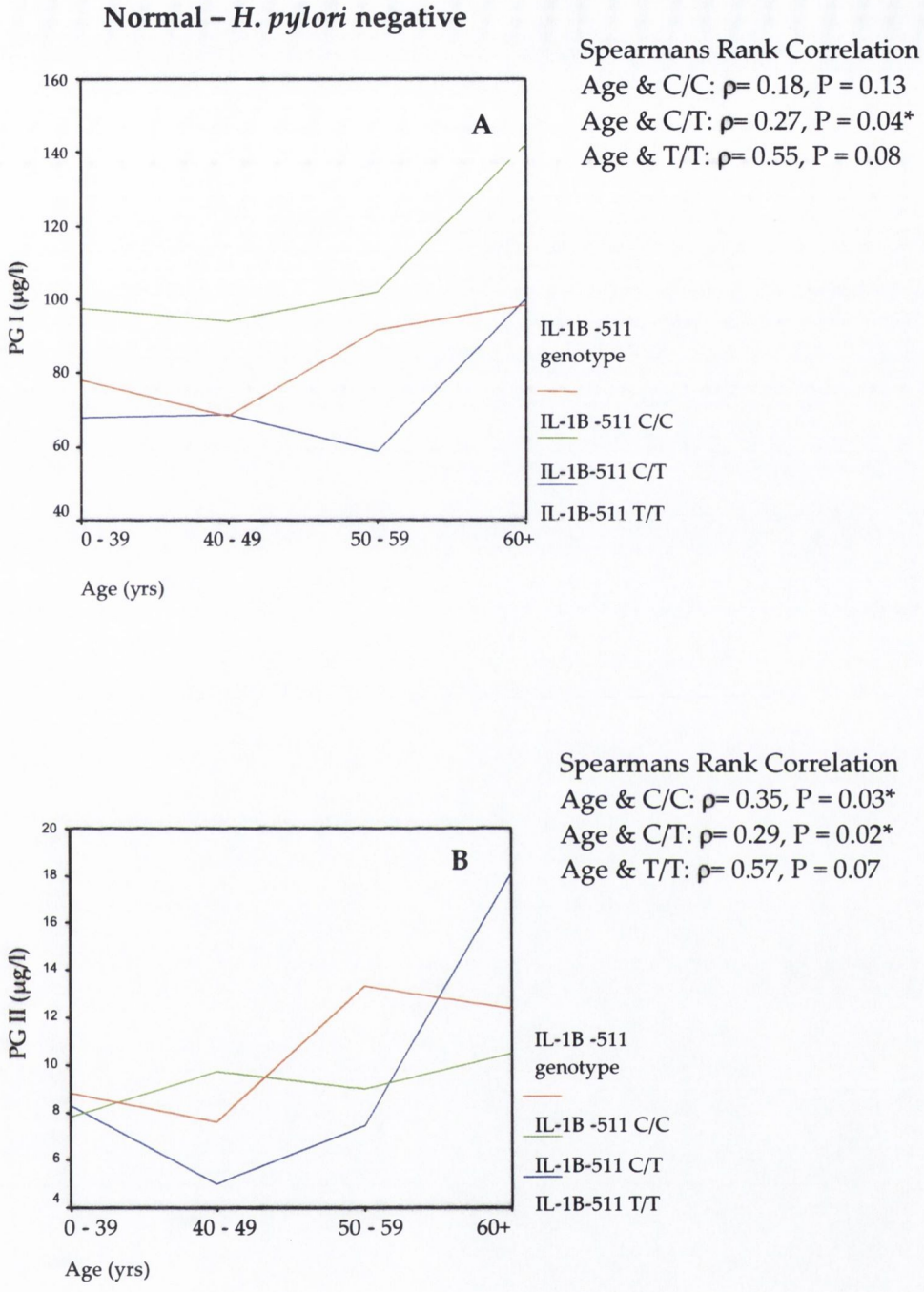
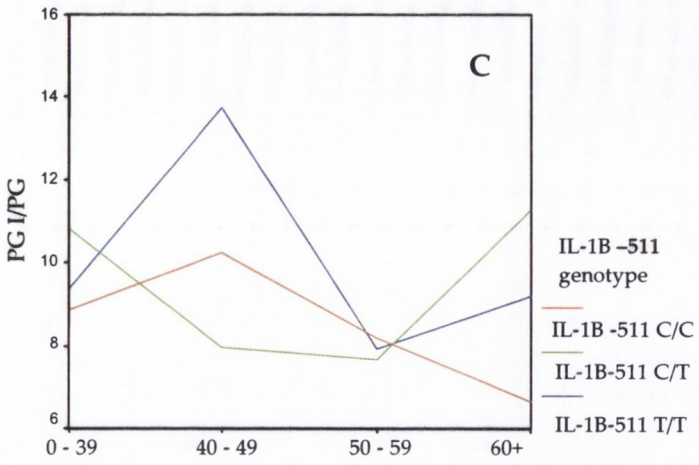
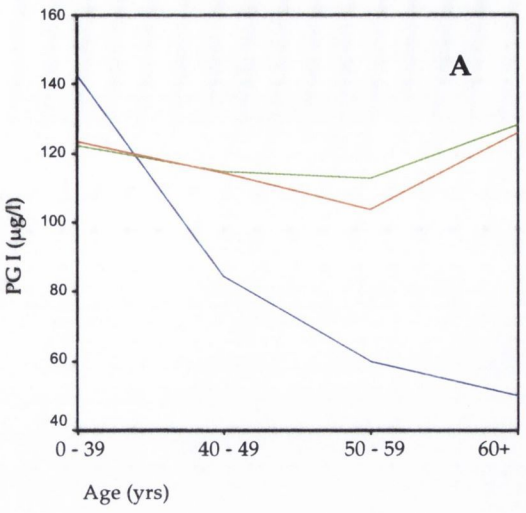


Figure 5.3: The effect of age (years) on serum PGI (A) and PGII (B) levels and PGI/PG II ratio (C) in different IL-1B –511 genotype groups of *H. pylori* negative patients. Data are expressed as medians. Age was categorized in 4 bands: ≤ 39, 40-49, 50-59, and ≥ 60 years.

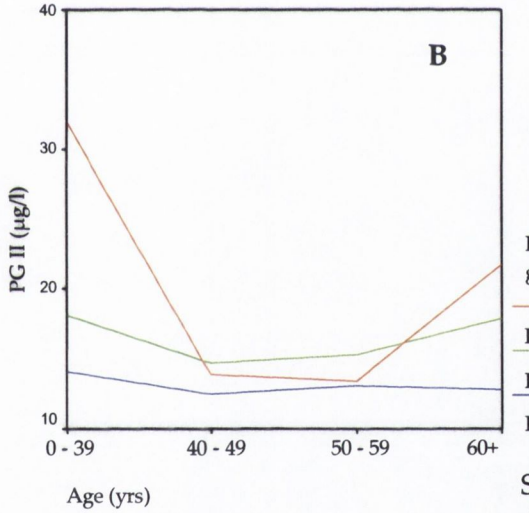


Spearmans Rank Correlation
Age & C/C: ρ= -0.17, P = 0.19
Age & C/T: ρ= -0.25, P = 0.05*
Age & T/T: ρ= -0.36, P = 0.19

