The role of gastrin in the development of gastric preneoplastic and neoplastic changes

'Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Medicine (MD) by *Senthil Vel Murugan Murugesan*.'

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List of abbreviations

ANOVA	One-Way Analysis of Variance
AFP	Alpha Fetoprotein
BMI	Body Mass Index
BRC	Biomedical Research Centre
cagA	Cytotoxin Associated Gene A
CGRP	Calcitonin Gene Related Peptide
CEA	CarcinoEmbryonic Antigen
CCK-2	Cholecystokinin-2
CgA	ChromograninA
CT	Computerised tomography
COPD	Chronic Obstructive Pulmonary Disease
DOH	Department of Health
DNA	Deoxyribonucleic Acid
ECL-cell	Enterochromaffin Cell Like-cells
EDTA	Ethylene Diamine TetraAcetic Acid
ENETS	European Neuroendocrine Tumour Society
ELISA	Enzyme Linked Immunoassay
EUS	Endoscopic Ultrasound
FAP	Familial Adenomatous Polyposis
FNA	Fine Needle Aspiration
GPC	Gastric Parietal Cell
GRP	Gastrin Releasing Peptide
H & E	Haematoxylin and Eosin
HB-EGF	Heparin-Binding Growth Factor

HDC	Histidine Decarboxylase
HDGC	Hereditary Diffuse Gastric Cancer
H. pylori	*Helicobacter pylori*
HGD	High Grade Dysplasia
H2 RA	Histamine 2 Receptor Antagonist
IGF	Insulin-like Growth Factor
IGF BP	Insulin-like Growth Factor Binding Protein
IL	Interleukin
IF	Intrinsic Factor
IM	Intestinal Metaplasia
LGD	Low Grade Dysplasia
mRNA	Messenger Ribonucleic Acid
MMP	Matrix Metalloproteinases
MALT	Mucosa Associated Lymphoid Tissue
MEN	Multiple Endocrine Neoplasia
NETs	Neuroendocrine Tumours
NICE	National Institute of Clinical Excellence
NIHR	National Institute of Health Research
NSAID	NonSteroidal Anti Inflammatory Drug
OD	Optical Density
PAI	Pathogenecity Island
PAS	Periodic Acid Shiff
PET	Positron Emission Tomography
PCR	Polymerase Chain Reaction
PPI	Proton Pump Inhibitors
PSA	Prostate Specific Antigen

RIA	Radioimmunoassay
RUT	Rapid Urease Test
SPEM	Spasmolytic Polypeptide-Expressing Metaplasia
TIMPs	Tissue Inhibitors of Matrix Metalloproteinases
TNF	Tumour Necrosis Factor
TNM	Tumour Node Metastasis
uPA	Urokinase Plasminogen Activator
vacA	Vacuolating Cytotoxin Gene A
VMAT-2	Vesicular Monoamine Transporter-2
WHO	World Health Organisation
ZES	Zollinger Ellison Syndrome

‘The role of gastrin in the development of gastric preneoplastic and neoplastic changes’

Senthil Murugesan

Abstract

The hormone gastrin regulates gastric acid secretion and through its effects on cell proliferation, apoptosis and angiogenesis also regulates gastric epithelial and enterochromaffin-like (ECL) cell growth. The influence of various factors (host, bacterial and environmental) upon fasting serum gastrin concentrations and to what extent these factors interact to influence the progression of gastric preneoplastic pathology is not fully understood.

Long standing hypergastrinaemia secondary to hypochlorhydria resulting from autoimmune gastritis can result in the development of ECL-cell hyperplasia. In some patients this progresses to type-1 gastric neuroendocrine tumour formation. The factors that influence this progression have not been fully characterised.

The management of type-1 gastric neuroendocrine tumours is dependent on their size. However, there is still controversy regarding the optimal management of larger (> 1cm) tumours. Antrectomy is one option and the results of an octreotide suppression test (to determine gastrin dependency of type-1 gastric neuroendocrine tumours in order to predict response to antrectomy) have been reported in a single patient.

This aims of this thesis were to assess:

1. The interaction between various factors (host, bacterial and environmental) that may influence fasting serum gastrin concentrations and the development of gastric preneoplastic pathology.

2. The roles of certain factors in the progression of ECL-cell hyperplasia to type-1 gastric neuroendocrine tumours.

3. The role of an octreotide suppression test in identifying patients with type-1 gastric neuroendocrine tumours who may benefit from antrectomy.

In a large cohort of >1000 prospectively recruited patients, we demonstrated that in addition to *H. pylori* infection, the presence of a host factor (advancing age), a bacterial virulence factor (*cag*A) and elevated fasting serum gastrin concentrations (>100pM) were significantly associated with the presence of gastric preneoplastic pathology. Concurrent proton pump inhibitor therapy was however not associated with the presence of gastric preneoplastic pathology.

The interactions between *H. pylori* infection, proton pump inhibitor use and the presence of gastric preneoplastic pathology in determining fasting serum gastrin concentrations were found to be complex. In addition, other host and environmental factors also influenced fasting serum gastrin concentrations.

Although results from our study did not demonstrate any statistically significant associations, there did appear to be correlations between the presence of factors such as hypothyroidism, positive anti-gastric parietal and intrinsic factor antibodies and extent of gastric atrophy with the presence of more advanced degrees of gastric ECL-cell hyperplasia.

Although a positive octreotide suppression test was associated with tumour regression following antrectomy in four patients with type-1 gastric neuroendocrine tumours, a fifth patient who had a positive test did not show tumour regression and needed additional surgery.

In conclusion, gastrin appears to act as an important co-factor in the pathogenesis of epithelial and neuroendocrine neoplasia in the stomach, but interactions with other factors are complex.

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Chapter 1

1 Introduction

1.1 Stomach – anatomy and physiology

1.1.1 Gross anatomy of stomach

The human stomach is a ‘J’ shaped organ of the gastrointestinal tract continuous with the oesophagus proximally and duodenum distally. Acting primarily as a reservoir of ingested food, it ensures a controlled release of food into the distal duodenum (which is of a smaller calibre), thereby controlling digestion and absorption of nutrients. The stomach is divided into four main anatomical regions (Fig 1-1):

1. Cardia - located immediately distal to the oesophagogastric junction and the lower oesophageal sphincter
2. Fundus - this is a dome shaped elevation of the stomach, projecting upwards above the cardia and the oesophagogastric junction and lying in contact with the left hemidiaphragm
3. Corpus or body - the largest part of the stomach which lies immediately distal to the fundus
4. Antrum - the distal end of the stomach which continues into the duodenum through the pylorus.

The demarcation between the various regions is however often variable but these four regions correspond approximately with the mucosal histology.

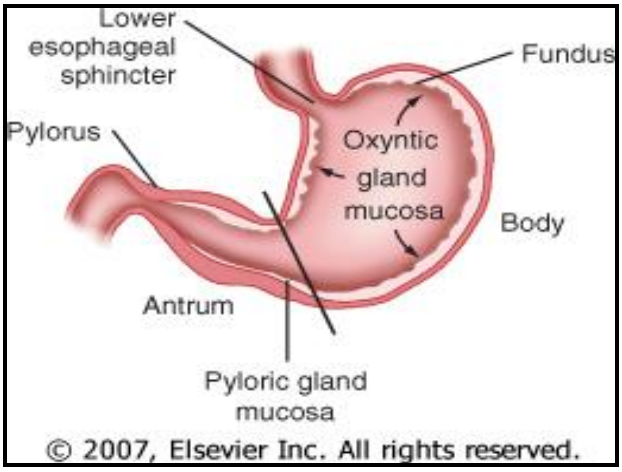


Figure 1-1: Cross sectional view of the human stomach showing the anatomic regions of the stomach.

The line is drawn from the incisura angularis along the lesser curvature to an indistinct border between the antrum and corpus along the greater curvature (adapted from Sleisenger and Fordtran's Gastrointestinal and Liver disease online 8th edition).

1.1.2 Tissue layers of the stomach

The stomach wall is composed of four layers:

1. Mucosa - innermost and lines the gastric lumen
2. Submucosa - lying immediately deep to the mucosa and containing connective tissue, arterioles, venules, lymphatics and the submucosal plexus
3. Muscularis propria - contains three muscle layers namely the inner oblique, middle circular and outer longitudinal

4. Serosa - final and outermost layer which is a continuation of the peritoneal layer

1.1.3 Mucosal cell types and function

The gastric mucosa is composed of a layer of columnar epithelial cells measuring 20 to 40µm in height. These form the surface mucosal cells which secrete mucus, and are the same throughout the stomach. This surface layer is invaginated by gastric pits providing access to the lumen for gastric glands. The different regions of the stomach contain different types of gastric glands (Owen, 1986).

1.1.3.1 Parietal or Oxyntic gland

These are predominantly located in the gastric fundus and the body (corpus) and consist of parietal, chief (or peptic), mucous neck and endocrine cells. The primary function of the oxyntic gland is secretion of acid, intrinsic factor and other enzymes. The principal cell of the oxyntic gland is the parietal cell which secretes acid at a rate of $3x10^6$ hydrogen ions per second, achieving a final hydrochloric acid concentration of 160mmol/L (Kopic et al., 2010). The parietal cells also secrete intrinsic factor which is essential for the absorption of vitamin B12.

A typical oxyntic gland is divided into three areas, namely the isthmus (where the surface mucous cells predominate), the neck (containing parietal and mucous neck cells) and the base (which consists predominantly of chief cells, but with some parietal and mucous neck cells) (Fig1-2).

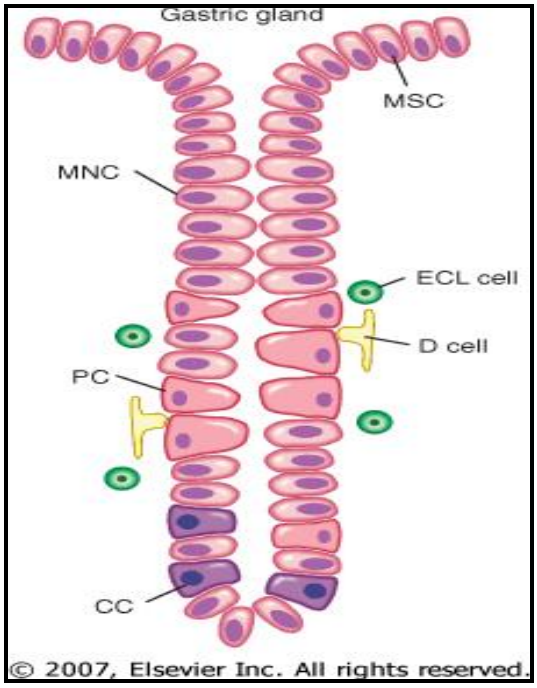


Figure 1-2: Schematic representation of oxyntic gland containing mucous surface cells (MSC), mucous neck cells (MNC), enterochromaffin–like cells (ECL), acid secreting parietal cells (PC), somatostatin secreting D cells (D cells) and pepsin secreting chief cells (CC).

(Adapted from Sleisenger and Fordtran's Gastrointestinal and Liver disease online 8th edition).

1.1.3.2 Antrum and pyloric glands

The antrum (and pylorus) is lined by coiled glands containing epithelial and endocrine cells. The epithelial cells are predominantly mucus secreting; a small number of pepsinogen-II- secreting oxyntic cells are also found (Owen, 1986).

1.1.3.3 Endocrine Cells

The stomach contains several types of endocrine and endocrine-like cells, many of which are involved in the regulation of gastric exocrine secretion. Gastrin cells (G cells) are located in the pyloric glands and somatostatin secreting D cells are located close to the G cells and are also

present in the corpus. Histamine secreting enterochromaffin-like cells (ECL cells) are seen only in the oxyntic glands in close proximity to parietal cells. ECL-like cells and mast cells which are located in the lamina propria of the gastric mucosa contain histamine, an important stimulator of gastric acid secretion.

1.2 Gastric secretion

The human stomach secretes water, electrolytes (including H^+, K^+, Na^+, Cl^- and $HCO3^-$), enzymes and glycoproteins (intrinsic factor and mucins). These are summarised in table 1-1.

Exocrine product	Function	Secretory cell type
Hydrochloric acid	• Provides optimal pH for pepsin and gastric lipase activation • Duodenal iron absorption • Negative feedback inhibition of gastrin release • Stimulates pancreatic $HCO3^-$ release • Defence against ingested microbes	Parietal cell, via the H^+/K^+ - ATPase (the proton pump)
Pepsins	• Hydrolysis of dietary proteins • Vitamin B_{12} separation	Chief cell
Gastric lipase	• Early hydrolysis of dietary triglycerides	Chief cell
Intrinsic factor	• Vitamin B_{12} binding, for absorption at specific receptors in the terminal ileum	Parietal cell
Mucin and $HCO3^-$	• Protection against HCl and pepsins	Mucous neck cell and chief cell

Table 1-1: Gastric exocrine secretory products, secretory cell type and physiological functions.

1.2.1 Gastric acid secretion

Hydrochloric acid secretion in the stomach occurs under basal and stimulated conditions. Basal secretion follows a circadian pattern, with highest secretion occurring at night and lowest secretion being during the early morning hours. Cholinergic stimulation via the vagus nerve and histamine stimulation are the major stimulants of basal acid secretion. Stimulated acid secretion occurs primarily in three phases, which are named cephalic, gastric and intestinal depending on the site where the signal originates (Soll and Walsh, 1979).

The cephalic phase refers to acid secretion in response to the sight, smell or taste of food by vagal nerve stimulation. The gastric phase is initiated as soon as food enters the stomach. This phase may be induced by two possible mechanisms. Firstly, the nutrients in the ingested food stimulate gastrin release, which in turn activates acid secretion (explained in detail in section 1.3.1) and secondly distension of the stomach stimulates stretch receptors and the subsequent stimulation of the vagal nerve results in acid secretion. The intestinal phase is initiated as food enters the intestine and is stimulated by intestinal distension and nutrient absorption (Feldman and Richardson, 1986).

During these phases, several series of pathways that inhibit acid secretion are also set into motion. These are predominantly mediated by somatostatin.

The acid secreting parietal cell, located in the oxyntic gland, expresses receptors for several of the acid stimulants, namely the

histamine (H2) receptor, CCK-2 receptor (gastrin receptor) and acetylcholine (muscarinic M3) receptor. Stimulation of the H2 receptor following binding by histamine leads to activation of adenylate cyclase and an increase in cyclic AMP. The stimulation of the CCK-2 and M3 receptors results in the activation of the protein kinase C and phosphoinositide signalling pathways. These regulate several downstream pathways that control the acid secreting pump-H^+/K^+-ATPase (Schubert, 2009).

1.2.2 The proton pump (H^+/K^+-ATPase) and acid secretion

This enzyme is responsible for generating the high concentration of H^+ (hydrogen) ions in the parietal cell. It is a membrane bound protein that consists of two subunits named α and β. The active catalytic site is found within the α-subunit.

The proton pump is located within the secretory canaliculus and also in the nonsecretory cytoplasmic tubulovesicles. It utilises the energy from ATP (Adenosine triphosphate) to transfer H^+ ions from the parietal cell cytoplasm to the secretory canaliculi in exchange for K^+ (potassium). The nonsecretory cytoplasmic tubulovesicles are impermeable to K^+ leading to an inactive pump at this location. In the resting state only 5% of the pumps are within the secretory canaliculus. Upon parietal cell stimulation, tubulovesicles are transferred to the secretory canalicular membrane, where about 60-70% of the pumps are activated. Proton pumps are then recycled back to the inactive state in cytoplasmic vesicles once parietal cell stimulation ceases (Schubert, 2009; Kopic et al., 2010).

Acid is generated within the parietal cell from the hydration of CO_2 to form H^+ and HCO_3^-, catalysed by the enzyme carbonic anhydrase. The H^+ formed in this reaction is secreted by the proton pump in exchange for K^+ and the HCO_3^- is exchanged for Cl^- thereby maintaining a slightly alkaline pH within the parietal cell (Fig 1-3).

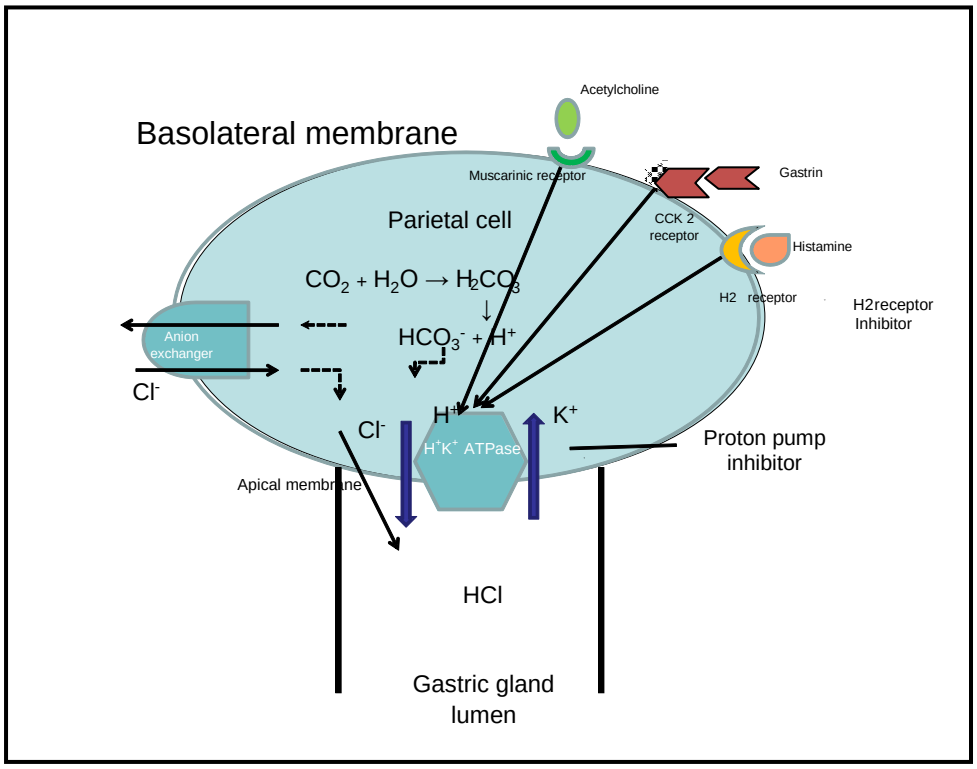


Figure 1-3: Model of gastric acid secretion by the parietal cell.

1.2.3 Regulation of H^+ secretion

Several factors are involved in the complex regulation of acid secretion and these include both stimulators and inhibitors of gastric acid secretion.

1.2.3.1 Stimulators of acid secretion

1.2.3.1.1 **Gastrin**

Gastrin secreted by the G-cells of the antrum is the most potent stimulator of acid secretion. Luminal amino acids stimulate gastrin release. Gastrin then acts via CCK-2 receptors located on gastric ECL-cells to stimulate the release of stored histamine. This released histamine subsequently stimulates acid secretion from gastric parietal cells through binding to H2 receptors. The role played by gastrin in the regulation of gastric physiology is described in more detail in section 1.3.

1.2.3.1.2 **Acetylcholine, GRP and other neurotransmitters**

Acetylcholine

Acetylcholine released from postganglionic neurons acts on the parietal cell muscarinic (M3) receptors to stimulate acid secretion. Its inhibition of somatostatin secretion by stimulation of M2 and M4 receptors on somatostatin D cells appears to be another mechanism by which it promotes acid secretion. Acetylcholine and cholinergic neurons also appear to mediate the acid secretion stimulated by gastric distension (gastric phase) and during the cephalic phase (Perez-Zoghbi et al., 2008).

Gastrin releasing peptide (GRP)

This is a 27-amino acid neuropeptide first recognised in 1975 (McDonald et al., 1978). GRP has been shown to directly stimulate gastrin

release (Giraud et al., 1987), but it also stimulates somatostatin release thereby exhibiting both stimulatory and inhibitory effects on acid secretion.

Other neurotransmitters

Calcitonin gene related peptide (CGRP) (Manela et al., 1995) and pituitary adenylate cyclase-activating peptide (PACAP) (Li et al., 2000) have also been shown to regulate gastric acid secretion in humans.

1.2.3.1.3 **Histamine and ECL-cells**

ECL-cells, located in the oxyntic mucosa, play a major role in gastric acid secretion. These cells synthesise histamine from histidine by the enzyme histidine decarboxylase (HDC). ECL-cells which have been stimulated by gastrin, cholecystokinin (CCK) or acetylcholine release stored histamine, which in turn stimulates parietal cells to produce acid via the H2 receptor (Fykse et al., 2006).

The major inhibitor of ECL-cells is somatostatin. Histamine acting via H3 receptors can also inhibit histamine secretion via autocrine inhibition (Vuyyuru and Schubert, 1997).

The major role played by histamine in the gastrin mediated stimulation of acid secretion has been demonstrated by studies in which depletion of histamine or targeted disruption of HDC eliminates the acid response to gastrin stimulation (Chen et al., 2006).

1.2.3.2 Inhibitors of acid secretion

Several factors and mechanisms play a key role in the inhibition of gastric acid secretion:

1.2.3.2.1 **Negative feedback inhibition**

When the pH in the lumen of the stomach falls below 3 as a result of gastrin mediated acid release, further release of gastrin from G cells is inhibited. Amino acids that are stimulants for gastrin release are ionised by the secreted acid, thereby reducing their stimulatory effect on further gastrin release.

1.2.3.2.2 **Somatostatin**

Somatostatin is the main inhibitor of gastric acid production and is produced by the somatostatin secreting D cells, located in the oxyntic mucosa, in response to both a fall in the intraluminal pH of the stomach and also by gastrin. Circulating CCK (cholecystokinin) also appears to stimulate D-cells via CCK-1 receptors. Somatostatin probably inhibits parietal cells directly, but its major effects on acid secretion are mediated by inhibition of ECL-cells and G-cells.

Somatostatin is synthesised from a 92-aminoacid precursor preprosomatostatin. This precursor is processed to somatostatin-14 and somatostatin-28 subunits, the former being predominantly distributed in the stomach and pancreatic islet cells, whereas the latter is present in the small intestine. In the stomach, somatostatin cells are closely coupled to their target cells and this provides a tonic restraint on acid secretion (Schubert et al., 1987). Somatostatin exerts its inhibitory effect on gastrin

secretion through its effects on the somatostatin subtype-2 receptor via the induction of menin, a tumour suppressor gene, which has been shown to directly inhibit gastrin gene expression (Mensah-Osman et al., 2008).

1.2.3.2.3 **Secretin**

Secretin has been shown to decrease acid secretion by its ability to suppress the release of gastrin and also by direct non-competitive inhibition of the action of gastrin at the parietal cell. Low luminal pH following meal stimulated acid secretion stimulates duodenal S cells to release secretin(Johnson and Grossman, 1969). The main effect of secretin is to stimulate the release of alkaline pancreatic juice which helps neutralise the acidic juice from the stomach. This results in negative feedback inhibition of further secretin release and also inhibition of antral gastrin release. Secretin receptors have also been shown on the surface of other neuroendocrine cells including gastrinomas and secretin can stimulate these cells to release gastrin. This forms the basis of the secretin stimulation test for diagnosing Zollinger Ellison syndrome (Berna et al., 2006a).

1.2.3.2.4 **Nitric oxide and Dopamine**

Both nitric oxide and dopamine have been shown in animal studies to inhibit acid secretion. Ito *et al*. have shown increased acid production in rats in the presence of a nitric oxide synthase inhibitor (Ito et al., 2008). Similarly D_2 receptors have been isolated in the stomach and the D_2 receptor agonist quinpirole has been shown to inhibit histamine and carbachol stimulated acid secretion in rats (Eliassi et al., 2008).

1.3 The hormone gastrin and its roles in the regulation of gastric acid secretion and development of gastric tumours

The hormone gastrin has a well established role in the regulation of acid secretion. However it is now known that gastrin also plays a major part in regulating gastric epithelial cell proliferation, apoptosis, migration and invasion as well as gastric tumour angiogenesis.

1.3.1 Gastrin synthesis and release

The gene encoding gastrin is located at chromosome 17q21 (Ito et al., 1984). Gastrin is synthesised in its precursor form, preprogastrin, in the endoplasmic reticulum of gastrin (G)-cells, located in the gastric antrum. The hormone is mainly expressed in the gastric antrum, but there is evidence to suggest that the gene is also expressed in some colorectal tumours, pituitary gland and testis although at lower concentrations at the latter two sites (Wang and Dockray, 1999). Preprogastrin is subsequently enzymatically cleaved to progastrin which is stored in storage vesicles prior to secretion. In these storage vesicles, progastrin undergoes phosphorylation and amidation to form both G34 and G17 amidated gastrins (Fig 1- 4).

The amidated forms bind specifically to CCK-1 and CCK-2 (or CCK-B) receptors. These belong to the G-protein coupled receptor family and gastrin exhibits greater binding affinity to the CCK-2 rather than the CCK-1 receptor. CCK-2 receptors are located on the surface of parietal cells and

enterochromaffin-like (ECL) cells in the gastric corpus, as well as in certain cells in the pancreas, smooth muscle and brain. The effect of direct gastrin binding to CCK-2 receptors located on gastric parietal cells is still under investigation, but recent evidence suggests that it plays a role in the regulation of parietal cell maturation (Chen et al., 2000; Kirton et al., 2002).

Gastrin is released in its amidated form from antral G-cells by various stimuli, including distension of the stomach following a meal, luminal contents especially aromatic amino acids, calcium and amines present in ingested food, vagal stimulation mediated by acetylcholine and by gastrin releasing peptide (GRP), a neurotransmitter (Schubert, 2009).

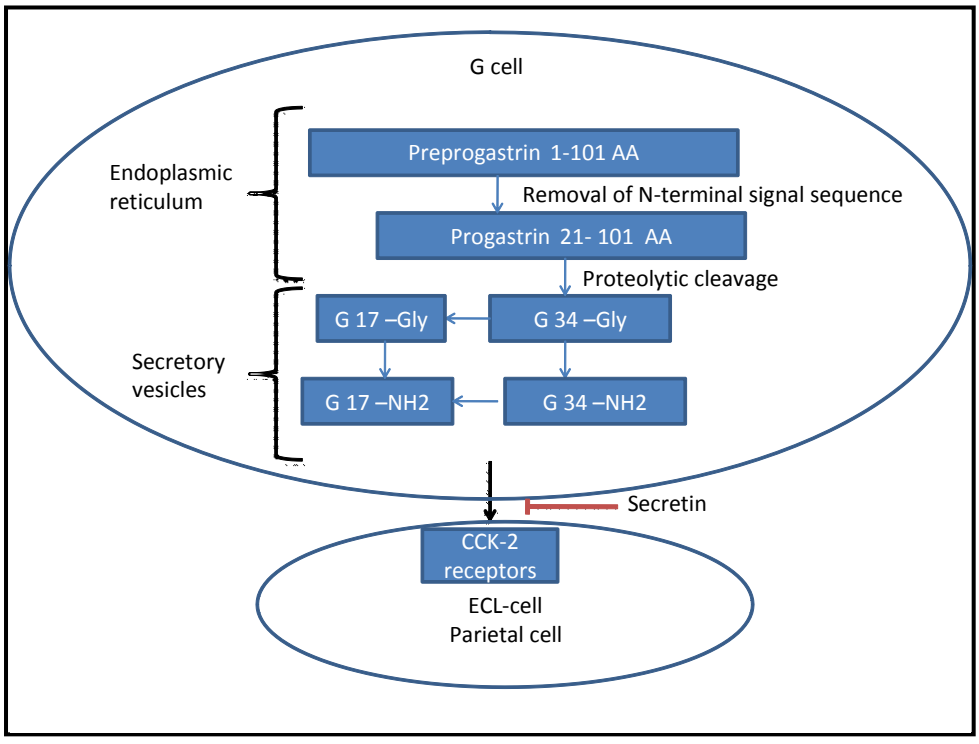


Figure 1-4: Gastrin synthesis and secretion

1.3.2 Roles of Gastrin

1.3.2.1 Acid secretion

As discussed above, gastrin stimulates histamine release by stimulation of ECL-cells located in the region of the oxyntic glands through binding to CCK-2 receptors. The released histamine subsequently binds to the H2-receptors on parietal cells, which in turn are stimulated to release hydrochloric acid (Dockray, 2004; Schubert, 2009). This appears to be the main route by which gastrin stimulates acid secretion (Fig1-5). Gastrin also stimulates the synthesis and storage of histamine by ECL cells, by inducing histidine decarboxylase expression and also promotes the sequestration of histamine in secretory vesicles by stimulating the expression of vesicular monoamine transporter-2 (VMAT-2) in ECL cells (Dockray et al., 2001). As explained in section 1.3 and outlined in fig 1-5, there are many factors that stimulate the release of gastrin.

Both somatostatin and secretin are thought to inhibit gastrin release. Somatostatin is released by gastric D cells in response to an acidic pH and therefore gastrin secretion is controlled by negative feedback inhibitory mechanisms. Secretin on the other hand acts through non-competitive inhibition of gastrin at the gastric parietal cell (Johnson and Grossman, 1969).

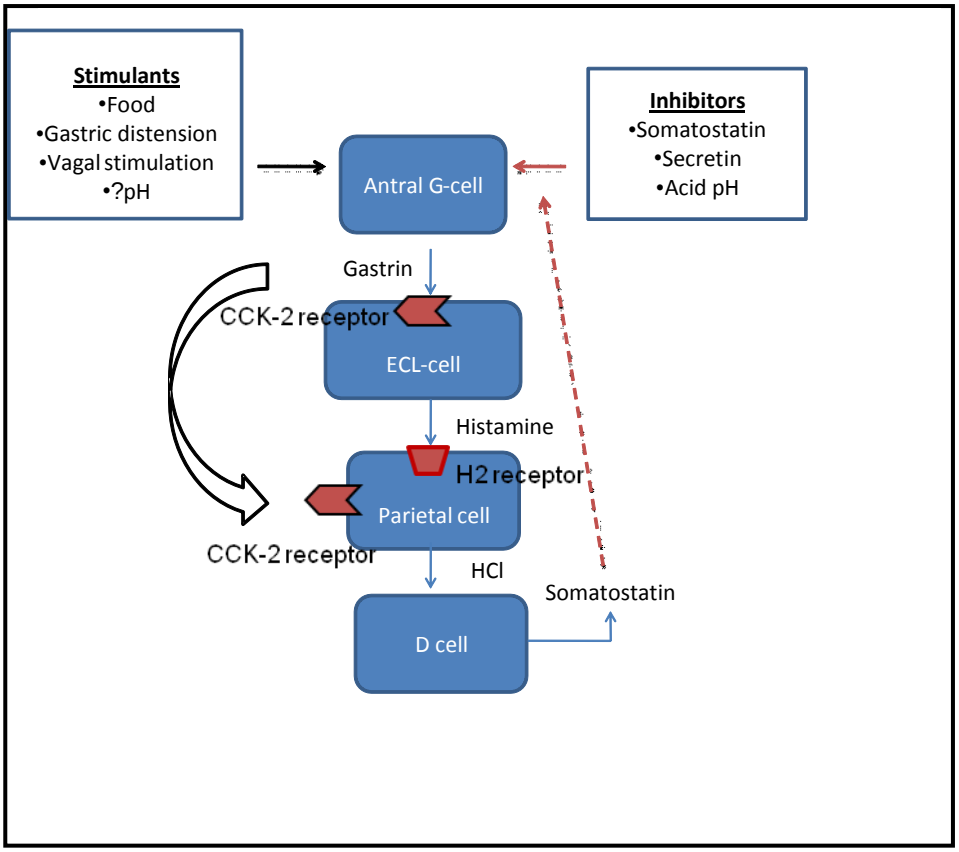


Figure 1-5: Role of inhibitors and stimulants of acid release

1.3.2.2 Cellular proliferation, apoptosis, migration and angiogenesis

Many of the effects of gastrin have been studied in a range of animal models and cell lines (Burkitt et al., 2009). Gastrin has been found to stimulate gastric epithelial cell proliferation and gastric gland elongation in hypergastrinaemic rodents (Kidd et al., 2000). It has also been shown to stimulate the proliferation of epithelial cells resulting in either parietal cell or ECL-cell hyperplasia. This may be secondary to a direct effect on ECL-cells through CCK-2 receptor stimulation or by paracrine mechanisms mediated through signalling messengers and growth factors such as heparin-binding growth factor (HB-EGF) (Miyazaki et al., 1999). The effect

of gastrin on the angiogenic potential of endothelial cells has also been described. Gastrin acting through the CCK-2 receptor has been shown to enhance endothelial cell activity, probably mediated through enhanced expression of HB-EGF (Clarke et al., 2006). This pro-angiogenic effect may contribute to the role played by gastrin in gastric tumour development.