Intestinal metaplasia – *H. pylori* positive

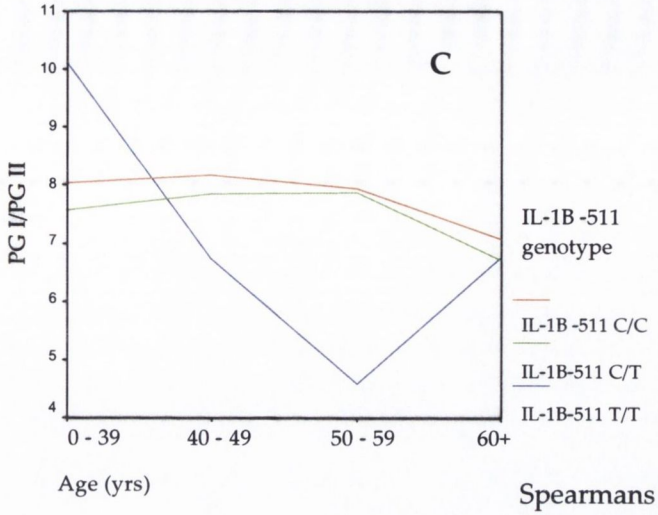


Spearmans Rank Correlation
Age & C/C: ρ= 0.17, P = 0.19
Age & C/T: ρ= -0.13, P = 0.28
Age & T/T: ρ= -0.54, P = 0.08



Spearmans Rank Correlation
Age & C/C: ρ= 0.35, P = 0.03*
Age & C/T: ρ= -0.05, P = 0.4
Age & T/T: ρ= -0.16, P = 0.36

Figure 5.4: The effect of age (years) on serum PGI (A) and PGII (B) levels and PGI/PG II ratio (C) in different IL-1B –511 genotype groups of *H. pylori* positive intestinal metaplasia patients. Data are expressed as medians. Age was categorized in 4 bands: ≤ 39, 40-49, 50-59, and ≥ 60 years.



Spearmans Rank Correlation
Age & C/C: ρ= -0.17, P = 0.19
Age & C/T: ρ= -0.96, P = 0.33
Age & T/T: ρ= -0.19, P = 0.32

5.4 Discussion

This study investigated a pro-inflammatory profile of genetic polymorphisms in IL-1B, IL-1RN, IL-10, TNF-A and TLR4 in a patient population of normal controls and *H. pylori* related gastric disease groups. Carriage of the TLR4+896G allele and homozygosity for the IL-10 ATA haplotype conferred significantly increased risk of developing non-cardia gastric cancer with odds ratios of 4.92 (95% CI, 1.11, 21.79) and 5.93 (95% CI, 0.99, 35.4) respectively.

The increased risk of developing non-cardia gastric cancer conferred on those carrying the TLR4+896G allele is supported by a recent report, which found the same allele to be associated with increased risk of developing gastric mucosal atrophy and hypochlorhydria in first degree relatives of patients with gastric cancer[159]. The present study found no association between carriage of the TLR4+896G allele and risk of intestinal metaplasia development. This may be due to the relative 'benign' or 'focal' nature of the metaplasia expressed in this group. None of the patients in the *H. pylori* positive population were found to have profound gastric mucosal atrophy.

The increased risk of gastric cancer development conferred by homozygotes for the IL-10 ATA haplotype, in this study, is supported by a previous study[158] which reported a similar degree of risk (OR 4.8, 95% CI, 1.8, 12.8). In a recent study, Rad *et al.* investigated the frequency of the IL-10-592, -819 and -1082 SNPs and reported no association between carriage of the rare or 'risk' alleles (or associated haplotype combinations) and intestinal

metaplasia or atrophic gastritis[97], in agreement with the results of the present study.

This study finds that the frequently reported associations between carriage of the IL-1B, IL-1RN and TNF polymorphisms[113, 155, 158, 160-163] and increased risk of gastric cancer did not confer an increased risk in this patient population. Given that these 'risk' alleles *were* observed in the population but conferred no increase in risk, the strength of the associations with the IL-10 and TLR4 loci which were found and the adequate power of the study, it can be assumed that the lack of association reported here for the IL-1B/IL-1RN and TNF SNPs is indeed representative of this cohort. The disparity between the results of this study and previous studies reporting significant risk associated with carriage of these alleles can only be accounted for by population differences and highlights to importance of investigating genetic, in particular SNP factors in a variety of ethnic groups regardless of how similar the cohort in different studies may appear.

The mechanism by which the TLR4+896 SNP and IL-10 ATA haplotype may influence carcinogenesis is, as yet, largely undetermined. The Rad *et al.*[97] study, investigated IL-10 mRNA expression in gastric mucosal biopsy tissue and reported IL-10 expression to correlate with expression of the IL-10 ATA haplotype. In this study, the highest levels of IL-10 mRNA were observed in patients with a haplotype other than ATA while carriage of the ATA haplotype was associated with the lowest IL-10 mRNA levels. This lends much credence to the hypothesis of cytokine genotype influencing gastric mucosal inflammatory phenotype. As IL-10 lowers gastric inflammation[164], the

disturbance in the immune response imposed by a 'low-producing' IL-10 genotype could 'tip the scales' in favour of a pro-longed, unimpeded inflammatory response to an immune challenge such as *Helicobacter pylori* infection. To observe increased risk of cancer development with an IL-10 ATA haplotype, regardless of risk previously associated with the inflammatory genes IL-1B, IL-1RN or TNF, emphasizes the prominence of IL-10 in modulation of the immune response.

The interpretation of TLR4 as a possible risk determinant in cancer development is far more controversial. The role of TLR in *Helicobacter pylori* is complex and not fully understood. A number of studies have reported that TLR4 regulates the immune response to lipopolysacchardide (LPS) and to *H. pylori* LPS, in particular[150, 165-169]. The TLR4 polymorphisms at loci +896 and +936 (also known as amino acid sequence Asp299Gly and Thr399Ile) have been associated with functional changes, specifically a blunted physiological response to inhaled LPS. In the context of *H. pylori* related gastric disease and gastric carcinogenesis, it is likely that the influence of TLR4 on gastric cancer is tied to initial *H. pylori* infection and response to this infection, the blunted response to LPS associated with the polymorphisms could allow *H. pylori* not only to colonise the gastric mucosa but allow rapid spread of infection. *H. pylori* colonisation is then established and the adaptive immune response including the up-regulation of cytokines begins. Polymorphisms in the genes controlling expression of immune mediators such as IL-10 are now influential in determining degree and duration of response to infection.

In conclusion, this study found that, in this Irish patient population, carriage of TLR4+896G and homozygosity for IL-10ATA haplotype confers significantly increased risk of non-cardia gastric cancer. This increase in risk may be due to an altered inflammatory response to initial *H. pylori* colonisation and subsequent infection.

Chapter 6

General Discussion

6. Discussion

This principle finding of this study was that carriage of the TLR4+896G allele and homozygosity for the IL-10 ATA haplotype confer (independent) increased risk of non-cardia gastric cancer development with odds ratios of 4.92 (95% CI: 1.11, 21.79) and 5.93 (95% CI: 0.99, 35.4), respectively. No increase in risk was found for either carriage, or homozygosity, of the IL-1B-31C, IL-1B-511T, IL-1RN*2, TNF-238A, TNF-308A or TLR4+936T alleles.