The effects of gastrin on the gastric mucosa have recently become better understood by studying various transgenic and knockout mice. Mice in which either the gastrin gene (GAS KO mice) or the CCK-2 receptor have been deleted exhibit similar phenotypes, with decreased numbers of gastric parietal and ECL-cells (Langhans et al., 1997). Gastric pH is elevated in such mice and they exhibit a poor acid secretory response to externally administered gastrin or histamine (Friis-Hansen et al., 1998; Miyazaki et al., 1999). By contrast, mice that over express gastrin (INS-GAS–Insulin-Gastrin transgenic mice that over express gastrin in pancreatic β-cells and therefore have elevated serum concentrations of amidated gastrin) initially show increased parietal cell proliferation and acid secretion. Older INS-GAS mice (>20 months) show a tendency to developing gastric cancer and this process is greatly accelerated by concurrent infection with *Helicobacter felis* or *Helicobacter pylori* (Wang et al., 2000; Fox et al., 2003). Progression to gastric atrophy was associated with decreased parietal cell numbers and subsequent hypochlorhydria. At 20 months the INS-GAS mice showed increased gastric metaplasia, dysplasia and invasive carcinoma. This was associated with increased expression of growth factors including heparin binding epidermal growth factor (HB-EGF) and transforming growth factor alpha (TGFα). There was

also an associated decrease or cessation in the previously noted increase in ECL-cell numbers. *Helicobacter felis* co-infection led to accelerated progression to invasive carcinoma by 6 months (Wang et al., 2000).

Gastrin also appears to modulate apoptosis by signalling through CCK-2 receptors. It appears to increase the susceptibility of normal gastric epithelial cells to apoptosis. *Mastomys* (rodents) treated with H2-receptor antagonists have been shown to have a 2-fold increase in apoptosis in the presence of hypergastrinaemia when compared to controls (Kidd et al., 2000). Both Cui *et al* and Przemeck *et al* have also demonstrated that hypergastrinaemia increases the susceptibility to apoptosis in INS-GAS mice following exposure to either *Helicobacter* bacteria or gamma irradiation (Cui et al., 2006; Przemeck et al., 2008). In their study, Przemeck *et al* compared the effects of irradiation on gastric epithelial apoptosis in wild-type FVB/N mice and INS-GAS mice. INS-GAS mice consistently developed greater amounts of apoptosis both 6 hours and at 48 hours following irradiation. This effect was diminished in those mice which were pre-treated with the CCK-2 receptor antagonist YM022, suggesting that the increased susceptibility to apoptosis rendered by gastrin was mediated via the CCK-2 receptor. What was also demonstrated in this study was that the precursor forms of gastrin namely progastrin and glycine-extended gastrins did not significantly increase gastric epithelial susceptibility to apoptosis. However glycine-extended gastrin has been shown to modulate the effects of concurrent *Helicobacter* infection in INS-GAS mice (Wang et al., 2000). In humans, those with moderate hypergastrinaemia (defined in the study as a fasting serum

gastrin concentration of >150pM) were also shown to have more apoptosis in gastric corpus biopsies in the presence of *Helicobacter pylori* infection (Przemeck et al., 2008).

The effect of gastrin on AGS_{Gr} cells (a gastric adenocarcinoma cell line stably expressing the CCK-2 receptor) suggests that gastrin also regulates cellular migration and invasion. Upon gastrin stimulation, AGS_{Gr} cells show changes in migration mediated via the CCK-2 receptor. This was observed when AGS_{Gr} cells where co-cultured in the presence of gastrin; however this effect was not seen in untransfected AGS cells (Noble et al., 2003). These effects on migration and invasion appear to be at least partially mediated by members if the matrix metalloproteinase (MMP) family of proteins especially MMP-7 and -9, both of which show increased expression in response to gastrin stimulation (Wroblewski et al., 2002; Varro et al., 2007).

1.3.2.3 The development of ECL-cell hyperplasia and gastric carcinoid tumours

Gastric neuroendocrine tumours (type-1 carcinoid tumours) develop in some cases in response to chronic hypergastrinaemia (Fig 1-6). They arise from ECL-cells which are normally present in the oxyntic corpus mucosa of the stomach. Although hypergastrinaemia stimulates ECL-cells to increase in numbers, this stimulus alone appears to be insufficient to cause progression to carcinoid tumour formation. Pernicious anaemia or autoimmune atrophic gastritis is typically associated with the

hypergastrinaemia that is responsible for the development of type-1 gastric carcinoid tumours. In this condition, there is a net reduction in parietal cell numbers leading to gastric atrophy and subsequent hypo/achlorhydria. This results in 'secondary' hypergastrinaemia due to the loss of negative feedback inhibition of gastrin secretion (by acidic gastric juice). Achlorhydria results in an increase in the number of antral G-cells and subsequent hypergastrinaemia. Fasting serum gastrin concentrations are often >1000pM and result in ECL-cell hyperplasia. Prolonged stimulation results in progression of ECL hyperplasia to linear, nodular and adenomatoid hyperplasia and finally results in the development of frank carcinoid tumours.

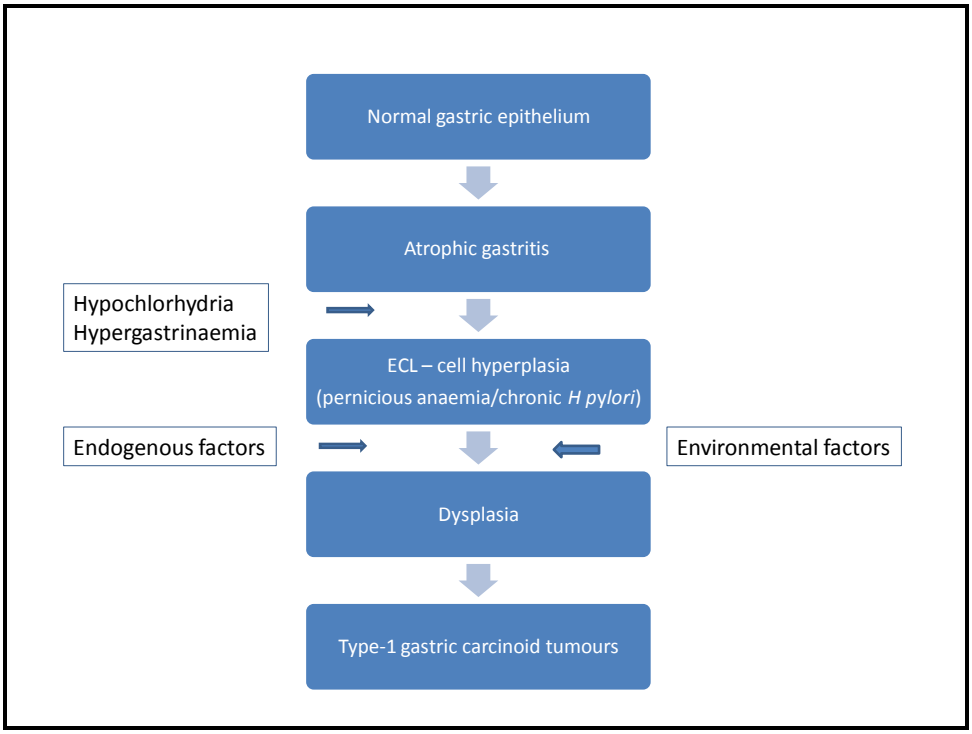


Figure 1-6: Development of atrophic gastritis and progression to type-1 gastric carcinoid tumours

Type-2 gastric neuroendocrine tumours are seen in some patients with mutations in the MEN1 gene (Wermer, 1954). This gene codes for the 610-amino acid protein menin which acts as a tumour suppressor and mutations in this gene result in either a reduced expression or complete absence of menin (Piecha et al., 2008). In type-2 gastric neuroendocrine tumours, there is inappropriate hypergastrinaemia in the presence of an acidic gastric juice as a result of a gastrinoma and Zollinger Ellison syndrome. Type-2 gastric neuroendocrine tumours are seen in about 20% of patients with MEN-1 and Zollinger Ellison syndrome (Gibril et al., 2004).

Type-3 gastric neuroendocrine tumours, which are seen more commonly in men, are sporadic lesions which are larger than typical type-1 and 2 tumours. They are associated with a normal gastric juice pH and a normal fasting serum gastrin concentration. These tumours have the greatest malignant potential compared to the type-1 and -2 tumours and are therefore associated with a poorer prognosis.

Type-1 and -2 gastric neuroendocrine tumours are therefore associated with hypergastrinaemia. In type-1 tumours, ECL-cell hyperplasia is thought to be secondary to the direct proliferative effects of gastrin on ECL-cells. This hypergastrinaemia is secondary to the achlorhydria that results from gastric corpus atrophy. However, there are several other conditions that can also result in chronic hypergastrinaemia, including chronic PPI (proton pump inhibitor) use, vagotomy, chronic renal failure (table 1-2). However such conditions have not been shown to be associated with the development of gastric carcinoid tumours in humans, although rats exposed to chronic PPIs can develop gastric carcinoid

tumours. This therefore suggests that there are other co-factors involved in the progression of ECL-cell hyperplasia to frank carcinoid tumours. Factors that may promote this progression include concurrent bacterial colonisation of the stomach (due to the loss of the protective acid milieu), nitroso compounds in the ingested food and unopposed bile reflux. Gastrin has also been suggested to play an important role in mesenchymal signalling and in the remodelling of the extracellular matrix as discussed in section 3.2.2.

1.3.2.4 Precursor gastrins and their role in gastrointestinal pathology

The immediate precursors of amidated gastrin namely progastrin and the glycated gastrin (Gly-gastrin) have been identified in gastric neuroendocrine tumours and epithelial gastrointestinal cancers (Nemeth et al., 1993; Van Solinge et al., 1993). This finding suggests that the precursors of gastrin may play a role in the development of gastric neoplastic pathology. Although increased concentrations of Gly-gastrin have been demonstrated in adenomatous colonic polyps, so far no relationship has been established between the concentrations of Gly-gastrin and stage of colorectal cancer or with serum gastrin concentrations (Siddheshwar et al., 2001).

Animal studies have demonstrated the proliferative effect of Gly-gastrin and mediation of action via receptors distinct from CCK-2 but this has not been demonstrated in human studies (Seva et al., 1994).

So far there have been no reliable assays to measure serum concentrations of total gastrins. Most assays measure the biologically

active ‘amidated gastrin’ concentrations which mediate their effects by binding to the CCK-2 receptor.

1.3.2.5 Causes of hypergastrinaemia

The normal upper limit of fasting serum gastrin concentration in humans is usually recognised to be 40pM (Varro and Ardill, 2003). There are several causes of hypergastrinaemia which are summarised in the following table.

Acidic gastric pH 1. Gastrinoma 2. Antral predominant *H. pylori* infection 3. Pyloric obstruction 4. Renal failure and uraemia 5. Post gastric resection with an intact antrum (eg. Billroth type II)
Elevated gastric pH 1. Chronic atrophic gastritis associated with pernicious anaemia (autoimmune gastritis) and chronic *H. pylori* infection (corpus predominant infection). 2. Proton pump inhibitor (PPI) therapy 3. H2 receptor antagonist therapy 4. Post vagotomy

Table 1-2: Causes of hypergastrinaemia in relation to the pH of the stomach.

1.3.2.5.1 **Hypergastrinaemia associated with an acidic gastric pH:**

Hypergastrinaemia in the presence of an acidic gastric pH is most often due to a gastrin secreting tumour as seen in Zollinger Ellison syndrome (ZES). First described in 1955, the syndrome is characterised by the occurrence of multiple gastric and duodenal ulcers and diarrhoea in

association with the presence of pancreatic neuroendocrine tumours (Zollinger and Ellison, 1989). ZES may be sporadic or associated with the MEN (Multiple Endocrine Neoplasia) type-1 syndrome. The latter is characterised by adenomas in the pituitary and parathyroid glands as well as in the pancreas. MEN type-1 syndrome is inherited as an autosomal dominant disorder and results from the loss of menin, a tumour suppressor gene (Wermer, 1954). Fasting serum gastrin concentrations >1000pM in the presence of an acidic gastric pH are virtually confirmatory of ZES, but many patients have less profound hypergastrinaemia (Berna et al., 2006b).

Patients with pyloric obstruction have also been reported to have markedly elevated fasting serum gastrin concentrations and relief of obstruction has been associated with normalisation of the elevated serum gastrin concentration (Feurle et al., 1972). Fasting serum gastrin concentrations are often found to be elevated from around twice the upper limit of normal to approximately 380pM (Tani and Shimazu, 1977). This suggests the possible role of mechanical factors, including stretch receptors, in influencing serum gastrin concentrations (Feurle et al., 1972).

Most patients with chronic renal failure also exhibit hypergastrinaemia with about 10% exhibiting serum gastrin concentrations in the range often seen in ZES (Hallgren et al., 1978). Gastrin is partly cleared from the circulation by renal metabolism and the degree of hypergastrinaemia is often proportional to the severity of renal impairment (Korman et al., 1972).

Excluded gastric antrum following incomplete excision of this portion of the stomach has also been found to be associated with

hypergastrinaemia, which is usually mild to moderate (2 to 3 times upper limit of normal). However with changes in surgical practice, this diagnosis is now extremely rare (Friesen and Tomita, 1981; Webster et al., 1978).

1.3.2.5.2 **Hypergastrinaemia associated with an elevated gastric pH**

Chronic atrophic gastritis secondary to either autoimmune gastritis (often associated with pernicious anaemia) or chronic *H. pylori* infection is the most common cause of elevated fasting serum gastrin concentrations (Orlando et al., 2007). The degree of hypergastrinaemia associated with both these conditions is usually moderate, although it can range from mild to severe (Korman et al., 1970).

Both these conditions result in hypochlorhydria or achlorhydria due to the net loss of acid secreting parietal cells. The resulting loss of negative feedback inhibition causes unopposed gastrin release. Prolonged hypergastrinaemia stimulates ECL-cell hyperplasia and in some patients especially those with pernicious anaemia, this can progress to type-1 gastric carcinoid tumour formation as detailed in section 3.2.3 (Annibale et al., 2001a; Burkitt and Pritchard, 2006).

Proton pump inhibitor (PPI) therapy has also been associated with modest elevations in fasting serum gastrin concentrations (usually less than three times the upper limit of normal), with only about 2% of patients on a PPI developing serum gastrin concentrations greater than 400pM (Jensen, 2006). It has been reported that PPI therapy does not appear to increase further the elevated fasting serum gastrin concentrations seen in

patients with ZES (Hirschowitz and Haber, 2001). Lamberts *et al.* demonstrated moderate hypergastrinaemia in patients treated with omeprazole. There were however no subsequent changes reported in the fasting serum gastrin concentrations in these patients during an 8-year follow up (Lamberts et al., 2001). Elevated fasting serum gastrin concentrations associated with PPI therapy take approximately one week to return to the normal range once the medication has been discontinued (Festen et al., 1984).

1.4 *Helicobacter pylori* and gastric adenocarcinoma

It has been postulated for over a century that certain bacteria colonise the human stomach. Initially these bacteria were considered to be contaminants from ingested food rather than true colonizers of the human stomach. With the discovery and successful isolation of a spiral bacterium from the human stomach about 20 years ago by the Nobel Laureates Barry Marshall and Robin Warren, it has now been conclusively established that *Helicobacter pylori* organisms colonise the human stomach (Marshall and Warren, 1984). *Helicobacter pylori* is now implicated in the development of duodenal and gastric ulcers as well as in the development of gastric atrophy, gastric adenocarcinoma and Mucosa Associated Lymphoid Tissue (MALT) lymphoma.

Helicobacter pylori belongs to the family *Helicobacteraceae* and the genus *Helicobacter* consists of over 20 recognised species with some still awaiting formal recognition (Fox, 2002). All members of this genus are

microaerophilic, most are catalase and oxidase positive and many are also urease positive.

Helicobacter species can be divided into two major groups depending on the major organ of colonisation. Gastric and enterohepatic groups predominantly colonise the gastric or colonic and biliary systems respectively. They demonstrate a high level of organ specificity and are unable to colonise other sites (Solnick and Schauer, 2001).

1.4.1 Enterohepatic *Helicobacter* species

These bacteria, such as *Helicobacter hepaticus*, colonise the lower gastrointestinal tract (ileum and colon) and the biliary tree of humans and some other mammals. Infection has been reported to cause a chronic inflammatory reaction and may predispose to neoplastic lesions in the hepatobiliary tree (Solnick and Schauer, 2001; Verhoef et al., 2003; Avenaud et al., 2000). In addition, enterohepatic *Helicobacter* species have also been reported to be associated with the development of colitis in animal models. Fox and colleagues initially reported the possible association between *Helicobacter hepaticus* infection and intestinal inflammation in immunodeficient mice (Ward et al., 1996) and this has subsequently been confirmed in other studies (Kullberg et al., 2006).

1.4.2 Gastric *Helicobacter* species

Gastric *Helicobacter* tend to be motile with flagella and are all urease positive. The urease enzyme allows survival for a short time in the

acid milieu of the stomach. The spiral morphology and flagella related motility assist penetration into the viscous mucous layer of the stomach, where the more neutral pH allows growth. Hence both motility and urease production are required for gastric colonisation.

H. pylori lacks clonality because it is genetically heterogeneous, suggesting that every *H. pylori* positive patient carries a distinct strain (Kansau et al., 1996). However organisms cultured from related family members are genetically similar. This heterogeneity may be the result of adaptation by *H. pylori* to survive the gastric conditions as well as the immune response of the host. The cagPAI described in section 1.4.4 is a striking example of this heterogeneity.

1.4.3 *Helicobacter pylori*

1.4.3.1 Morphology

H. pylori is a spiral shaped gram-negative bacterium measuring 2 to 4 µm in length and 0.5 to 1 µm in width (Fig1-7). It can be seen in coccoid and rod shapes (usually after prolonged *in vitro* culture or antibiotic treatment) (Kusters et al., 1997). *H. pylori* is a fastidious organism and requires complex growth conditions. This explains why it was initially difficult to obtain cultures from gastric biopsies. It is microaerophilic and growth occurs at a pH range of 5.5 to 8.0 although it survives brief exposures to pH<4 (Scott et al., 2002).

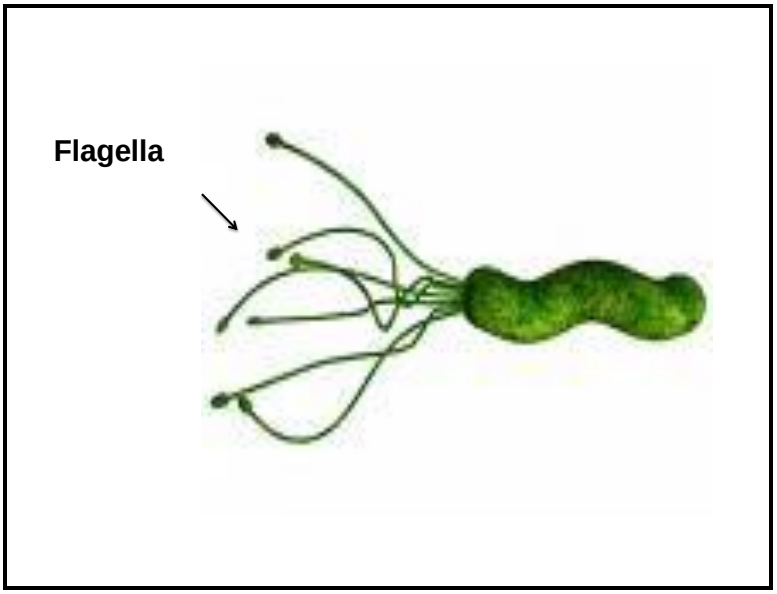


Figure 1-7: *Helicobacter pylori*
(adapted from www.h pylori.com.au/h pylori-16.png)

1.4.3.2 Epidemiology

The prevalence of *Helicobacter pylori* shows large geographical variations throughout the world. More than 80% of the population in developing countries are *Helicobacter pylori* positive, whereas the prevalence in developed countries is approximately 40% (Pounder and Ng, 1995). Prevalence is also rapidly declining as a result of eradication by use of antibiotics, improved hygiene and sanitation, less overcrowding and improved housing conditions. The incidence of new *H. pylori* infection in the Western world is less than approximately 0.5% per year (Roosendaal et al., 1997). Hence the prevalence of *H. pylori* infection in industrialised countries currently increases markedly with age (Parsonnet, 1995).

New infection occurs predominantly in childhood and unless treated usually lasts for life. Transmission is by direct person to person infection

usually from close family members. There is no evidence to suggest zoonotic transmission.

1.4.3.3 Colonisation in the gastric lumen

H. pylori is the main organism that survives and inhabits the normal human stomach (Blaser and Atherton, 2004). However, it remains susceptible to low pH. Elucidation of the *H. pylori* genome and post genomic techniques have demonstrated several factors that are important for successful gastric colonisation (Wen et al., 2003).

Acid resistance is crucial and one of the many genes that determine acid resistance encodes the urease protein, which hydrolyses urea to ammonia and carbon dioxide (Weeks et al., 2000). This transiently buffers the acidic environment, lowering the pH to below 5.0 which allows survival for long enough for the bacteria to colonise the gastric mucosa (Marshall et al., 1990). This also forms the basis of two of the commonly used diagnostic tests for *H. pylori* – the invasive biopsy test (Rapid Urease test) and the non-invasive Urea breath test.

However, the presence of urease is insufficient for long term survival and the bacteria have evolved other mechanisms to minimize exposure to a low pH, in particular they remain in very close proximity to the surface of the epithelium where the pH is near neutral. Their natural habitat is located below the gastric mucous layer, attached to the mucus secreting epithelial cells lining the stomach. Most *H. pylori* are free

swimming. They utilise their flagellar motility and chemotactic responses for colonisation.

H. pylori inhabits only gastric type mucus and hence does not colonise duodenal or oesophageal mucosa unless these have undergone gastric metaplasia. Approximately 20% of *H. pylori* organisms in the stomach are found adherent to epithelial cells by means of adhesions (Hessey et al., 1990). This adhesion often shows a tropism for the intercellular junctions and to the expression of Trefoil factor 1 to which *H. pylori* binds strongly and specifically (Clyne et al., 2004). The best studied adhesins are the outer membrane proteins that bind to host cell glycoproteins. The three Hop (Hsp Organising Proteins) proteins are the best characterised and are detailed below.

a) BabA (HopS): this is probably the best characterized *H. pylori* adhesion protein. It is a 78-KDa protein encoded by the *bab*A gene. BabA mediates binding to the fucosylated Lewis b (Le^b) blood group antigen in human host cells (Ilver et al., 1998). Animal studies have suggested that for colonisation and pathogenesis of *H. pylori,* BabA mediated adhesion is essential (Rad et al., 2002). Some studies have suggested that BabA plays a role in *H. pylori* virulence due to the strong association demonstrated between the *bab*A2 allele and gastric inflammation and adenocarcinoma (Gerhard et al., 1999). However other studies have not confirmed this association (Yamaoka et al., 2002). It is possible that BabA may not be an independent marker, as the presence of the *bab*A2 allele is linked to *vac*As1 and *cag*A alleles.

b) Oip (HopH): Oip (proinflammatory outer membrane protein) was originally described as a proinflammatory response inducing protein. The gene encoding this protein is present in all *H. pylori* strains. It is believed to play a role in gastric inflammation (Yamaoka et al., 2002).

c) SabA (HopP): this is thought to play a role in chronic inflammation and atrophic gastritis disease stages. It mediates binding to sialic acid containing glycoconjugates. It has been shown that *H. pylori* induced gastric inflammation and carcinogenesis is associated with the replacement of non sialylated Lewis antigens by sialylated antigens (Mahdavi et al. 573-78). This probably explains the role of SabA in gastric inflammation and carcinogenesis (Yamaoka et al., 2006).

However no individual adhesin is essential for attachment to the gastric mucosa and the expression of adhesins is diverse between several strains and has also been found to be variable within the same strain over time(de et al., 2004).

Three hypotheses have therefore been proposed to explain why *H. pylori* adhere to epithelial cells (Amieva and El-Omar, 2008).

1. Cellular damage and inflammation induced by adhesion:

   This hypothesis suggests that *H. pylori* adhere to epithelial cells to cause damage, deliver toxins and induce inflammation. The delivery of the major *H. pylori* virulence factors (CagA and VacA) is also dependent on this adhesion. There also remains the possibility of *H. pylori* deriving nutrients from damaged cells.

2. Promotion of invasion and persistence and avoidance of clearance: Adhesion protects against mechanical clearance in the gastric lumen.
3. Possible use of adhesion site for bacterial replication

1.4.3.4 Clinical consequences of *H. pylori* infection:

Helicobacter colonisation of the gastric mucosa is in itself not a disease, but a predisposing condition to various diseases of the stomach.

Symptoms of childhood infection and its subsequent immediate effect on gastric acid secretion are unknown. In adults, when colonisation occurs for the first time, it can result in a gastritis associated with hypochlorhydria, dyspepsia and nausea (Graham et al., 1988; Morris and Nicholson, 1987). There is an estimated subsequent 10 to 20% lifetime risk of developing peptic ulcer disease and a 1 to 2% chance of developing gastric adenocarcinoma (Kuipers et al., 1995).

Chronic infection with *Helicobacter pylori* results in two different types of pathology depending upon the distribution of gastritis and resultant changes in acid secretion:

If acid secretion remains intact, then *H. pylori* organisms are often confined to the gastric antrum resulting in an antral-predominant gastritis (type 1). On the other hand in patients with decreased acid secretion (such as those taking Proton Pump Inhibitor drugs), there is a more even distribution of the bacteria in the antrum and corpus resulting in pan-gastritis (corpus-predominant).

Chronic antral-predominant gastritis can result in a rebound increase in acid secretion by direct stimulation of gastrin secreting G cells. This, as well as changes in the duodenal mucosa induced by excess acid may predispose to the development of duodenal ulcers.

On the other hand, chronic corpus-predominant gastritis may result in the development of gastric atrophy and this may progress to intestinal metaplasia and dysplasia and eventually gastric adenocarcinoma (Fig1-8).

Not all people with *H. pylori* infection however develop disease. Several bacterial and host factors influence disease development and progression including:

a) Virulence factors of the infecting *H. pylori* (section 1.4.4)

b) Host immune response to the infection (section 1.4.5)

c) Environmental cofactors (section 1.5)

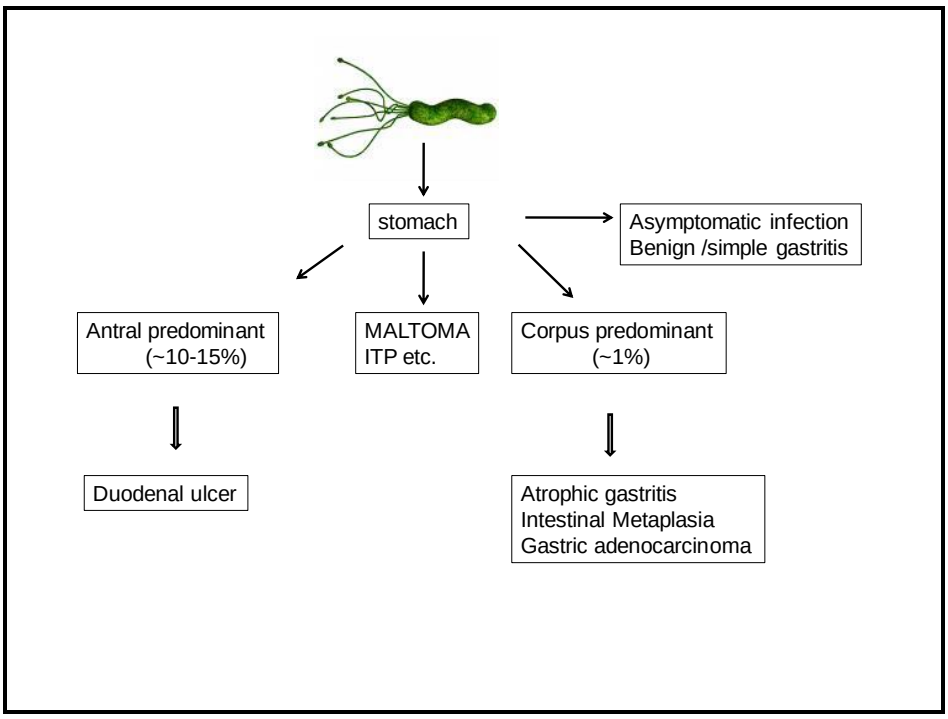


Figure 1-8: Clinical consequences of *H. pylori* infection

1.4.4 Bacterial Virulence Factors

The two best characterised *H. pylori* virulence factors are the cytotoxin associated gene A (*cag*) pathogenecity island (PAI) and the vacuolating cytotoxin gene (*vac*A). These are described in detail below. Pathogenecity islands (PAIs) are large sections of DNA acquired by bacterial species over time that render them pathogenic.

1.4.4.1 *cag*PAI

Initial observations that some *H. pylori* infected patients developed symptoms whereas others did not led to the hypothesis that there might be differences in the virulence of the infecting *H. pylori* organisms. Further investigations showed that some *H. pylori* strains were more virulent and caused morphological changes, vacuolisation and degeneration of *in vitro*

cells. This activity was subsequently linked to the presence of a 140 kDa protein named cagA (cytotoxin associated gene A). This protein is encoded by the *cag*A gene and is present in about 50 to 70% of *H. pylori* strains. Studies using Mongolian gerbils have indicated that in the presence of a functioning *cag* PAI and *cag*A, there is a subsequent increased progression to atrophic gastritis (Rieder et al., 2005). *H. pylori* strains that carry the *cag*PAI (Pathogenecity Island) are referred to as *cag*A^+ and those that lack it, either in its entirety or in parts, are referred to as *cag*A^-. Those strains that are *cag*A^+ are associated with a greater inflammatory response and more significant outcome such as peptic ulcer or gastric cancer. In a nested case-control study, Japanese–American men with gastric and duodenal ulcers and matched controls were tested for antibodies to *H. pylori* and for antibodies to cagA. Patients who tested positive for *H. pylori* had an odds ratio of 4.0 for the development of gastric ulcer and an odds ratio of 2.5 for duodenal ulcer. If seropositive for both *H. pylori* and cagA then the odds ratio for gastric ulcer was 4.4 and for duodenal ulcer was 5.8 suggesting that cagA positivity increases the risk of developing both gastric and duodenal ulcers above that of *cag*- strains (Nomura et al., 2002). Using the same cohort, in a subsequent study the authors sought to assess the association between *cag*A^+ and the risk of development of gastric adenocarcinoma. 103 patients with *H. pylori* positivity and gastric cancer were matched with 103 *H. pylori* positive subjects without gastric cancer (controls) and their sera were analysed for the presence of cagA antibodies. The odds ratio of developing gastric adenocarcinoma if patients had evidence of *cag*A positive *H. pylori*

infection was 1.9 and for the intestinal subtype this was 2.3. Interestingly, the authors also found that age < 72 years and advanced tumour stage at the time of diagnosis were also associated with *cag*A positivity (Blaser et al., 1995).

The *cag* Pathogenecity Island comprises 30 genes and these encode proteins that comprise a type IV secretory system (T4SS) (Censini et al., 1996) (Fig1-10). *H. pylori* utilises this T4SS to translocate products into the host cell's cytoplasm. This is often seen microscopically as a sheathed rigid needle linking *H. pylori* to the host cell (Tanaka et al., 2003). CagA protein is subsequently translocated into the gastric epithelial cell where it undergoes phosphorylation (Odenbreit et al., 2000). In the host cell this stimulates cell signalling pathways resulting in changes in cell morphology and shape. *In vitro* the most commonly seen changes are of cell elongation and the development of long processes – termed the 'hummingbird phenotype' (Moese et al., 2004).

CagA also stimulates the expression of transcription factors involved in cellular proliferation. Unregulated *cag*A signalling can also result in apoptosis. However activated cagA inhibits the SRC kinase that phosphorylates cagA in the first instance, thereby providing a negative feedback inhibition of further *cag*A expression (Moss et al., 2001). *Cag*A is also thought to disrupt the apical junctional complexes between epithelial cells resulting in loss of barrier function (Amieva et al., 2003). This may also help the bacteria to derive nutrients from the host cell.

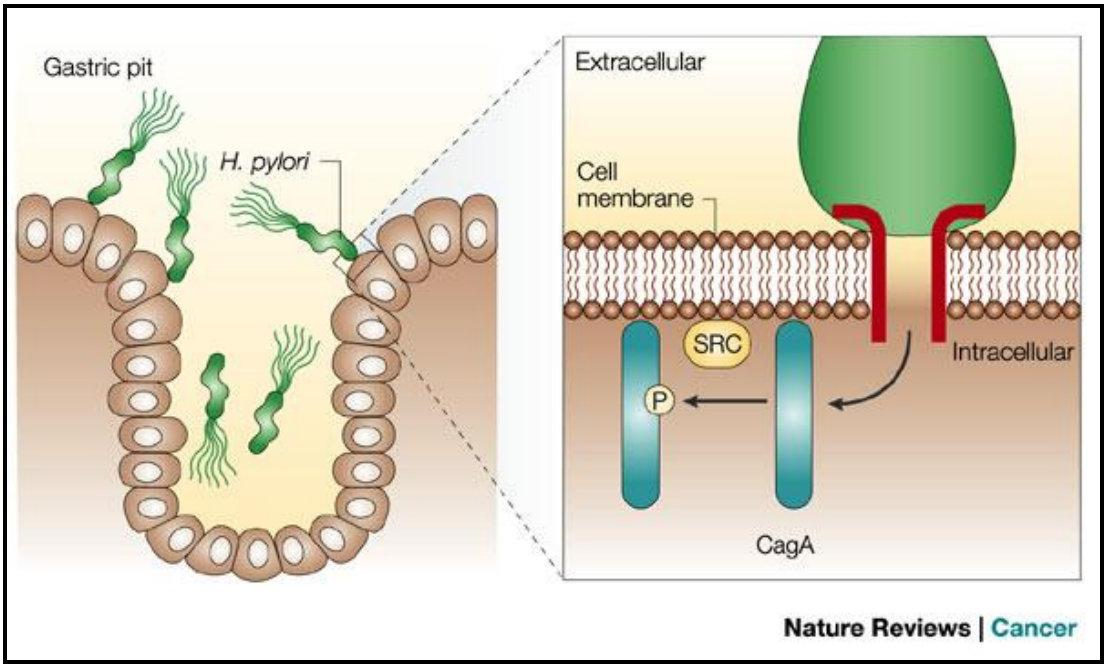


Figure 1-9: CagA is translocated into the cytoplasm of the gastric epithelial cell through the type IV secreting system following the attachment of the cagA+ *H. pylori*

(www.nature.com; Nature Reviews Cancer 4, 688-694 [September 2004] doi: 10.1038/nrc1433).