The findings of this study are in agreement with some previous studies. El-Omar *et al.*, reported carriage of a number of pro-inflammatory alleles (including IL-10) to be associated with increased risk of developing non-cardia gastric cancer[158]. The same group noted TLR4+896G increased risk of development of *H. pylori* induced premalignant changes (gastric mucosal atrophy and hypochlorhydria)[159].

While the absence of an association for the IL-1B, IL-1RN and TNF polymorphisms reported in this study seems to contradict the findings of previous studies[113, 155, 158, 162, 170, 171] many groups have reported a similar lack of association, though these findings often remain as abstracts rather than full papers[98-103]. It is concerning to think a publication bias for studies reporting polymorphisms increasing risk of gastric disease may be influencing current opinion in the wider Gastroenterological Community concerning the magnitude of risk conferred. Evidence on both sides seems to support some role for host genetics in determining the outcome of *H. pylori* associated

gastric disease though the magnitude of this role and the specific polymorphisms involved can differ greatly from study to study emphasising the role for well designed, 'powerful' genetic studies, to be carried out in centres across the world to better understand this role.

The observed risk associated with carriage of polymorphisms in the TLR4 and IL-10 genes in this study maybe supported by their role in the immune response to *H. pylori* colonisation. Polymorphisms in both genes could allow first a blunted response to initial *H. pylori* challenge (TLR4 +896G+)[149, 159, 172] followed by an exaggerated response to infection where anti-inflammatory action of IL-10 is depressed by expression of a low IL-10 producing genotype (IL-10 ATA/ATA)[97, 148]. This prolonged, unimpeded, inflammatory challenge could allow the accumulation of genotoxic substances, as outlined by Correa[27, 38] and these genotoxic substances could induce the epigenetic changes essential in the transformation of normal or inflamed gastric mucosal tissue to neoplastic tissue and adenocarcinoma.

Given Correas proposed mechanism of carcinogenesis and the long lead-time thus required for eventual development of non-cardia gastric carcinogenesis, atrophy and intestinal metaplasia groups are essential in the study of possible risk factors for cancer development. It is concerning that so much controversy exists in diagnosis, particularly of gastric mucosal atrophy. Until more specific molecular/cellular markers are identified, it is difficult to see how this may be overcome. This study used serology testing for pepsinogen to strengthen diagnosis of

intestinal metaplasia and identify mucosal atrophy. In this context and in this patient population the result of this testing was disappointing. Firstly, the tests failed to identify any patients with atrophic gastritis amongst the *H. pylori* positive patients, though this may be a function of the patient population rather than the tests. Secondly, perhaps more worryingly, testing for serum pepsinogen levels failed to identify the intestinal metaplasia group. 7 of the 63 patients with intestinal metaplasia were identified as having a PG I/PG II ratio below 3. Of the 9 patients with histologically classified 'extensive' intestinal metaplasia only 2 had a PG I/PG II ratio below 3.

Serology testing for *H. pylori* IgGs was a strength of the study in that it identified recent, as well as previous, infection. Once the process of atrophy and metaplasia commences the gastric mileu is often changed so that the environment no longer sustains *H. pylori* so that patients with atrophy or IM often appear to be uninfected. This could lead to an underestimation of risk of development of these pre-cancerous changes associated with *H. pylori*.

Patients with chronic inflammatory conditions (eg. rheumatoid arthritis, coeliac disease and diabetes) which, in some cases have been previously associated with cytokine polymorphisms, were also excluded from the study. Together with the thorough testing for *H. pylori* and histopathological classification, it may be that the strict classification and screening processes throughout the study distinguish it from similar studies. This could, conceivably, account for absence of some previously reported associations (in particular those previously reported for IL-1B, IL-1RN and TNF).

There are many potential applications of this study and this cohort data. It is hoped further potential targets for investigation will be identified through microarray analysis using gastric mucosal tissue from many of the patients enrolled in this study (particularly patients with intestinal metaplasia). Planned analysis will encompass both SNP and gene expression microarray work in identification of targets for further investigation. In the vast number of targets which can be studied on a single gene chip it will be possible to develop a wider profile of SNPs which may influence disease and study the effect of SNP expression on mRNA levels of various immune modulators. Studies specific to gastric mucosal tissue are essential given the local changes that occur in *H. pylori* associated disease.

The technique developed in this study to allow DNA extraction from archival formalin-fixed, paraffin embedded gastric mucosal biopsy and surgical specimens will be further developed for use in microarray analysis.

Recruitment of patients with intestinal metaplasia and gastric cancer will continue in order to expand and update the patient database developed for this study. Increases in number in the gastric cancer group may also allow the investigation into the importance on 'type' of gastric cancer (intestinal or diffuse) and risk conferred. Prospective patient recruitment will also overcome a number of the difficulties reported in this study with respect to retrospective recruitment (lack of detailed medical history or *H. pylori* status, for example).

The identification of a patient group with intestinal metaplasia *without* concomitant *H. pylori* infection may allow further insight

into altered gene expression in this group relative to the *H. pylori* positive intestinal metaplasia patients.

It was not possible, during the course of this study, to genotype the infecting *H. pylori* strains. This type of analysis would allow further observations on the effects of the combined risks of virulent bacterial strains together with expression of 'risk' alleles. Increasing the number of factors under investigation is futile, however, without increasing the cohort size proportionally in order to conserve the power of the study.

Both this study and future work aim to better characterise, molecularly, the gastric mucosal changes that precede gastric cancer. Thus the pre-cancerous groups identified clinically and epidemiologically, could be molecularly assessed for potential risk of carcinogenesis, and surveillance could be tailored to degree of risk conferred.

"Despite advances in surgical treatment and chemotherapy, gastric cancer remains a major global health burden. The most recent estimates show that it is the fourth most common cancer and the second most common cause of cancer deaths worldwide."

Pelayo Correa et al.[173], 2004

Studies into both bacterial and host genotypes are, as yet, in their infancy; their value in the clinical setting, untested. Their potential, however, in identifying individuals for targeted surveillance and intervention is obvious. Though eradication therapy for *Helicobacter pylori* infection has high success rates and

non-cardia gastric cancer rates are globally declining, year on year, the model whereby *H. pylori* may initially induce an inflammatory reaction which subsequently leads to development of atrophic gastritis, dysplasia and neoplasia is an invaluable study of a bacterial, gene, environment interaction. Investigations into this interaction will have far reaching implications in all fields of cancer research.

Study Number:

Patient Group:

Reason for exclusion:

Hospital No.: _____

Name: _____ **Overall Hp Status**: ____

Address: _____

_____ UBT: _____

_____ Clo: _____

_____ Micro: _____

DOB: _____ Histology: _____

Age @ Diagnosis: _____ Serology: _____

Contact: _____ Date of last visit:_____

Gender: _____ Clinic: _____

History

C/o: _____

Previous Medical Hx.: _____

_____

_____

_____

Previous Surgical Hx.: _____

_____

_____

Relevant Family Hx: _____

_____

Social:_____

Medication: _____

_____

Endoscopy Report

Most recent (Date:)

Oesoph.:_____

Stomach:_____

Duoden.:_____

Previous (Date:)
Oesoph.:_____
Stomach:_____
Duoden.:_____

Laboratory Medicine:

Culture:_____ **Histology report attached:**
Antrum:_____
Corpus:_____ Yes ☐

No ☐

Biohit diagnostics: Biohit PLC

Helicobacter pylori IgG ELISA

Cat. No. 601 040.01

Protocol as per 'Instructions for Use' as follows

Preliminary Preparations

Allow all reagents and the microplate to reach room temperature (20…25°C). Warm the incubator to 37°C. Dilute the washing buffer concentrate 1 to 10 with distilled deionised water.