1.4.4.2 VacA

VacA is a pore forming cytotoxin initially discovered when cultured cells developed abnormal vacuolation following infection with *H. pylori* (Leunk et al., 1988). The vacuolating cytotoxin gene is present in all strains of *H. pylori,* although there is marked polymorphism.

VacA is a 140 KDa polypeptide that is trimmed at both ends during secretion from the bacterial cell. The amino terminus contains the signal sequence ‘s’ region of the gene. Those strains harbouring the s1 types of VacA are associated with gastric cancer and ulcers. The middle region of the gene is classified as the ‘m’ region – this shows allelic variations

similar to the ‘s’ region; with the m1 subtype also being strongly associated with increased vacuolation (Atherton et al., 1995).

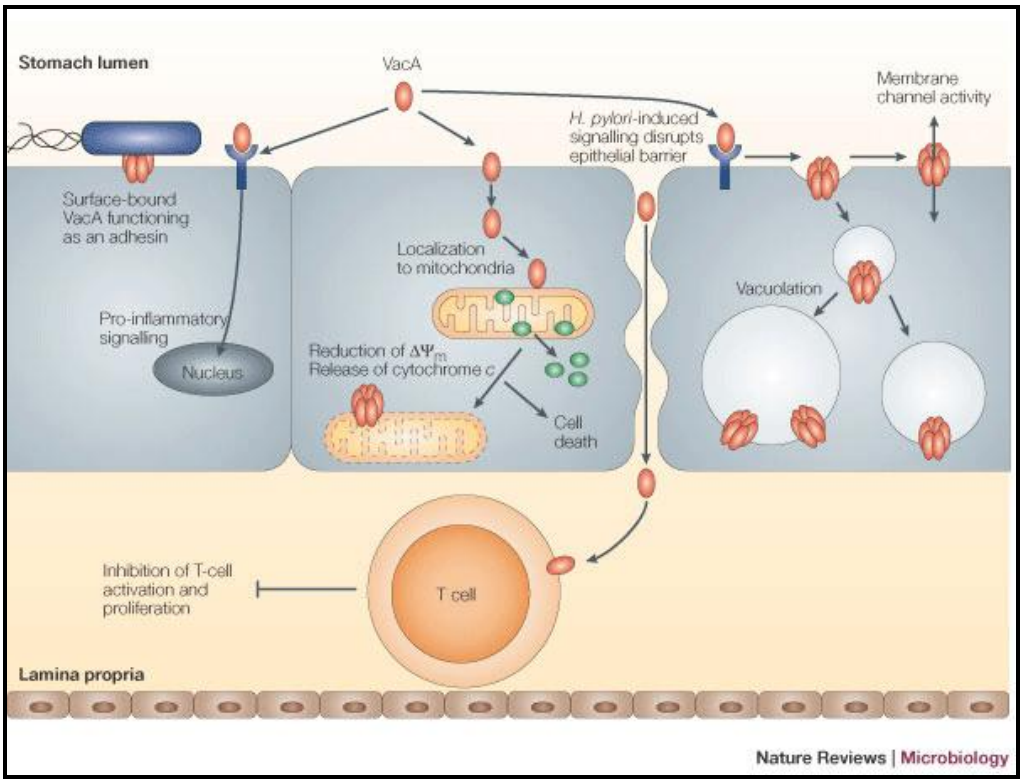


Figure 1-10: VacA and its effects on gastric epithelial cells include increased cellular permeability, apoptosis and altered mitochondrial membrane permeability.

It can also inhibit the activation of T-cell and its proliferation. (www.nature.com; Nature Reviews Microbiology 3, 320-332 [April 2005] doi: 10.1038/nrmicro1095)

In humans, the VacA s2 type is rarely associated with disease and whereas the s1m1 type is commonly seen in association with gastric adenocarcinoma. In Japan, the s1m1 type is ubiquitous (Ito et al., 1998).

Studies have shown that VacA is transferred into host cells by either secretion or by contact dependent transfer (Ilver et al., 2004). The pore forming effect of Vac A has been best studied *in vitro* and is thought to result from its effects on endosomal maturation. Other effects include

increased paracellular permeability, membrane channel formation, disruption of lysosomal and endosomal activity, immune modulation and induction of apoptosis through pore formation in mitochondrial membranes and also through activation of proapoptotic signal molecules (Papini et al., 1998; Cover et al., 2003) (Fig1-10). VacA has also been found to be a powerful inhibitor of T cell activation *in vitro* (Gebert et al., 2003). VacA is therefore thought to play a major role in regulating susceptibility to peptic ulceration and gastric adenocarcinoma development.

1.4.5 Host immune response

The immune response of the host to *H. pylori* is an important feature that helps to determine susceptibility to the diseases associated with infection with this organism. The Th1 response (mainly cell-mediated) is the predominant immune response induced by *H. pylori*.

The importance of this Th1 mediated response has been shown in various experiments. For example mice that are genetically prone [eg. C57B2/6 or IL 4-null mice (which have been manipulated to have a predominantly Th1 response)] develop more intense inflammation following infection with *H. pylori* (Smythies et al., 2000) than mice which develop a predominantly Th2 response (eg. Balb/c). However the Th1 response is not strong enough to achieve bacterial eradication and hence bacterial persistence occurs.

Many of the pathogenic effects of *H. pylori* result from chronic inflammation. It has been shown that various genetic polymorphisms in

the host can lead to alterations in the levels of these inflammatory mediators. This eventually influences the outcome of infection. Proinflammatory genetic polymorphisms tend to increase the risk of development of pre-neoplastic changes, leading to the development of gastric adenocarcinoma.

The best characterised of these host genetic polymorphisms is the IL-1 cytokine (El-Omar et al., 2001). The IL-1 cytokine is encoded by the gene cluster containing IL-1B, which encodes the IL-1β cytokine and IL-RN, encoding the IL-1 receptor antagonist. IL-1β is the most potent inhibitor of gastric acid secretion and is also a powerful pro- inflammatory cytokine. The IL-1 gene has been found to have several polymorphisms, all leading to elevated levels of expression of IL-1β. This leads to significant acid suppression and hence to a corpus predominant gastritis following *H. pylori* infection. This pattern of gastritis has been shown to be associated with the development of atrophic gastritis and thus an increased risk of gastric cancer (Furuta et al., 2002; Rad et al., 2004). Studies using IL-1β transgenic mice have demonstrated the step wise progression to gastric adenocarcinoma through the stages of spontaneous inflammation, metaplasia, dysplasia and carcinoma (Tu et al., 2008). This effect was mediated by activation and recruitment into the stomach of myeloid-derived suppressor cells (MDSCs) following IL-1β activation of the IL-1 RI/NF-κB pathway. The progression to gastric atrophy and adenocarcinoma was greatly accelerated by co-infection with *H. felis* infection in IL-1β transgenic mice. Antagonism of IL-1β receptor suppressed MDSCs mobilisation and inhibited development of gastric

preneoplastic changes, confirming the important role played by IL-1β in gastric carcinogenesis (Tu et al., 2008).

IL-8 has also been found to have effects on cellular proliferation, migration and angiogenesis and increases the risk of severe inflammation and pre-neoplastic changes (Troost et al., 2003).

Similar polymorphisms in the TNF-α gene and in IL-10 have also been studied. TNF-α is also an inhibitor of acid secretion and is produced in the gastric mucosa in response to infection with *H. pylori.* IL-10 on the other hand down regulates IL-1β, TNF-α and other proinflammatory cytokines. Hence relative deficiencies of IL-10 may result in a greater proinflammatory response with subsequent greater mucosal damage (Rad et al., 2004).

1.4.6 *H. pylori* and fasting serum gastrin concentrations

There are two proposed mechanisms by which *H. pylori* infection can cause elevations in fasting serum gastrin concentrations, which are often modest. In antral predominant *H. pylori* infection, inhibition of somatostatin secretion results in uninhibited gastrin secretion and the subsequent increased acid secretion is often associated with the development of duodenal ulcers.

Chronic corpus predominant *H. pylori* infection on the other hand sometimes results in gastric atrophy. The subsequent hypochlorhydria, due to the loss of acid secreting parietal cells, results in an appropriate G-cell hyperplasia and hypergastrinaemia (Troost et al., 2003).

Eradication of *H. pylori* has been shown to be associated with a normalisation of serum gastrin concentration by 12-15 months (Ohkusa et al., 2004). In several studies, a gradual decrease and subsequent normalisation of serum gastrin concentrations was observed within about 6 months following *H. pylori* eradication (El-Omar et al., 1997; Iijima et al., 2000). An increase in serum gastrin concentrations has also been observed following reinfection with *H. pylori* (Chen et al., 1994).

1.5 Distal gastric adenocarcinoma

1.5.1 Epidemiology

1.5.1.1 World statistics

Adenocarcinoma is the most common tumour of the human stomach. It is the fourth most frequent cancer worldwide and accounts for 7.8% of new cancer cases. It also remains the second most common cause of cancer related deaths worldwide (after lung and breast cancer) (http://globocan.iarc.fr/).

988,000 new diagnoses of gastric cancer were made in 2008 and there were 736,000 worldwide deaths. The incidence is approximately 22 per 100,000 in men and 10.3 per 100,000 in women, with associated mortality reaching 14.3 and 8.3 per 100,000 respectively.

Gastric adenocarcinoma is currently the fourth most common cancer in males (after lung , prostate and colorectal cancers) and fifth in women (after breast, cervix, colorectal and lung) (Parkin et al., 2005).

The lowest global rates for gastric cancer are in North America and Western Europe and the highest rates occur in East Asia, South America and Eastern Europe. Japan and Korea have the highest incidence. In 2006, 86,000 new cases were diagnosed in the EU (Cancer statistics registration, 2008).

1.5.1.2 UK statistics

In 2009, approximately 7500 cases of gastric adenocarcinoma were diagnosed in the UK with 5178 deaths (UK Mortality 2010). It is the 9th most common cause of cancer in men in the UK with 4900 new cases per year and 14th most common cancer in women with 2600 new cases a year. The incidence of gastric adenocarcinoma is approximately 12.3 per 100,000 men and 5.1 per 100,000 women in England and Wales, of which more than 80% of cases occur in patients aged 65 years and over and less than 8% of cases occur below 55 years of age (Cancer statistics registration, 2008).

There has also been a change in the anatomical site of gastric adenocarcinoma development over recent years. The incidence of tumours in the gastric cardia (proximal stomach) is increasing compared to the previously more commonly occurring distal gastric carcinoma (Cancer statistics registration, 2008).

1.5.2 Risk factors for the development of distal gastric adenocarcinoma

There are several known risk factors for the development of gastric adenocarcinoma. These include:

1.5.2.1 Gastritis

Chronic atrophic gastritis is a recognised risk factor for the development of gastric adenocarcinoma. This may result from autoimmune conditions such as pernicious anaemia or be secondary to corpus predominant *Helicobacter pylori* infection. Atrophic gastritis stimulates metaplasia of the gastric epithelium to the intestinal type. Metaplastic glands have been shown to consistently surround the periphery of gastric adenocarcinomas in pathology specimens. Intestinal metaplasia itself is a dynamic process. Younger individuals often exhibit ‘small-intestinal type’ metaplasia whereas older patients exhibit dysplasia and patients at high risk of gastric cancer consistently show ‘colonic type’ metaplasia (Cancer statistics registration, 2008). Intestinal metaplasia is classified as either complete or incomplete (Jass and Filipe, 1980). The complete type of intestinal metaplasia (type 1) is characterised by the presence of Paneth cells, absorptive cells and mucin secreting goblet cells, resembling small intestinal epithelial cells. The incomplete type (type 2 and type 3) on the other hand is characterised by the presence of columnar cells and goblet cells. The other important feature associated with type 3 intestinal metaplasia is the distortion of columnar cells and absence of Paneth cells. Gastritis eventually progresses to gastric atrophy over years or decades in

some individuals (Cancer statistics registration, 2008). The presence of SPEM (spasmolytic polypeptide-expressing metaplasia) has recently been more strongly associated with the development of gastric adenocarcinoma than intestinal metaplasia and this entity may well be the precancerous lesion (Schmidt et al., 1999). In addition newer studies have assessed the potential of gene expression profiling (Serial Analysis of Gene Expression, SAGE) of intestinal metaplasia and SPEM in identifying not only useful prognostic markers of early gastric cancer but also metaplasia biomarkers. Studies have also demonstrated that cumulative expressions of such metaplasia biomarkers provide a better prognostic marker rather than single individual markers (Suh et al., 2012).

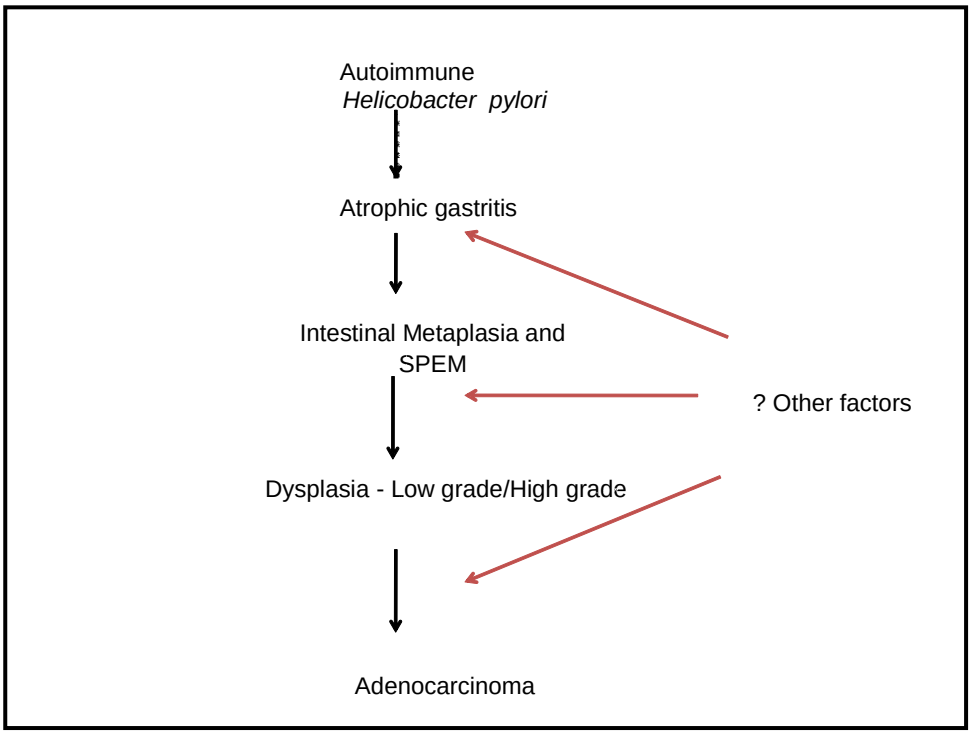


Figure 1-11: Progression from atrophic gastritis to adenocarcinoma (Correa Pathway). SPEM (spasmolytic polypeptide-expressing metaplasia) has been found to be more strongly associated with gastric cancer than intestinal metaplasia.

1.5.2.2 Pernicious anaemia

Chronic atrophic gastritis and gastric polyps are commonly seen in pernicious anaemia (Schmidt et al., 1999). Screening by endoscopy has shown a high incidence of gastric epithelial dysplasia in this condition, hence pernicious anaemia is considered to be a precancerous condition. Some studies have shown up to a threefold increase in the risk of developing gastric adenocarcinoma (Siurala and Seppala, 1960; Schmidt et al., 1999) in affected subjects.