Mix all reagents well.

Specimen dilution

Dilute the mixed serum or plasma samples to 1 to 200 with the diluent buffer, mix well.

Step 1

Mix and pipette 100 µl of the diluent buffer (Blank), the calibrator (CAL), the negative control (NC), positive control (PC) and the diluted samples into the microplate wells as duplicates. Cover the plate with the incubation cover. Incubate for 30 minutes at 37°C.

Washing

Wash the strips with 3 x 350 µl of the diluted washing buffer and gently tap the inverted plate a few times on a clean paper towel.

Step 2

Pipette 100 µl of the mixed dilution (1 to 100) of conjugate solution into the wells with an 8-channel pipette. Cover the plate with the incubation cover. Incubate for 30 minutes at 37°C.

Washing

As before

Step 3

Pipette 100 µl of the substrate solution into the wells with an 8-channel pipette. Start the incubation timer after pipetting the substrate solution into the first strip and continue the incubation for 30 minutes at room temperature (20…25°C). Avoid direct exposure to light during incubation.

Step 4

Pipette 100 µl of the stop solution with an 8-channel pipette into the wells.

Measuring

Measure the absorbance at 450 nm within 30 minutes.

Taqman Assays: Protocol for IL-1B-31, -511 & +3954

IL-1B-31

Cocktail x 1:

	µl
Mastermix:	6.25
Fam (75nM)	0.1875
Tet (200nM)	0.5
H_20	2.0625
For. Primer (300nM)	0.375
Rev. Primer (900nM)	1.125
Total rxn volume	**10.5**

+2µl DNA (neat) per well
NTC: +2µl H_20
NAC: +2µl SDS
PCR conditions: 50°C – 2mins, 95°C – 10mins, 40 cycles of 95°C for 15s & 62°C for 1 min.

IL-1B-511

Cocktail x 1:

	µl
Mastermix:	6.25
Fam (80nM)	0.2125
Tet (200nM)	0.5
H_20	2.7875
For. Primer (300nM)	0.375
Rev. Primer (300nM)	0.375
Total rxn volume	**10.5**

+2µl DNA (neat) per well
NTC: +2µl H_20
NAC: +2µl SDS

PCR conditions: 50°C – 2mins, 95°C – 10mins, 40 cycles of 95°C for 15s & 62°C for 1 min.

IL-1B+3954

Cocktail x 1:

	µl
Mastermix:	6.25
Fam (200nM)	0.5
Tet (250nM)	0.625
H_2O	2.375
For. Primer (300nM)	0.375
Rev. Primer (300nM)	0.375
Total rxn volume	**10.5**

+2µl DNA (neat) per well
NTC: +2µl H_2O
NAC: +2µl SDS

PCR conditions: 50°C – 2mins, 95°C – 10mins, 40 cycles of 95°C for 15s & 62°C for 1 min.

Taqman Assays: Protocol for TNF -308 & -238

TNF-308

Cocktail x 1:

	µl
Mastermix:	12.5
Fam (75nM)	0.25
Tet (200nM)	0.875
H_2O	7.875
For. Primer (300nM)	0.75
Rev. Primer (900nM)	0.75
Total rxn volume	**23**

+2µl DNA (neat) per well
NTC: +2µl H_2O
NAC: +2µl SDS

PCR conditions: 50°C – 2mins, 95°C – 10mins, 40 cycles of 95°C for 15s & 58°C for 1 min, holding cycle of 15°C.

TNF-238

Cocktail x 1:

	µl
Mastermix:	12.5
Fam (75nM)	0.25
Tet (200nM)	0.625
H_2O	5.125
For. Primer (300nM)	2.25
Rev. Primer (900nM)	2.25
Total rxn volume	**23**

+2µl DNA (neat) per well
NTC: +2µl H_2O
NAC: +2µl SDS

PCR conditions: 50°C – 2mins, 95°C – 10mins, 40 cycles of 95°C for 15s & 58°C for 1 min, holding cycle of 15°C.

Taqman Assays: Protocol for TLR4+896

TLR4+896

Cocktail x 1:

	µl
Mastermix:	12.5
Fam (75nM) **ALLELE 1**	0.375
Tet (200nM) **ALLELE 2**	1.25
H_2O	4.375
For. Primer (300nM)	2.25
Rev. Primer (900nM)	2.25
Total rxn volume	**23**

+2µl DNA (neat) per well

NTC: +2µl H_2O

NAC: +2µl SDS

PCR conditions: 50°C – 2mins, 95°C – 10mins, 40 cycles of 95°C for 15s & 62°C for 1 min, holding cycle of 15°C.

List of reagents/equipment used

Blood collection

Centrifuge	
Serum tubes	Vacuette Z serum clot activator®, Greiner bio-one, UK
Whole blood tubes	BD Vacutainer™, Preanalytical Systems, UK

ELISA Kits

Pepsinogen I	Pepsinogen I ELISA; Cat. No. 601 010.01, Biohit Plc., Helsinki, Finland.
Pepsinogen II	Pepsinogen II ELISA; Cat. No. 601 020.01, Biohit Plc., Helsinki, Finland.
Helicobacter pylori	Helicobacter pylori IgG ELISA; Cat No. 601 040.01, Biohit Plc., Helsinki, Finland.

DNA extraction from whole blood

QIAamp® DNA Blood Mini Kit	Qiagen Ltd., UK, Cat. No. 51104(06)

DNA extraction from formalin fixed paraffin embedded tissue

Microtome	Leica, RM2125, Germany, Laboratory Instruments & Supplies, Dublin
Vortex	Vortex Genie 2, Sparks Lab Supplies, Dublin

Extraction Kit	Puregene™ Mouse Tail Kit, Gentra Systems, MN 5541, USA, Part No. D-7000B
Isopropanol 99+%	Sigma-Aldrich GmbH, Germany, Cat.No.I9516
Glycogen (20mg/ml)	Roche Diagnostics GmbH, Germany, Cat. No. 901 393
Ethanol (Absolute)	Alrich, MI, USA, Cat. No. E702-3
Xylene	Analytical grade (ACS), Scharlau Spain, Cat. No. Xi0057
DNA ladder	Lambda HIND III, 199µg/ml, Sigma-Aldrich, UK, Cat. No.:D9780

Taqman® reagents

Taqman® Universal Master Mix	Applied Biosystems, UK. Product No. 4304437
ABI PRISM™ 96-Well Optical Reaction Plate	Applied Biosystems, UK. Product No. 4326659
ABI PRISM™ Optical Caps, (8 caps/strip)	Applied Biosystems, UK. Product No. 4323032
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich GmbH, Germany, Cat. No. 71727

Fluorescence Detection

Assays carried out on ABI PRISM™ 7200 Sequence DetectionSystem, Applied Biosystems, UK.

Software – Primers & Probes Design

PrimerExpress®, Software v2.0 Applications based primer design software, Applied Biosystems, UK.

Fluorescent Probes

R-Q Probe	Crude >30moles, Scandinavian Gene Synthesis AB (SGS, Sweden)

PCR primers

HPSF Base	0.05µmol scale, MWG Biotech (UK) Cat. No. 1210-005100

Thermal Cycler

GeneAmp® PCR System 9600Applied Biosystems, UK.

IL-1B-31C/T

http://snp500cancer.nci.nih.gov/snp.cfm?both_snp_id=IL1B-03

dbSNP ID: **rs1143627**/SNP500Cancer ID: IL1B-03, Note: aka -31

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TTGTACCCATTTATCCATCTGTAAGTGGGAAGATTCCTAAACTTAAGTACAA
AGAAGTGAATGAAGAAAAGTATGTGCATGTATAAATCTGTGTGTCTTCCACT
TTGTCCCACATATACTAAATTTAAACATTCTTCTAACGTGGGAAAATCCAGT
ATTTTAATGTGGACATCAACTGCACAACGATTGTCAGGAAAACAATGCATAT
TTGCATGGTGATACATTTGCAAAATGTGTCATAGTTTGCTACTCCTTGCCCT
TCCATGAACCAGAGAATTATCTCAGTTTATTAGTCCCCTCCCCTAAGAAGCT
TCCACCAATACTCTTTTCCCCTTTCCTTTAACTTGATTGTGAAATCAGGTAT
TCAACAGAKAAATTTCTCAGCCTCCTACTTCTGCTTTTGAAAGC(**C/T**)ATA
AAAACAGCGAGGGAGAAACTGGCAGATAMCMAACCTCTTCGAGGCAKAAGG
CACAACAGGCTGCTCKGGGATTCTCTTCAGCCAATCTTCATTGCTCAAGTYTG
AYTTTAATCTTCCTTACAACTAGGTGCTAAGGGAGTCTCTCTGTCTCTCTGC
CTCTTTGTGTGTATGCATATTCTCTCTCTCTCTCTCTTTCTTTCTCTGTCTC
TCCCTCTCCTTCCCTCTCTGCCTCCCTCTCTCAGCTTTTTGCAAAAATGCCA
GGTGTAATATAATGCTTATGACTCGGGAAATATTCTGGGAATGGATACTGCT
TATCTAACAGCTGACACCCTAAAGGTTAGTGTCAAAGCCTCTGCTCCAGCTC
TCCTAGCCAATACATTGCTAGTTGGGGTTTGGTTTAGCAAATGCTTTTCTCT
AGACCCAAAGGACTTCTCTTTCACACATTCATTCATTTACTCAGAGATCATT
TCTTTGCATGACTGCCATGCACTGGATGCTGAGAGAAATCACACATGAACGT
AGCCGTCATGGGGAAGTCACTCATTTTCTCCTTTTTACACAGGTGTCTGAAG
CAGCCATGGCAGAAGTACCT

IL-1B-511C/T

http://snp500cancer.nci.nih.gov/snp.cfm?both_snp_id=16944

dbSNP ID: **rs16944**/SNP500Cancer ID: IL1B-01, Note: aka -511

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ACTGTTCATAACCTGGACTCTCATTCATTCTACAAATGGAGGGCTCCCCTGG
GCAGTACCCTGGAGCAGGCACTTTGCTGGTGTCTCGGTTAAAGAGAAACTGA
TAACTCTTGGTTGGTATTACCAAGAGATAGAGTCTCAGATGGATATTCTTAC
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CTCATCTGGCATTGATCTGGTTCATCCATGAGATTGGCTAGGGTAACAGCAC
CTGGTCTTGCAGGGTTGTGTGAGCTTATCTCCAGGGTTGCCCCAACTCCGTC
AGGAGCCTGAACCCTGCATACCGTATGTTCTCTGCCCCAGCCAAGAAAGGTC
AATTTTCTCCTCAGAGGCTCCTGCAATTGACAGAGAGCTCC(**C/T**)GAGGCA
GAGAACAGCACCCAAGGTAGAGACCCACACCCTCAATACAGACAGGGAGGGC
TATTGGCCCTTCATTGTACCCATTTATCCATCTGTAAGTGGGAAGATTCCTA
AACTTAAGTACAAAGAAGTGAATGAAGAAAAGTATGTGCATGTATAAATCTG
TGTGTCTTCCACTTTGTCCCACATATACTAAATTTAAACATTCTTCTAACGT
GGGAAAATCCAGTATTTTAATGTGGACATCAACTGCACAACGATTGTCAGGA
AAACAATGCATATTTGCATGGTGATACATTTGCAAAATGTGTCATAGTTTGC
TACTCCTTGCCCTTCCATGAACCAGAGAATTATCTCAGTTTATTAGTCCCCT
CCCCTAAGAAGCTTCCACCAATACTCTTTTCCCCTTTCCTTTAACTTGATTG
TGAAATCAGGTATTCAACAGAKAAATTTCTCAGCCTCCTACTTCTGCTTTTG
AAAGCYATAAAAACAGCGAGGGAGAAACTGGCAGATAMCMAACCTCTTCGA
GGCAKAAGGCACAACAGGCTGCTCKGGGATTCTCTTCAGCCAATCTTCATTGC
TCAAGTYTGAYTTTAATCTT

IL-1B+3954 C/T

http://snp500cancer.nci.nih.gov/snp.cfm?both_snp_id=1143634

dbSNP ID: **rs1143634**/SNP500Cancer ID: IL1B-02, Note: aka +3954

AGAAGGTAGTTAGCCAAGAGCAGGCAGTAGATCTCCACTTGTGTCCTCTTGG
AAGTCATCAAGCCCCAGCCAACTCAATTCCCCCAGAGCCAAAGCCCTTTAAA
GGTAGAAGGCCCAGCGGGGAGACAAAACAAAGAAGGCTGGAAACCAAAGCA
ATCATCTCTTTAGTGGAAACTATTCTTAAAGAAGATCTTGATGGCTACTGACA
TTTGCAACTCCCTCACTCTTTCTCAGGGGCCTTTCACTTACATTGTCACCAG
AGGTTCGTAACCTCCCTGTGGGCTAGTGTTATGACCATCACCATTTTACCTA
AGTAGCTCTGTTGCTCGGCCACAGTGAGCAGTAATAGACCTGAAGCTGGAAC
CCATGTCTAATAGTGTCAGGTCCAGTGTTCTTAGCCACCCCACTCCCAGCTT
CATCCCTACTGGTGTTGTCATCAGACTTTGACCGTATATGCTCAGGTGTCCT
CCAAGAAATCAAATTTTGCCRCCTCGCCTCACGAGGCCTGCCCTTCTGATTT
TATACCTAAACAACATGTGCTCCACATTTCAGAACCTATCTTCTT(**C/T**)GA
CACATGGGATAACGAGGCTTATGTGCACGATGCACCTGTACGATCACTGAAC
TGCACGCTCCGGGACTCACAGCAAAAAAGCTTGGTGATGTCTGGTCCATATG
AACTGAAAGCTCTCCACCTCCAGGGACAGGATATGGAGCAACAAGGTAAATG
GAAACATCCTGGTTTCCCTGCCTGGCCTCCTGGCAGCTTGCTAATTCTCCAT
GTTTTAAACAAAGTAGAAAGTTAATTTAAGGCAAATGATCAACACAAGTGAA
AAAAAATATTAAAAAGGAATATACAAACTTTGGTCCTAGAAATGGCACATTT
GATTGCACTGGCCAGTGCATTTGTTAACAGGAGTGTGACCCTGAGAAATTAG
ACGGCTCAAGCACTCCCAGGACCATGTCCACCCAAGTCTCTTGGGCATAGTG
CAATGTCAATTCTTCCACAATATGGGGTCATTTGATGGACATGGCCTAACTG
CCTGTGGGTTCTCTCTTCCTGTTGTTGAGGCTGAAACAAGAGTGCTGGAGCG
ATAATGTGTCCATCCCCCTCCCCAGTCTTCCCCCCTTGCCCCAACATCCGTC
CCACCCAATGCCAGGTGGTT