1.5.2.3 Previous gastric resection

Gastric stump carcinoma is defined as gastric adenocarcinoma developing in the remnant gastric stump at least 5 years following gastric resection for benign peptic ulcer disease (Helsingen and Hillestad, 1956; Safatle-Ribeiro et al., 1998). This risk increases with time since surgery, with lifetime risk exceeding that of the general population 15 years following gastric surgery(Helsingen and Hillestad, 1956). Several factors have been proposed to account for this increased susceptibility to carcinogenesis.

a) Chronic duodenogastric reflux resulting from gastric resection and anastomosis. This may result in intestinal metaplasia, dysplasia and adenomatous changes in the gastric stump (Kondo, 2002; Lorusso et al., 2000; Mason et al., 1988; Miwa et al., 1995; Tanigawa et al., 2002).

b) Bile acids in the refluxate may also exert a pro-carcinogenic effect. In animal models, nitrated bile acid derivatives – namely glycocholic and taurocholic acids have been shown to be carcinogenic in the stomach (Kondo, 2002; Tanigawa et al., 2002).

c) The gastric stump is often associated with gastritis and atrophy resulting in chronic hypochlorhydria and possible increased bacterial growth. Some bacteria (especially anaerobes) may be associated with the production of carcinogens (Muscroft et al., 1981).

d) Unrecognised *Helicobacter pylori* infection is another factor that predisposes to gastric atrophy and progression to adenocarcinoma.

The risk seems to be higher in those patients who have undergone gastric resection for gastric ulcer rather than duodenal ulcer. This risk was 3 fold within the first 20 years following surgery and 5.5 fold after this time period. In contrast, in patients with duodenal ulcer, the risk of gastric stump cancer was increased only 3 fold more than 20 years following surgery (Caygill et al., 1986). This could be explained by the observation that gastritis and gastric atrophy occur more frequently in patients with gastric ulcer disease, whereas duodenal ulcers are associated with antral predominant *Helicobacter pylori* infection and increased gastric acid production.

Patients with Billroth–II reconstruction had a 4 fold increased risk of gastric stump cancer compared with patients who had undergone Billroth-I

surgery, further suggesting that duodenal reflux plays a role in the development of gastric stump cancer (Muscroft et al., 1981).

Atrophy resulting from deficiency of gastrin following antrectomy has also been thought to play a role in the development of gastric stump cancer. This has also been suggested following vagotomy with the resultant loss of vagus mediated growth regulation (Kaminishi et al., 1995; Fisher et al., 1993).

1.5.2.4 Diet

There is evidence to suggest that reduced consumption of fresh fruit and vegetables is associated with an increased risk of developing gastric cancer. Dietary antioxidants (seen in fruits and vegetables) may play an important role in cancer prevention as shown in the study by Munoz *et al*., where a diet enriched with vitamins C and E and carotene lowered the incidence of intestinal metaplasia in a susceptible population (Plummer et al., 2007).

Increased consumption of salt and preserved food has also been postulated to increase the risk of gastric cancer (Hill, 1998). In a recent publication by the World Cancer Research Fund and the American Institute for Cancer Research, it has been suggested that there is a probable increased risk of stomach cancer with consumption of salt and salt-preserved food. There, is also a probable decreased risk associated with the consumption of non-starchy and allium vegetables (for example garlic and onions) and fruits (World Cancer Research Fund., 2007).

1.5.2.5 Alcohol

There is no convincing evidence suggesting an increased risk of gastric cancer associated with alcohol consumption. In a recently published study based on the Netherlands Cohort Study (NLCS), there was no association seen between alcohol consumption (>30gms/day compared to abstaining) and gastric non-cardia, cardia or oesophageal adenocarcinoma (relative risk of 1, 0.9 and 1.04 respectively, compared to a relative risk of 4.61 for oesophageal squamous cell carcinoma) (Steevens et al., 2010).

1.5.2.6 Smoking

As with other cancers, there is an increased risk of developing gastric cancer in smokers, but this association is not as strong as with lung cancer (Tredaniel et al., 1997). In a recently published study, an increased risk of gastric cancer (relative risk of 1.6) was noted in current smokers, with risk being in proportion to the duration of smoking (gastric noncardia cancers only) (Steevens et al., 2010).

1.5.2.7 Genetic predisposition

Approximately 5 to 10% of gastric cancer cases have a familial association. This has been well documented for about 200 years. For example Napoleon Bonaparte died from stomach cancer and so did

several of his family members – father, grandfather, brother and three sisters (Kubba and Young, 1999).

This familial risk is more common with the diffuse type of gastric adenocarcinoma than the intestinal variant, conferring a 7-fold increased risk amongst relatives of patients with diffuse-type gastric cancer compared to a 1.4 fold increased risk in the relatives of patients with intestinal-type gastric adenocarcinoma (Lehtola, 1978).

The term ‘Hereditary diffuse gastric cancer’ (HDGC) was coined to include patients who have an autosomal dominant inheritance pattern for a diffuse-type gastric cancer syndrome. The International Gastric Cancer Linkage Consortium (IGCLC) has established criteria to define HDGC as well as guidelines for the clinical management of such patients and their families (Park et al., 2000; Guilford et al., 1999). HDGC is an autosomal dominant inherited cancer syndrome characterised clinically by either

(1) ≥ 2 documented diffuse gastric cancer cases in first or second degree relatives with at least one case being diagnosed before the age of 50 years; or

(2) ≥ 3 cases of documented diffuse gastric cancer in first or second degree relatives irrespective of age of onset (Park et al., 2000).

E-cadherin gene mutations (CDH1 mutation) have been demonstrated in about 30-50% of HDGC cases and the penetrance is about 75-80%, suggesting that carriers of the CDH1 mutation have a 75-80% lifetime chance of developing gastric cancer by the age of 80 (Pharoah et al., 2001).

The E-cadherin gene is located at chromosome 16q22.1. It is a type-1 cadherin found in all epithelial tissues. Type-1 cadherins are glycoproteins that medicate adhesion at junctions. In addition, it also plays a vital role in the regulation of cell division and inhibits both apoptosis and proliferation (Grunwald, 1993). Mutation therefore results in loss of cell adhesion, proliferation, invasion and metastasis (Pharoah et al., 2001; Becker et al., 1994; Norton et al., 2007).

Genetic screening for HDGC is often reserved until patients are old enough to be able to give informed consent as the implication of the diagnosis is far reaching (Oliveira et al., 2003). There is currently no role for routine endoscopic screening. Prophylactic total gastrectomy 5 years before the youngest affected family member is advocated in relatives (Chun et al., 2001; Newman and Mulholland, 2006). Apart from breast cancer screening, routine screening for other cancers is not currently advocated in HDGC.

1.5.2.8 *Helicobacter pylori*

With the discovery of *Helicobacter pylori* in 1983 by Warren *et al*., our understanding of gastric diseases has vastly improved (Marshall and Warren, 1984). *Helicobacter pylori* has been recognised as a type-1 carcinogen and is associated with the development of gastric adenocarcinoma as well as mucosa associated lymphoma (MALT) (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994) (see section 1.4).

1.5.3 Pathological classification of distal gastric adenocarcinoma

Various different classification systems have been suggested for gastric adenocarcinoma including:

a) Ming – which classifies the tumour border as being either infiltrative or expansile (Ming, 1977);

b) WHO – based on histopathology (papillary, tubular, signet ring cell, mucinous and undifferentiated);

c) Goseki – classifying tumours depending on whether tumours show evidence of good tubal formation and intracellular mucin (Goseki et al., 1992)

- group I – tubular differentiation well, mucous in cytoplasm – poor;
- group II – tubular differentiation well, mucous in cytoplasm – rich;
- group III – tubular differentiation poor, mucous in cytoplasm – poor;
- group IV – tubular differentiation poor, mucous in cytoplasm – rich

d) Lauren classification – diffuse, intestinal or mixed types. The Lauren classification is currently the most widely used.

- Intestinal-type: these tumours tend to be well differentiated with cells being arranged in tubular or glandular structures. The

terms tubular, papillary and mucinous are all within the spectrum of intestinal-type gastric cancer.

- Diffuse-type: the cells are undifferentiated and lack glandular structure. Clinically the diffuse type tends to infiltrate the gastric wall (linitis plastica).
- Mixed-type: Features of both intestinal-type and diffuse-type cellular architecture are consistently seen.

1.5.4 Clinical features associated with distal gastric adenocarcinoma

Approximately 70% of patients with so called ‘early gastric cancer’ (confined to the mucosa or submucosa) have symptoms of uncomplicated dyspepsia (Hallissey et al., 1990; Eckardt et al., 1990).

Patients with advanced gastric cancer often present with alarm symptoms including weight loss, anaemia, vomiting, haemetemesis and abdominal pain (Fuchs and Mayer, 1995; Bramble et al., 2000). The concurrent use of proton pump inhibitors has been thought to potentially mask and delay the diagnosis of gastric cancer and hence the use of these medications should be delayed in individuals ‘at risk’, prior to establishing a diagnosis (Allum et al., 2002).

Currently in the UK the following are considered as alarm symptoms in patients of any age with dyspepsia. Such patients are advised to have an urgent upper GI endoscopy to rule out underlying gastric cancer (NICE Guidelines) (National Institute for Health and Clinical Excellence, 2004):

- Chronic gastrointestinal bleeding
- Progressive unintentional weight loss
- Progressive swallowing difficulties
- Iron deficiency anaemia
- Persisting vomiting
- Epigastric mass
- Suspicious barium meal

1.5.5 Diagnosis of distal gastric adenocarcinoma

The diagnosis of gastric adenocarcinoma is usually made by examination of biopsy specimens obtained at gastroscopy (Fig1-12). Additional investigations to stage the disease include CT scan of the abdomen and Endoscopic ultrasound. There is currently no role for PET (Positron Emission Tomography) CT in the routine diagnosis of gastric cancer (Shoda et al., 2007), although there may be roles for this modality in the diagnosis of distant metastases (Rosenbaum et al., 2006) and in evaluating the response to chemotherapy (Sun et al., 2007). Staging of the disease is usually based on the TNM classification (table1-3).

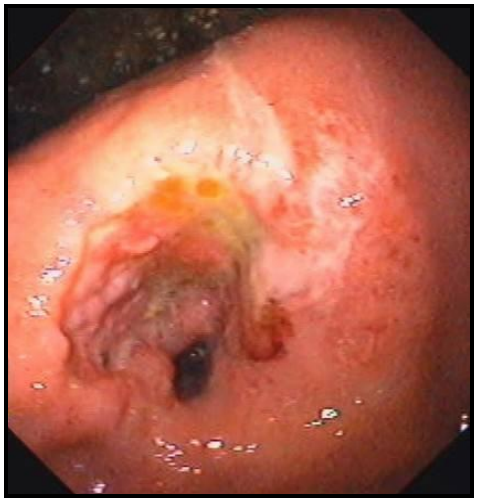


Figure 1-12: Endoscopic appearance of distal gastric adenocarcinoma

Tumour staging	Lymph node Involvement	Metastasis
T1 Lamina propria, Submucosa	N0 No nodes	M0 No metastasis
T2 Muscularis propria, Subserosa	N1 1-6 nodes	M1 Distant metastasis
T3 Penetrates serosa	N2 7-15 nodes	
T4 Adjacent structures	N3 >15 nodes	
Tumour staging		
Stage 1	T1N1M0; T2NOMO	
Stage 2	T1N2M0; T2N1M0; T3N0M0	
Stage 3	T2N2M0; T3N1-2M0; T4N0-1M0	
Stage 4	T4N2M0; T1-4N3	

Table 1-3: TNM classification of oesophageal and gastric cancer and staging (Greene and Sobin 119-20).

1.5.6 Treatment of distal gastric adenocarcinoma

Surgery (partial or total gastrectomy) is the treatment of choice for gastric cancer. The most important variable that determines tumour resectability and surgical outcome is the stage of disease at presentation. However multiple factors determine the outcome of curative surgery including a patient's age, co-morbidities and fitness for operation in addition to the tumour stage at presentation. There is a higher post operative mortality with total gastrectomy than subtotal gastrectomy. Results from specialist units in the UK report an operative mortality of 4.5% (The NHS Information Centre, 2010).

The role of adjuvant chemotherapy has been addressed in several studies and meta-analyses and this modality has been shown to confer a survival advantage (Mari et al., 2000; Sakuramoto et al., 2007). In a recent trial (MAGIC Trial), pre and post-operative chemotherapy for localised disease using a combination of epirubicin, cisplatin and 5-FU demonstrated significantly improved 5-year survival rates (36.3%) compared to surgery alone (23%) (Cunningham et al., 2006). This strategy of peri-operative chemotherap for localised gastric cancer has increasingly become the standard of care in the UK and in some centres in Europe (Cunningham and Oliveira, 2008; Allum et al., 2011).

Palliative chemotherapy is widely used in patients with inoperable and advanced disease. Several randomised studies have demonstrated survival benefits and improved quality of life with palliative chemotherapy (Pyrhonen et al., 1995; Glimelius et al., 1997; Assersohn et al., 2004). The drugs most commonly used are a combination of epirubicin, cisplatin and a

continuous intravenous infusion of 5-flurouracil which has a 65% response rate and a 11% complete response rate (Findlay et al., 1994). A recent Cochrane review has also highlighted the benefits of combination chemotherapy in a palliative setting (Wagner et al., 2005).

1.5.7 Prognosis of distal gastric adenocarcinoma

Most patients in the UK are diagnosed with stage 4 disease and their 5-year survival rate is less than 5%. On the contrary, patients with tumours measuring less than 5 cm in diameter, with no serosal invasion or lymph node metastasis, have a 5-year survival rate approaching 80% (Gatta et al., 2006; Bowles and Benjamin, 2001). In a retrospective study conducted in our centre the median survival for patients with gastric adenocarcinoma was 246 $\pm$ 39 days. Patients presenting as an emergency were found to have more advanced tumour stage. Such patients not only had lower surgical resection rates but also had reduced survival rates compared to those patients whose diagnosis was made through non-emergency routes (Sehgal et al., 2009).

1.5.8 Cardia or Proximal Gastric Cancer

There is increasing recognition of another distinct type of gastric cancer namely ‘proximal’ or ‘cardia’ cancer. This is considered to be an entirely different entity from distal gastric cancer, as tumours at this site differ in their epidemiology and pathogenesis. Two types of cardia cancers have been recognised –

- Type A - associated with atrophic gastritis and resembling the more common distal gastric cancer and
- Type B - resembling oesophageal adenocarcinoma, occurring in people with an acid secreting stomach and possibly arising as a consequence of chronic gastro-oesophageal reflux.

The gastric cardia is the most proximal part of the stomach adjoining the oesophagus and located below the gastro-oesophageal junction. The mucosa of the cardia is similar to that seen in the gastric antrum and is lined by columnar cells and contains branched mucous secreting cells with a relative absence of parietal and chief cells. There has been much debate regarding the origin of cardia type mucosa. Analysis of autopsy samples suggests the presence of cardia-type mucosa in only about 50% of specimens. The presence and also the total length of cardia type mucosa increases with age. It has therefore been suggested that cardia-type mucosa may be acquired secondary to gastro-oesophageal reflux (Chandrasoma et al., 2000). However, a study of the histology of the gastro-oeosophageal junction in 21 neonates, found a short segment of the mucosa resembling cardia-type mucosa in all specimens, suggesting that this is part of the normal anatomy of the stomach (De et al., 2003). However, it still remains unclear as to whether the cardia-type mucosa seen in neonates represents true gastric type epithelium or a primitive developing mucosa, as it appears to be different from adult-type cardia mucosa.

Various hypotheses have been proposed to explain the mechanisms responsible for cardia mucosa development. The

oesophageal squamous epithelium is unable to withstand acid reflux and hence in some cases undergoes metaplastic change to gastric columnar type epithelium upon persistent acid exposure. Hence cardia mucosa might arise from metaplasia of the very distal end of the oesophageal squamous epithelium. However this does not differentiate between true cardia-type mucosa and metaplastic squamous epithelium (Barrett's). Another possible aetiology involves changes secondary to chronic *H. pylori*-induced gastric atrophy involving the oxyntic mucosa. This can stimulate metaplasia in the atrophic oxyntic mucosa leading to the formation of cardia-type mucosa (Van Zanten et al., 1999).

It is currently difficult to define adenocarcinoma of the cardia and the term cardia cancer is therefore applied to those tumours (adenocarcinomas) arising in the proximal portion of the stomach near to or involving the gastro-oesophageal junction. 80% of these tumours are of the intestinal type (Palli et al., 1992). There has been an increase in the incidence of cardia cancers worldwide, while the incidence of distal gastric adenocarcinomas has been decreasing (Botterweck et al., 2000; Neugut et al., 1996). This suggests that different pathophysiological mechanisms are involved.

Gastro-oesophageal reflux is an important risk factor for oesophageal adenocarcinoma and hence type B cardia adenocarcinoma may have a similar aetiology and pathogenesis (Lagergren et al., 1999), involving exposure to both acid reflux and luminal carcinogens (McColl, 2006). There also appears to be a higher prevalance of cardia cancer in men,

similar to that observed in oesophageal adencarcinoma (Brewster et al., 2000).