IL-1RN +2018

dbSNP ID: **rs419598**

BLASTed: FAM probe from Am J Respir Crit Care Med 2000,162;2:755-758

AATAGAAGAAAATTTAAAATCCAGACCCTTGGTCACACTATCCACATTTA
AAGAGGTCAATAGCCACATGTGGTTAGTGGCCACCCTATTGGGCAGTGCA
GCTACAGAACATTTTTGCATCCCAGAAAGTTCTTTTGGATGTTGCTGCTC
TACAGCATGCTTTGCTGAAACAGAAGTGCCTTCCCTGGGAATCTCAGATG
GGAAGCAAGTAAGGAGGGGAGTCAAATGTGGGCTCACTGCTCACCAGCTG
TGAGGGTTGGGCCTGCCTCTTAACCATTGTCAGCCTCAGTCTTCTCATCC
ATGCATGCCGTGGGTATACTAAAATACTATACCCCTGGAAGAGCTGGATG
CAAATTTGACAAGTTCTGGGGGACACAGGAAGGTGCCAAGCACAAGGCTG
GGCACATGGTGGCTGTGCACTACAGCTGAGTCCTTTTCCTTTTCAGAATC
TGGGATGTTAACCAGAAGACCTTCTATCTGAGGAACAACCAACTAGTTGC
[113]GGATACTTGCAAGGACCAAATGTCAATTTAGAAGGTGAGTGGTTG
CCAGGAAAGCCAATGTATGTGGGCATCACGTCACTTTGCCCGTCTGTCTG
CAGCAGCATGGCCTGCCTGCACAAACCCTAGGTGCAATGTCCTAATCCTT
GTTGGGTCTTTGTATTCAAGTTTGAAGCTGGGAGGGCCTGGCTACTGAAG
GGCAC

TNF –308 A/G

http://snp500cancer.nci.nih.gov/snp.cfm?both_snp_id=1800629

dbSNP ID: **rs1800629,** SNP500Cancer ID: TNF-02, Note: aka -308

GACAGGGCCATGTAGAGGGCCCCAGGGAGTGAAAGAGCCTCCAGGACCTCCA
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GAGAAGTGAGAGCTTCATGAAAAAAATCAGGGACCCCAGAGTTCCTTGGAAG
CCAAGACTGAAMCMAGCATTATGAGTCTCCGGGTCAGAATGAAAGAAGARGG
CCTGCCCCAGTGGGGTCTGTGAATTCCCGGGGGTGATTTCACTCCCCGGGGC
TGTCCCAGGCTTGTCCCTGCTACCCSCACCCAGCCTTTCCTGAGGCCTCAAG
CCTGCCACCAAGCCCCCAGCTCCTTCTCCCCGCAGGGACCCAAACACAGGCC
TCAGGACTCAACACAGCTTTTCCCTCCAACCCCGTTTTCTCTCCCTCAAGGA
CTCAGCTTTCTGAAGCCCCTCCCAGTTCTAGTTCTATCTTTTTCCTGCATCC
TGTCTGGAARTTAGAAGGAAACAGACCACAGACCTGGTCCCCAAAAGAAATG
GAGGCAATAGGTTTTGAGGGGCATG(**A/G**)GGACGGGGTTCAGCCTCCAGGG
TCCTACACACAAATCAGTCAGTGGCCCAGAAGACCCCCCTMRGAATCRGAGC
AGGGAGGATGGGGAGTGTGAGGGGTATCCTTGATGCTTGTGTGTCCCCAACT
TTCCAAATCCCCGCCCCCRCGATGGAGAAGAAACCGAGACAGAAGGTGCAGG
GCCCACTACCGCTTCCTCCAGATGAGCTCATGGGTTTCTCCACCAAGGAAGT
TTTCCGCTGGTTGAATGATTCTTTCCCCGCCCTCCTCTCGCCCCAGGGACAT
ATAAAGGCAGTTGTTGGCACACCCAGCCAGCAGACGCTCCCTCAGCAAGGAC
AGCAGAGGACCAGCTAAGAGGGAGAGAAGCAACTACAGACCCCCCCTGAAA
ACAACCCTCAGACGCCACATCCCCTGACAAGCTGCCAGGCAGGTTCTCTTCCT
CTCACATACTGACCCACGGCTYCACCCTCTCTCCCCTGGAAAGGACACCATG
AGCACTGAAAGCATGATCCGGGACGTGGAGCTGGCCGAGGAGGCGCTCCCCA
AGAAGACAGGGGGGCCCCAGGGCTCCAGGCGKTGCTTGTTCCTCAGCCTCTT
CTCCTTCCTGATCGTGGCAG

TNF –238 A/G

http://snp500cancer.nci.nih.gov/snp.cfm?both_snp_id=361525

dbSNP ID: **rs361525,** SNP500Cancer ID: TNF-04, Note: aka -238

GCCCCAGGGAGTGAAAGAGCCTCCAGGACCTCCAGGTATGGAATACAGGGGA
CRTTTAAGAAGATATGGCCACACACTGGGGCCCTGAGAAGTGAGAGCTTCAT
GAAAAAAATCAGGGACCCCAGAGTTCCTTGGAAGCCAAGACTGAAMCMAGC
ATTATGAGTCTCCGGGTCAGAATGAAAGAAGARGGCCTGCCCCAGTGGGGTCT
GTGAATTCCCGGGGGTGATTTCACTCCCCGGGGCTGTCCCAGGCTTGTCCCT
GCTACCCSCACCCAGCCTTTCCTGAGGCCTCAAGCCTGCCACCAAGCCCCCA
GCTCCTTCTCCCCGCAGGGACCCAAACACAGGCCTCAGGACTCAACACAGCT
TTTCCCTCCAACCCCGTTTTCTCTCCCTCAAGGACTCAGCTTTCTGAAGCCC
CTCCCAGTTCTAGTTCTATCTTTTTCCTGCATCCTGTCTGGAARTTAGAAGG
AAACAGACCACAGACCTGGTCCCCAAAAGAAATGGAGGCAATAGGTTTTGAG
GGGCATGRGGACGGGGTTCAGCCTCCAGGGTCCTACACACAAATCAGTCAGT
GGCCCAGAAGACCCCCCTMRGAATC(**A/G**)GAGCAGGGAGGATGGGGAGTGT
GAGGGGTATCCTTGATGCTTGTGTGTCCCCAACTTTCCAAATCCCCGCCCCC
RCGATGGAGAAGAAACCGAGACAGAAGGTGCAGGGCCCACTACCGCTTCCTC
CAGATGAGCTCATGGGTTTCTCCACCAAGGAAGTTTTCCGCTGGTTGAATGA
TTCTTTCCCCGCCCTCCTCTCGCCCCAGGGACATATAAAGGCAGTTGTTGGC
ACACCCAGCCAGCAGACGCTCCCTCAGCAAGGACAGCAGAGGACCAGCTAAG
AGGGAGAGAAGCAACTACAGACCCCCCCTGAAAACAACCCTCAGACGCCACA
TCCCCTGACAAGCTGCCAGGCAGGTTCTCTTCCTCTCACATACTGACCCACG
GCTYCACCCTCTCTCCCCTGGAAAGGACACCATGAGCACTGAAAGCATGATC
CGGGACGTGGAGCTGGCCGAGGAGGCGCTCCCCAAGAAGACAGGGGGGCCCC
AGGGCTCCAGGCGKTGCTTGTTCCTCAGCCTCTTCTCCTTCCTGATCGTGGC
AGGCGCCACCACGCTCTTCT