H. pylori induced gastric atrophy has also been shown to be positively associated with cardia cancer in a case-controlled study. There was a significant association between *H. pylori* sero-positivity and the risk of developing cardia-type cancer if there was also associated gastric atrophy. *H. pylori* infection on its own however was negatively associated with the development of cardia cancer in this study (Hansen et al., 2007).

1.6 Gastric Neuroendocrine tumours (NETs)

Gastric neuroendocrine tumours (gastric carcinoid tumours) are rare tumours occurring in 1 to 2 per 1,000,000 persons per year and account for 8.7% of all gastrointestinal neuroendocrine tumours (Modlin et al., 2003a). This tumour type was discussed initially in section 3.2.3.

1.6.1 Classification of gastric NETs

Clinically gastric carcinoid tumours are classified depending on the background gastric pathology as follows (Rindi et al., 1993) (table1-5):

1. Type-1: associated with atrophic body gastritis
2. Type-2: seen in Zollinger Ellison syndrome when associated with the MEN-1 syndrome and
3. Type-3: sporadic tumours which develop in the absence of any specific gastric pathology

This clinical classification is important not only in detecting co-existing diseases such as atrophic gastritis and Zollinger Ellison syndrome, but also in predicting tumour growth and prognosis.

Histological classification of these tumours (WHO classification – table 1-4) is based on tumour differentiation namely:

1. Well-differentiated, which includes most type-1 and -2 gastric carcinoid tumours and
2. Poorly-differentiated, which are usually type-3 gastric carcinoid tumours

In addition, a third type namely 'mixed endocrine-exocrine' tumours has also been recognised (Rindi et al., 2000). Although type-3 tumours are often poorly-differentiated they can also occur as well-differentiated or as mixed tumours.

Well-differentiated tumours develop from enterochromaffin-like cells (ECL-cells) which are located mainly in the fundus and the corpus of the stomach. Gastrin is considered to be the main stimulant for the development of type-1 and -2 tumours and the ECL-cell appears to be the main proliferative target for gastrin. Type-3 tumours on the other hand grow independently to extrinsic gastrin stimulation ('gastrin-independent').

WHO classification	Tumour type	Metastases	Invasion beyond submucosa	Histological differentiation	Tumour size, cm	Vascular invasion	Ki 67 %
Group 1	Benign (low risk)	–	–	well-differentiated	≤ 1	–	<2
	Benign or low-grade	–	–	well-differentiated	> 1	±	<2

	malignant						
Group 2	Low-grade malignant	+	+	well-differentiated	> 2	+	>2
Group 3	High-grade malignant	+	+	poorly differentiated	any	+	>15

Table 1-4: WHO classification of gastric neuroendocrine tumours.

Ki 67 is the prototypic nuclear protein that is expressed in all active phases of the cell cycle and hence expressed by proliferating cells and absent in resting cells. The Ki 67 index is the ratio between the numbers of Ki 67 expressing cells to the total number of resting cells. The correlation between low Ki 67 index and low grade tumours is strong (Pelosi et al. 1124-34; Scholzen and Gerdes 311-22).

1.6.1.1 Type-1 gastric NETs

These are the most common form of gastric carcinoid and account for 70-80% of gastric NETs (Bordi, 1999). They are usually small (less than 1 to 2cms in size), often multiple, occur predominantly in the fundus and corpus of the stomach, are associated with atrophic body gastritis and are most frequently seen in women aged >50 years (Annibale et al., 2001a) (fig 1-15).

It has been proposed that persistent achlorhydria (resulting from complete loss of parietal cells or atrophic body gastritis secondary to autoimmune gastritis such as pernicious anaemia) and the subsequent loss of negative feedback inhibition of gastrin secretion stimulate G-cell hyperplasia. The resulting hypergastrinaemia promotes ECL-cell growth and carcinoid tumour formation (Annibale et al., 2001a) (Fig1-13). Approximately 5% of patients with autoimmune atrophic body gastritis will develop type-1 gastric carcinoid tumours (Fig1-14). These tumours are

associated with a good long term prognosis with 5 year survival rates quoted at 96% (Hosokawa et al., 2005).

Type-1 gastric carcinoid tumours are fairly indolent and are often found incidentally at gastroscopy. There is associated hypergastrinaemia and an elevated gastric pH. These tumours are often polypoidal or intramucosal, are well differentiated and considered to be of low grade, as demonstrated by low numbers of mitotic cells (or absent mitosis) and a low Ki 67 index. Metastatic potential is low, with rates of ~5% observed in most studies and in most cases metastases have been found to be limited to local lymph nodes (Rindi et al., 1996; Rindi et al., 1999b).

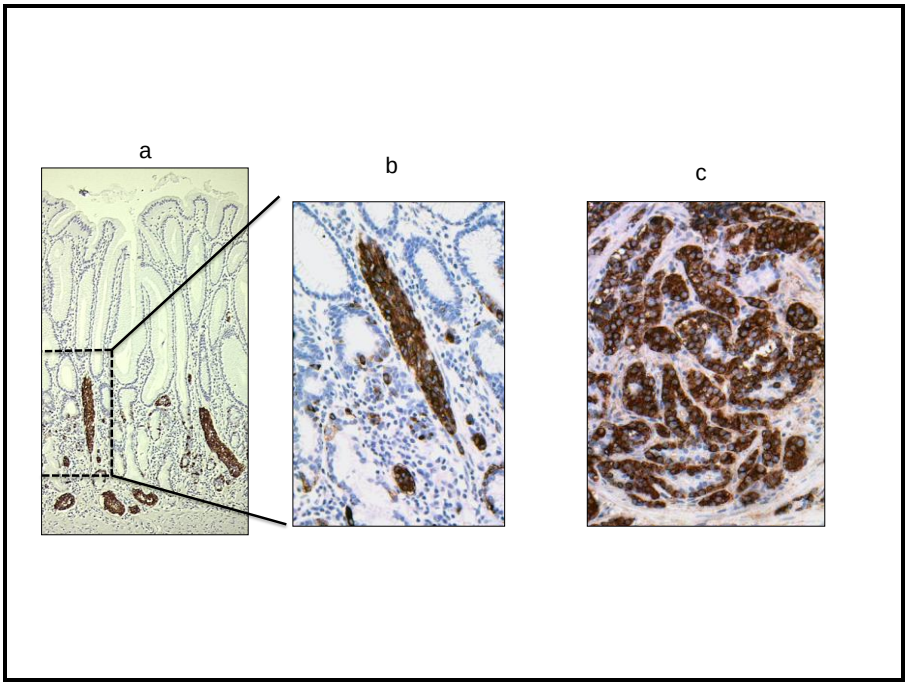


Figure 1-13: Histological characteristics of gastric neuroendocrine tumours. (a) Atrophic gastritis; (b) Linear ECL-cell hyperplasia and (c) Nodular ECL-cell hyperplasia.

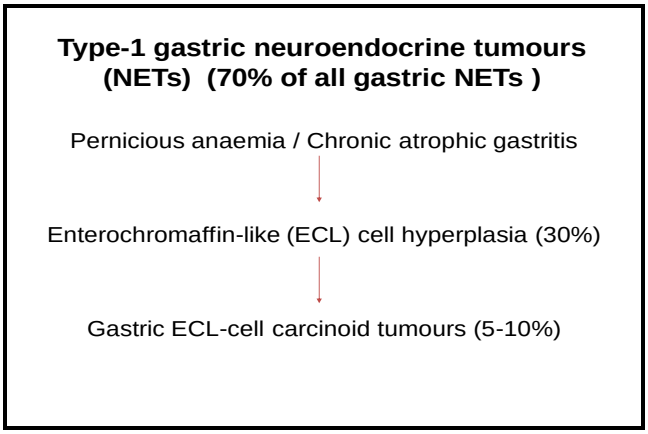


Figure 1-14: Progression of atrophic gastritis to ECL-cell hyperplasia and carcinoid tumour formation.

1.6.1.2 Type-2 gastric NETs

Type-2 gastric carcinoid tumours account for approximately 5 to 6% of gastric NETs (Bordi, 1999) and tend to occur in Zollinger Ellison syndrome associated with MEN-1 syndrome. Patients with the ZES/MEN-1 syndrome have a 29 to 34% risk of developing type-2 gastric carcinoid tumours in contrast with patients with sporadic ZES (not associated with MEN-1), who have a much lower risk at 0 to 1% (Gibril et al., 2004).

Type-2 gastric carcinoid tumours, like the type-1 tumours, are usually small, often multiple and are most frequently located in the fundus/corpus of the stomach. However, tumours have also been reported to occur in the antrum (Bordi et al., 2001) and the rate of metastasis has been shown to be higher than that seen with type-1 tumours, occurring in about 10 to 30% of cases (Norton et al., 2004). Type-2 gastric carcinoid tumours are usually polypoidal and well-differentiated and there is associated hypergastrinaemia in the presence of an acidic gastric pH.

1.6.1.3 Type-3 gastric carcinoid tumours

These tumours account for approximately 14 to 25% of gastric NETs and often develop in the absence of hypergastrinaemia or specific gastric pathology (Bordi, 1999). These tumours are often large (>2 cm), single, polypoidal or ulcerated at presentation and are more frequently seen in men aged > 50years. Metastasis at presentation is observed in approximately 50 to 70% of patients with well-differentiated tumours and in almost 100% of poorly-differentiated tumours (Rindi et al., 1996). Patients with type-3 carcinoid tumours have a relatively poorer prognosis due in part to the metastatic nature of the disease. Studies have shown 5-year survival rates at 30% (Borch et al., 2005; Rappel et al., 1995).

Features	Type-1	Type-2	Type-3
% of gastric NETs	70-80%	5-6%	14-25%
Associated gastric pathology	Atrophic Body gastritis	None	None
Associated diseases	Autoimmune gastritis; Pernicious anaemia	ZES and MEN-1	None
Site of tumours	Fundus	Fundus (antrum occasionally)	Antrum or fundus
Typical size	Usually <1cm	<1cm	>2 cms
Numbers	Multiple	Multiple	Solitary
Serum gastrin concentration	High	High	Normal
pH of stomach juice	Neutral	Acidic	Acidic
Prognosis	Good	Mostly good (some aggressive)	Poor
Treatment options	Endoscopic resection, antrectomy, surveillance	Endoscopic resection, gastrinoma resection, somatostatin analogues	Partial or total gastrectomy

Table 1-5: Types of gastric carcinoid tumours

1.6.2 Management of gastric NETs

Accurate clinical assessment and evaluation of the pathological features of gastric NETs are essential for the management of affected patients. Detecting the presence of associated conditions namely atrophic body gastritis and ZES/MEN-1 is crucial in classifying the tumours into the different clinical types and deciding upon the most appropriate treatment.

1.6.2.1 Investigations to aid tumour classification

In order to detect the presence of atrophic body gastritis and therefore to diagnose type-1 carcinoid tumours, all patients should undergo haematological and immunological assessment for the presence of autoimmune gastritis and pernicious anaemia. Investigations should include measurement of serum vitamin B12 levels and assessment for the presence of anti Gastric Parietal cell (anti-GPC) and anti Intrinsic factor (anti-IF) antibodies. Histological assessment should be made not only of the tumour and its surrounding gastric mucosa but also the antral mucosa for assessment of G-cell hyperplasia, the source of hypergastrinaemia in these patients.

In patients with type-2 tumours, hypergastrinaemia should be confirmed by estimating fasting serum gastrin concentration and demonstrating the presence of an acidic gastric pH (pH<2 is confirmatory of ZES). Investigations to assess the presence of the other components of the MEN-1 syndrome (eg. evaluation of the pancreas, pituitary and

parathyroid glands) and mutations in the MENIN gene should also be performed.

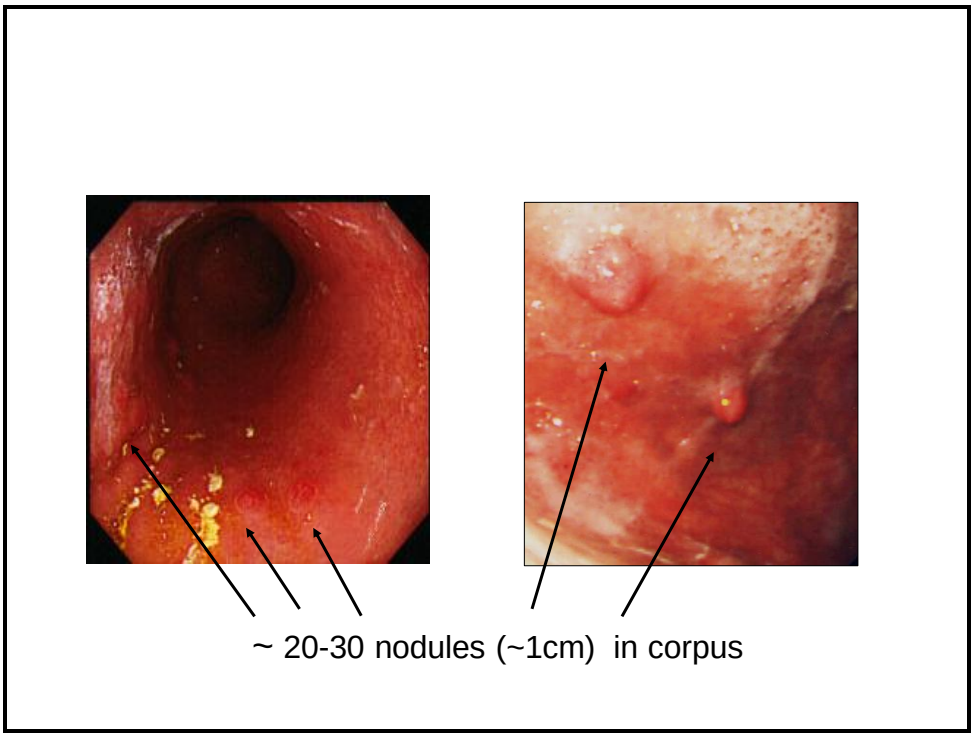


Figure 1-15: Endoscopic appearances of gastric type-1 carcinoid tumours in a background of atrophic gastritis.

1.6.2.2 Estimation of disease extent and localisation of associated neuroendocrine tumours

Given that metastatic gastric carcinoid tumours carry a worse prognosis, it is imperative to assess disease extent at presentation. Multislice CT (computerised tomography) scans help not only to identify metastatic disease, but can also locate type-2 gastric carcinoid tumours or other associated gastrinomas (Ba-Ssalamah et al., 2003).

However, CT scans do not provide any indication of functionality of tumours. Diffuse lesions could therefore be missed if CT scans were used

as the only imaging modality. Approximately 85% of gastric carcinoid tumours express somatostatin receptors and hence in these cases somatostatin receptor scintigraphy (octreotide scan) can be a useful technique to assess disease extent (Gibril et al., 2000). Anatomic localisation of tumours using somatostatin receptor scintigraphy on the other hand can be difficult, especially for distinguishing pancreatic and gastric tumours (Gibril et al., 2000).

Positron emission tomography scans (PET) may also help improve localisation of difficult lesions (Eriksson et al., 2000a). Using ^{11}C-5-hydroxytryptophan as a tracer rather than the conventional ^{18}F-fluorodeoxyglucose may be of more value in evaluating neuroendocrine tumours (Eriksson et al., 2005). Newer PET techniques using ^{68}Ga-DOTATOC have been shown to have a sensitivity of 97%, a specificity of 92% and a greater overall diagnostic accuracy in the diagnosis of neuroendocrine tumours compared to somatostatin receptor scintigraphy and conventional CT scan (Gabriel et al., 2007).

In combination, the three modalities namely CT scan, somatostatin receptor scintigraphy and ^{11}C-5-hydroxytryptophan PET scan may help to localise tumours accurately (Orlefors et al., 2005).

In patients with type-2 gastric carcinoid tumours, localisation of the gastrin secreting tumour is also essential for determining treatment strategies. Although gastroscopy may help to localise such tumours, particularly as they are often located in the duodenum, the combined use of CT, ^{68}Ga-DOTATOC PET scans and somatostatin receptor scintigraphy often provides more valuable information. Gastrin secreting

neuroendocrine tumours are often also detected by endoscopic ultrasound (EUS) and a histological diagnosis can be achieved using EUS guided fine needle aspiration (FNA) in approximately 90% of cases (Vander, III et al., 2004). On rare occasions, angiography and selective venous sampling may also be necessary to identify the location of gastrin producing tumours (Jackson, 2005).

1.6.2.3 Confirming tumour dependency on gastrin

As previously mentioned, both type-1 and -2 gastric carcinoid tumours are gastrin driven tumours. Removal of the source of hypergastrinaemia (eg. antrectomy in type-1 and gastrinoma resection in type-2 gastric carcinoid tumours) may well provide the treatment required to stop the growth of such tumours. It therefore becomes essential to confirm the dependency of these tumours on gastrin for their growth. This has been described in a single case study utilising the ‘octreotide suppression test’ (Higham et al., 1998). This will be described in more detail in chapter 8.