TLR4+896

BLAST: TAG ACT ACT ACC TCG ATG ATA TTA TTG ACT TAT TTA ATT C (FAM probe Read et al.)

dbSNP ID: **rs4986790**/SNP500Cancer ID: TLR4-01, Ex4+636A>G

(Amino acid change: D299G)

CTGGTTTAGAAGTCCATCGTTTGGTTCTGGGAGAATTTAGAAATGAAGGAAA
CTTGGAAAAGTTTGACAAATCTGCTCTAGAGGGCCTGTGCAATTTGACCATT
GAAGAATTCCGATTAGCATACTTAGACTACTACCTCGATG(**A/G**)TATTATT
GACTTATTTAATTGKTTGACAAATGKTTCTTCATTTTCCCTGGTGAGTGTGA
CTATTGAAAGGGTAAAAGACTTTTCTTATAATTTCGGATGGCAACATTTAGA
ATTAGTTAACTGTAAATWTGGACAGTTTCCCACATTGAAACTCAAATCTCTC
AAAAGGCTTACTTTCACTTCCAACAAAGGTGGGAATGCTTTTTCAGAAGTTG
ATCT

TLR4+936

dbSNP ID: **rs4986791**/SNP500Cancer ID: TLR4-04, Amino acid change: T399I

CTGTAAATWTGGACAGTTTCCCACATTGAAACTCAAATCTCTCAAAAGGCTT
ACTTTCACTTCCAACAAAGGTGGGAATGCTTTTTCAGAAGTTGATCTACCAA
GCCTTGAGTTTCTAGATCTCAGTAGAAATGGCTTGAGTTTCAAAGGTTGCTG
TTCTCAAAGTGATTTTGGGACAA**(C/T)**CARCCTAAAGTATTTAGATCTGAG
CTTCAATGGTGTTATTACCATGAGTTCAAACTTCTTGGGCTTAGAACAACTA
GAACATCTGGATTTCCAGCATTCCAATTTGAAACAAATGAGTGAGTTTTCAG
TATTCCTATCACTCAGAAACCTCATTTACCTTGACATTTCTCATACTCACAC
CAGAGTTGCTTTCAATGGCATCTTCAATGGCTTGTCCAGTCTCGAAGTCTTG
A

IL-10-592

dbSNP ID: **rs1800872/SNP500Cancer ID: IL10-02** (-626A>C)

CACANNNNNNNNAAATCCAAGACAACACTACTAAGGCTTCTTTGGGARGGGG
AAGTAGGGATAGGTAAGAGGAAAGTAAGGGACCTCCTATCCAGCCTCCATGG
AATCCTGACTTCTTTTCCTTGTTATTTCAACTTCTTCCACCCCATCTTTTAA
ACTTTAGACTCCAGCCACAGAAGCTTACAACTAAAAGAAACTCTAAGGCCAA
TTTAATCCAAGGTTTCATTCTATGTGCTGGAGATGGTGTACAGTAGGGTGAG
GAAACCAAATTCTCAGTTRGCACTGGTGTACCCTTGTACAGGTGATGTAAYA
TCTCTGTGCCTCAGTTTGCTCACTATAAAATAGAGACGGTAGGGGTCATGGT
GAGCACTACCTGACTAGCATATAAGAAGCTTTCAGCAAGTGCAGACTACTCT
TACCCACTTCCCCCAAGCACAGTTGGGGTGGGGGACAGCTGAAGAGGTGGAA
ACATRTGCCTGAGAATCCTAATGAAATCGGGGTAAAGGAGCCTGGAACACAT
CCTGTGACCCCRCCTGT**(A/C)**CTGTAGGAAGCCAGTCTCTGGAAAGTAAAA
TGGAAGGGCTGCTTGGGAACTTTGAGGATRTTTAGCCCACCCCCTCATTTTT
ACTTGGGGAAACTAAGGCCCAGAGACCTAAGGTGACTGCCTAAGTTAGCAAG
GAGAAGTCTTGGGTATTCATCCCAGGTTKGGGGGACCCAATTATTTCTCAAT
CCCATTGTATTCTGGAATGGGCAATTTGTCCACGTCACTGTGACCTAGGAAC
ACGCGAATGAGAACCCACAGCTGAGGGCCTCTGCGCACAGAACAGCTGTTCT
CYCCAGGAAATCAACTTTTTTTAATTGAGAAGCTAAAAAATTATTCTAAGAG
AGGTAGCCCATCCTAAAAATAGCTGTAATGCAGAAGTTCATGTTCAACCAAT
CATTTTTGCTTACGATGCAAAAATTGAAAACTAAGTTTATTAGAGAGGTTAG
AGAAGGAGGAGCTCTAAGSAGAAAAAATCCTGTGCCGGGAAACCTTGATTGT
GGCTTTTTAATGAATGAAGAGGCCTCCCTGAGCTTACAATATAAAAGGGGGA
CAGAGAGGTGAAGGTCTACACATCAGGGGCTTGCTCT

IL-10-819

dbSNP: **rs1800871/SNP500Cancer ID: IL10-01 (**-853C>T)

TTACAGTCTAAACTGGAATGGCAGGCAAAGCCCCTGTGGAAGGGGAAGGTGA
AGGCTCAATCAAAGGATCCCCAGAGACTTTCCAGATATCTGAAGAAGTCCTG
ATGTCACTGCCCCGGTCCTTCCCCAGGTAGAGCAACACTCCTCGCCGCAACC
CAACTGGCTCCCCTTACCTTCTNNNNACACNNNNNNNNNNNNNNACACACA
ACACACACACACACACACACACANNNNNNNNAAATCCAAGACAACACTACT
AAGGCTTCTTTGGGARGGGGAAGTAGGGATAGGTAAGAGGAAAGTAAGGGAC
CTCCTATCCAGCCTCCATGGAATCCTGACTTCTTTTCCTTGTTATTTCAACTT
CTTCCACCCCATCTTTTAAACTTTAGACTCCAGCCACAGAAGCTTACAACTA
AAAGAAACTCTAAGGCCAATTTAATCCAAGGTTTCATTCTATGTGCTGGAGA
TGGTGTACAGTAGGGTGAGGAAACCAAATTCTCAGTTRGCACTGGTGTACCC
TTGTACAGGTGATGTAA**(C/T)**ATCTCTGTGCCTCAGTTTGCTCACTATAAA
ATAGAGACGGTAGGGGTCATGGTGAGCACTACCTGACTAGCATATAAGAAGC
TTTCAGCAAGTGCAGACTACTCTTACCCACTTCCCCCAAGCACAGTTGGGGT
GGGGGACAGCTGAAGAGGTGGAAACATRTGCCTGAGAATCCTAATGAAATCG
GGGTAAAGGAGCCTGGAACACATCCTGTGACCCCRCCTGTMCTGTAGGAAGC
CAGTCTCTGGAAAGTAAAATGGAAGGGCTGCTTGGGAACTTTGAGGATRTTT
AGCCCACCCCCTCATTTTTACTTGGGGAAACTAAGGCCCAGAGACCTAAGGT
GACTGCCTAAGTTAGCAAGGAGAAGTCTTGGGTATTCATCCCAGGTTKGGGG
GACCCAATTATTTCTCAATCCCATTGTATTCTGGAATGGGCAATTTGTCCAC
GTCACTGTGACCTAGGAACACGCGAATGAGAACCCACAGCTGAGGGCCTCTG
CGCACAGAACAGCTGTTCTCYCCAGGAAATCAACTTTTTTTAATTGAGAAGC
TAAAAAATTATTCTAAGAGAGGTAGCCCATCCTAAAA

IL-10-1082

dbSNP ID: **rs1800896/SNP500Cancer ID: IL10-03 (**-1116A>G)

GATGCAGAAAGGTGAAGAACTAGCAGAACACTGGAAATGCCCTCCATCTGGG
TCCATGGCTACTTAAGCTCAATGYTCCCTGGCAGGCAGGAGGACAGGTGCTA
TTGCCCTGTTGGGACAGATGAAAAACAGACACAGGGAGGATGAGTGATTTGC
CCTGACTATAGAGTGGCAGGGCCAAGGCAGAGCCCAGGCCTCCTGCACCTAG
GTCARTGTTCCTCCCAGTTACAGTCTAAACTGGAATGGCAGGCAAAGCCCCT
GTGGAAGGGGAAGGTGAAGGCTCAATCAAAGGATCCCCAGAGACTTTCCAGA
TATCTGAAGAAGTCCTGATGTCACTGCCCCGGTCCTTCCCCAGGTAGAGCAA
CACTCCTCGCCGCAACCCAACTGGCTCCCCTTACCTTCTNNNNACACNNNNN
NNNNNNNNNACACACACACACACACACACACACACACACACANNNNNNNNA
AATCCAAGACAACACTACTAAGGCTTCTTTGGGA**(A/G)**GGGGAAGTAGGGATA
GGTAAGAGGAAAGTAAGGGACCTCCTATCCAGCCTCCATGGAATCCTGACTTC
TTTTCCTTGTTATTTCAACTTCTTCCACCCCATCTTTTAAACTTTAGACTCC
AGCCACAGAAGCTTACAACTAAAAGAAACTCTAAGGCCAATTTAATCCAAGG
TTTCATTCTATGTGCTGGAGATGGTGTACAGTAGGGTGAGGAAACCAAATTC
TCAGTTRGCACTGGTGTACCCTTGTACAGGTGATGTAAYATCTCTGTGCCTC
AGTTTGCTCACTATAAAATAGAGACGGTAGGGGTCATGGTGAGCACTACCTG
ACTAGCATATAAGAAGCTTTCAGCAAGTGCAGACTACTCTTACCCACTTCCC
CCAAGCACAGTTGGGGTGGGGGACAGCTGAAGAGGTGGAAACATRTGCCTGA
GAATCCTAATGAAATCGGGGTAAAGGAGCCTGGAACACATCCTGTGACCCCR
CCTGTMCTGTAGGAAGCCAGTCTCTGGAAAGTAAAATGGAAGGGCTGCTTGG
GAACTTTGAGGATRTTTAGCCCACCCCCTCATTTTTACTTGGGGAAACTAAG
GCCCAGAGACCTAAGGTGACTGCCTAAGTTAGCAAGG

IL-1B–31 Probes

FAM (C) 5′ - CCT CGC TGT TTT TAT AGC TTT CAA AAG CAG- 3′

TET (T) 5′ -TCG CTG TTT TTA TGG CTT TCA AAA GCA G- 3′

IL-1B –31 Primers

Forward 5′ -CCC TTT CCT TTA ACT TGA TTG TGA- 3′

Reverse 5′ -GGT TTG GTA TCT GCC AGT TTC TC- 3′

IL-1B –511 Probes

FAM (C) 5′ -CTG TTC TCT GCC TCA GGA GCT CTC TGT C- 3′

TET (T) 5′ -TGT TCT CTG CCT CGG GAG CTC TCT G- 3′

IL-1B –511 Primers

Forward 5′ -TCC TCA GAG GCT CCT GCA AT- 3′

Reverse 5′ -TGT GGG TCT CTA CCT TGG GTG- 3′

IL-1B+3954 Probes

FAM (T) 5′ -CAG AAC CTA TCT TCT TTG ACA CAT GGG ATA ACG- 3′

TET (C) 5′ -CAG AAC CTA TCT TCT TCG ACA CAT GGG ATA AC- 3′

IL-1B+3954 Primers

Forward 5′ -CCT AAA CAA CAT GTG CTC CAC ATT- 3′

Reverse 5′ -TGC ATC GTG CAC ATA AGC CT- 3′

TLR4+896 Probes

FAM (G) 5′ -TAG ACT ACT ACC TCG ATG ATA ATT ATT GAC TTA TTT AAT TC- 3′

TET (A) 5′ -ACT ACT ACC TCG ATG GTA TTA TTG ACT TAT TTA ATT GT- 3′

TLR4+896 Primers

Forward 5′ -CCA TTG AAG AAT TCC GAT TAG CAT- 3′

Reverse 5′ -GGG AAA ATG AAG AAA CAT TTG TCA A-3′

TNF-238 Probes

FAM (A) 5′ -CCT CCC TGC TCC GAT TCC G- 3′

TET (G) 5′-TCC TCC CTG CTC TGA TTC CGA-3′

TNF-238 Primers

Forward 5′ -GCA TCA AGG ATA CCC CTC ACA-3′

Reverse 5′-ATC AGT CAG TGG CCC AGA AGA-3′

TNF-308 Probes

FAM (G) 5′-AAC CCC GTC CTC ATG CCC C-3′

TET (A) 5′-ACC CCG TCC CCA TGC CC-3′

TNF-308 Primers

Forward 5′-CAA AAG AAA TGG AGG CAA TAG GTT-3′

Reverse 5′-GCC CAC TGA CTG ATT TGT GTG T-3′

**TAMRA quencher was attached to all Taqman probes

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"Here's to the crazy ones, the misfits, the rebels, the troublemakers, the round pegs in the square holes, the ones who see things differently. They're not fond of rules, and they have no respect for the status quo. You can quote them, disagree with them, glorify or villify them...about the only thing you can't do is ignore them, because they change things, they push the human race forward. And while some may see them as the crazy ones, we see genius, because the people who are crazy enough to think they can change the world are the ones who do."

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