1.6.2.4 Monitoring disease progression and tumour burden

Since most gastric carcinoid tumours are functionally non-secretory, the usual method of measurement of secreted hormones in neuroendocrine tumours is replaced by alternative serological markers. Serum chromogranin A (CgA) is one such marker that has been studied in considerable detail (Giovanella et al., 1999). It is a protein produced by all

cells derived from the neural crest (Tomassetti et al., 2001) and is produced in large amounts by neuroendocrine tumour cells irrespective of their secretory status (Eriksson et al., 2000b). Serum concentrations of CgA have been shown to correlate with ECL-cell mass in type-1 gastric carcinoid tumours (Borch et al., 1997), hence CgA is considered to be useful in monitoring disease extent and tumour burden in patients with metastatic disease as suggested by the European Neuroendocrine Tumour Society (ENETS) guidelines (Delle et al., 2012).

1.6.2.5 Treatment of gastric NETs

Treatment approaches for gastric carcinoid tumours depend on the type of tumour. It is appropriate for small type-1 gastric carcinoid tumours, due to their low metastatic potential, to be monitored by regular surveillance as outlined in the ENETS guidelines (Plockinger et al., 2004). Those type-1 tumours not suitable for simple surveillance may benefit from endoscopic resection. Type-1 carcinoid tumours that are amenable to endoscopic resection are usually small (<1cm) and few in number (3-5) (Ichikawa et al., 2003). Alternatively, removal of the source of gastrin by antrectomy or distal partial gastrectomy has also been advocated (Higham et al., 1998; Dakin et al., 2006). By utilising the inhibitory effect of somatostatin on ECL-cell hyperplasia (mediated by its direct antiproliferative effect on the ECL-cell and indirectly through the inhibition of gastrin synthesis), long acting somatostatin analogues have also been shown to be useful in the treatment of type-1 gastric carcinoid tumours (Fykse et al., 2004; Grozinsky-Glasberg et al., 2008b). However, follow up studies on patients following cessation of long acting somatostatin

analogues have demonstrated regression of tumours, suggesting that long term continuation of such therapy may be required (Jianu et al., 2011).

A similar approach of removing the source of hypergastrinaemia by gastrinoma resection may be used for type-2 gastric carcinoid tumours. In addition to endoscopic or surgical resection of type-2 gastric NETs, the somatostatin analogue, octreotide, has also been demonstrated to cause marked regression in tumour size and a decrease in serum gastrin concentrations (Tomassetti et al., 2000). This therefore is a potential strategy for the management of those type-2 gastric carcinoid tumours that are multiple and not suitable for surgical resection (Ramage et al., 2005a).

Type-3 carcinoid tumours will require surgery if there is no evidence of metastatic disease. Surgery usually involves either a partial or total gastrectomy with lymph node dissection, not dissimilar to that offered for gastric adenocarcinoma (Ruszniewski et al., 2006).

1.7 Tumour biomarkers

Specific tumour markers are useful for assessing the presence or response to treatment of various tumour types. For example, CEA (carcino-embryonic antigen) is useful in colorectal cancer; CA19-9 in pancreatic cancer; AFP (alpha fetoprotein) in hepatocelluar carcinoma and seminomas and PSA (prostate specific antigen) in prostate cancer. However, although significantly elevated concentrations of such biomarkers may be associated with tumours, in most cases they are not particularly useful as diagnostic tools. Nonetheless they are sometimes

useful as markers for disease progression or recurrence following therapy (especially CEA in colorectal cancer and PSA in prostate cancer) and can aid in the detection of difficult to diagnose cases. No such markers have however been associated with gastric adenocarcinoma to date, although serum pepsinogen concentrations (low serum pepsinogen and low pepsinogen I/pepsinogen II ratio [PGI/II]) have been utilised as a non-invasive tool for the diagnosis of gastric atrophy and in predicting the risk of development of gastric adenocarcinoma (Oishi et al., 2006; Ren et al., 2009). Ideally biomarkers should not only be associated with the presence of a tumour itself, but ideally should also be elevated during the precancerous stages. This would permit risk stratification and thereafter appropriate surveillance. No such surveillance programmes are currently available for gastric adenocarcinoma even though gastric atrophy and intestinal metaplasia are recognised precancerous conditions. Even in Japan, where there is a very high prevalence of gastric cancer, mass screening for this cancer is performed using gastric fluoroscopy (Hamashima et al., 2008). Patients then subsequently undergo gastroscopy only when the screening fluoroscopy is abnormal. Although the evidence to support the routine use of endoscopy, serum pepsinogen or *H. pylori* antibody testing in gastric cancer screening is not strong, the possible role of endoscopic surveillance in the detection of early gastric cancers has been studied. In a prospective study conducted in the UK, it was observed that surveillance endoscopy of patients with atrophic gastritis and intestinal metaplasia enabled detection of early stage gastric cancers (Whiting et al., 2002). Such patients had a better outcome due to

the early detection of gastric cancer. However such endoscopic surveillance programmes are currently restricted only to those patients with a known high risk genetic disorder associated cancer (such as Hereditary non-polyposis colorectal cancer, Familial Adenomatous polyposis, MUTYH-associated polyposis, Juvenile polyposis and Peutz-Jegher syndrome) (Cairns et al., 2010).

The Liverpool NIHR-Biomedical Research Centre (BRC) funded project 'Identification of genes and proteins involved in promoting gastric cancer development in patients with chronic *Helicobacter pylori* infection' in which the author was involved, was designed to investigate specific biomarkers that may predict development or progression to gastric adenocarcinoma. The following are potential candidate biomarkers of gastric preneoplasia that are currently being assessed within that study (Table 1-6).

1.7.1 IGF family

The IGF (Insulin-like Growth Factor) family comprises the peptide hormones IGF-1 and IGF-2, their cell surface receptors namely IGFR1 and IGFR2 and the binding proteins IGFBP, of which IGFB1 to 6 have been identified. This family of proteins plays important roles in regulating cell growth and apoptosis.

1.7.1.1 IGF-1 and 2

These were initially characterised in 1970 (Rinderknecht and Humbel, 1978) and are homologous, small chain peptides of about 7.5kD

in size. The gene encoding IGF-1 is located on chromosome 12q22-q24.1 and that encoding IGF-2 is located to the insulin gene at 11p15.5. IGF-1, a trophic factor, is present in high concentrations in the serum. The main source is the liver and its production is modulated by both growth hormone and insulin (Yu and Rohan, 2000).

IGF-1 exerts its effects mainly by binding to the extracellular domain of the IGF receptor 1 and it also binds to the insulin receptor. IGF-1 has been shown to directly stimulate DNA synthesis. It also stimulates the expression of cyclin D1, which in turn accelerates the progression of the cell cycle from the G1 to the S phase. It also stimulates the expression of the Bcl-2 protein and suppresses the expression of Bax, thereby blocking the initiation of apoptosis (Sara and Hall, 1990).

IGF-2 on the other hand binds with high affinity to the IGF-2 receptor, with low affinity to the IGF-1 receptor and not at all to the insulin receptor. The expression of IGF-2 is increased in foetal tissue, where it is thought to play a major role in the regulation of growth as compared to the postnatal period. It is a regulatory peptide and most of its actions are mediated through IGFR1 (Yu and Rohan, 2000).

Both IGF-1 and -2 are mitogens regulating cell proliferation, differentiation and apoptosis. These effects are mediated predominantly through stimulation of IGFR1. A pattern of autocrine stimulation of malignant cell division through stimulation of the IGFR1 receptor by both IGF-1and -2 has been shown in *in vitro, in vivo* and clinical studies. Increased secretion of IGF-2 has also been demonstrated in gastric cancer cells of both the diffuse and intestinal variants. A corresponding increased

expression of mRNA for IGF-2 and IGFR1 and a decreased expression of IGFR2 mRNA have been shown in gastric cancer cells (Pavelic et al., 2003).

1.7.1.2 IGFR

As mentioned above, IGF-1 and IGF-2 bind to the IGFR1 receptor and most of the effects of IGF-2 are mediated through binding to this receptor. IGF binds to the extracellular part of IGFR1. This results in a cytoplasmic signal cascade and a conformational change in the receptor through stimulation of phosphorylation. The subsequent downstream signalling results in activation of cell proliferation, differentiation and migration, and also inhibition of apoptosis (Yu and Rohan, 2000).

IGFR1 has been shown to be over expressed in some tumours and if this occurs independently of other peptides (such as associated over expression of IGF), it allows cells to develop a new phenotype of anchorage independent growth leading to tumour progression (Pavelic et al., 2003).

1.7.1.3 IGF B proteins and proteases

IGF Binding proteins (IGFBPs) 1 to 6 are six multifunctional proteins that were originally thought to be passive circulatory transport proteins for the IGFs. However, there is now sufficient evidence to suggest that they play major roles in the regulation of cell activity (Firth and Baxter, 2002).

IGFBP-3 is the most abundant circulatory IGFBP and acts as a carrier for over 70% of circulating IGF-1 and 2. The IGFBPs act via both autocrine and paracrine pathways. They modulate activation of IGFR1 by IGFs. They therefore influence cell motility and adhesion, apoptosis and cell survival as well as cell cycle progression. The expression of IGFBP-4 and 5 is regulated by IGF-1 in vascular smooth muscle cells and conversely IGFBP-4 inhibits IGF-1 and IGFBP-5 stimulates IGF-1. IGFBP-1 can either inhibit or stimulate IGF depending on the phosphorylation state of IGFBP-1 (when dephosphorylated the affinity of IGFBP-1 for IGF-1 is decreased six fold). IGFBP-2 on the other hand, by virtue of its increased affinity, often inhibits IGF-2. There is also evidence to suggest that the actions of IGFBPs may be independent of IGF binding (Firth and Baxter, 2002).

1.7.1.4 IGF and cancer

Several case studies and epidemiological studies have demonstrated associations between IGF and cancers of the lung, breast, colon and pancreas (Yu et al., 1999; Sugumar et al., 2004; Nomura et al., 2003; Lin et al., 2004). Increased concentrations of IGFs have been consistently seen in these cancers and elevated IGFBP-3 concentration has also been associated with a decreased cancer risk (Renehan et al., 2004; Yu and Rohan, 2000). However such an association has not been consistently established for stomach cancer.

Yatsuya *et al*., followed up 210 gastric cancer patients and 410 controls and determined the efficacy of IGF-1,-2, IGFBP-3, TGFβ-1, sFAS and SOD as potential biomarkers. Of the 6 biomarkers tested, only sFAS was found to be significantly elevated in cancer patients (and only in females) and none of the other biomarkers were consistently elevated (Yatsuya et al., 2005). However in a study including 97 patients, Zhang *et al*. found that IGFBP-2 was significantly elevated in patients with gastric cancer and this positively correlated with cancer progression (Zhang et al., 2007).

In patients with *H. pylori* infection, increased serum concentrations of IGFBP-2 and decreased concentrations of IGFBP-3 have been demonstrated (Baricevic et al., 2004). In a study from the UK, serum IGFBP-3 concentrations were found to be decreased in patients with gastric intestinal metaplasia, suggesting a potential role of IGFBP-3 in preventing the development of intestinal metaplasia and adenocarcinoma (Zhang et al., 2004). However conflicting results were seen in a study by Francois *et al*. in which serum samples from 26 patients with gastric cancer were analysed for concentrations of IGF-1 and IGFBP-3 before and after (15^{th} day) surgery. Significantly higher concentrations of IGF-1 were seen in patients with gastric cancer compared to controls and in all cancer patients the concentration of IGF-1 was above that of baseline normal. Following surgery, there was a significant decrease in IGF-1 concentrations, but no such change was seen with IGFBP-3, whose concentration was also higher at baseline compared to normal controls.

IGF-1 concentrations were not related to tumour extension or nodal involvement (Franciosi et al., 2003).

In addition, Pham *et al.* found no significant association of IGF-1/-2 with gastric cancer in a nested case control study. Although increased concentrations of IGFBP-3 were seen in patients with a lower risk of stomach cancer and decreased concentrations of IGFBP-3 were found in patients with stomach cancer, both of these associations were not statistically significant (Pham et al., 2007).

1.7.2 uPA (Urokinase Plasminogen Activator) system

The response of tissues to injury or inflammation often involves activation of the uPA system which includes uPA, its receptor (uPAR) and the plasminogen activator inhibitors (PAI-1,-2 and -3).

1.7.2.1 Urokinase Plasminogen Activator

uPA is one of several serine proteinases that are central to pericellular proteolysis through the conversion of plasminogen to plasmin.

The uPA system comprises a single chain polypeptide (sc-uPA) consisting of 411 amino acids. Plasmin confers proteolytic activity to sc-uPA after cleavage at the 157 site and plasmin is regarded as the most efficient method for converting the uPA to its active form. Other proteases including cathepsins, plasma kallikrien, nerve growth factor and mast cell tryptase can also activate uPA. Once activated enzymatically, the active

high molecular weight two chain form (HMWuPA) then converts plasminogen to plasmin. Plasmin is then able to amplify its own production by converting other sc-uPA into active HMWuPA (Dass et al., 2008).

1.7.2.2 Urokinase Plasminogen Activator Receptor (uPAR)

uPAR is a glycoprotein that binds to UPA through its growth factor like domain. It was first described as a receptor on human monocytes. uPAR is not a transmembrane protein and binds to both the single chained and double chained forms of uPA (Blasi and Carmeliet, 2002). This binding greatly increases the activation of plasminogen by uPA. uPAR can also bind to uPA which has been inactivated by PAI-1. This forms the uPA-uPAR-PAI complex, which is then rapidly endocytosed and the uPAR is recycled and delivered to a different site on the cell surface. This process is said to be directional and important for cell migration (Nykjaer et al., 1997)

1.7.2.3 Plasminogen Activator Inhibitors (PAI)

uPA mediated conversion of plasminogen to plasmin is regulated by two plasminogen activator inhibitors, PAI-1 and PAI-2. They exert their inhibitory effects by either binding to uPA in solution or to the uPA-uPAR complex present on the cell surface (Andreasen et al., 1990).

PAI-1 appears to be the more dominant inhibitor and is produced by platelets, endothelial cells and tumour cells (Schmitt et al., 1997). PAI-2 on the other hand is produced by phagocytic cells, trophoblasta and tumour cells (Schmitt et al., 1997).

Binding to the uPA-uPAR system, PAI-1 internalises the complex by stimulating endocytosis, resulting in subsequent degradation of the complex and recycling of uPAR to the cell surface at a different site (Conese et al., 1995).

The uPA system once activated following the binding of uPA to the uPAR may elicit several events linked to the conversion of plasminogen to plasmin. This probably includes extravascular fibrinolysis, activation of growth factors and matrix remodelling. These processes are integral to angiogenesis and metastasis, which are characteristic processes in malignant tissues. The uPA system is primarily associated with the degradation and regeneration of the basement membrane and the extracellular matrix and this therefore plays a major role in regulating metastasis, whereas its role as an anti-thrombolytic agent stimulates angiogenesis in tumour cells.

1.7.2.4 uPA and cancer

The various individual components of the uPA/uPAR system have been demonstrated to be differentially expressed in various cancer tissues compared to normal tissues. For example, in normal oesophageal tissue, uPA, uPAR and PAI-2 are not seen. uPAR was demonstrated in the border of oesophageal squamous carcinomas and uPA and PAI-2 were demonstrated throughout all the cancer cells (other than stromal cells) (Shiomi et al., 2000). Tumours expressing higher concentrations of uPA were associated with a higher rate of metastasis, whereas tumours with

higher PAI-2 concentrations were associated with lower degrees of metastasis. In colorectal cancers, greater concentrations of uPAR were associated with increased cancer related mortality (Stephens et al., 1999).

1.7.2.5 Gastric cancers

In a study by Ji *et.al*, 67 gastric tumour samples were analysed for the abundance of uPA and uPAR RNA by Northern Blot. Gastric cancer tissue expressed both UPA and uPAR in abundance and the presence of these proteins was associated with higher mortality and poorer prognosis (Ji et al., 2005). In another study of 105 gastric cancer specimens, increased expression of uPA and uPAR mRNA was seen in association with increased tumour invasion into the muscular and peritoneal layers. This was again associated with lower patient survival rates (Zhang et al., 2006).

uPAR, uPA and PAI-1 have all been shown to be associated with adverse outcomes in gastric cancer. Heis *et al.* noted an inverse correlation of levels of uPA, uPAR and PAI-1, but not PAI-2 with survival time. In their study, 203 consecutive patients with gastric cancer were recruited and the resection specimens were analysed by immunohistochemistry for the presence of uPA, uPAR and PAI-1 and –2. Expression was correlated with disease free and overall survival (Heiss et al., 1995). In another study, Ito et al. analysed specimens from 125 patients with gastric cancer by immunohistochemistry for uPA, PAI-1 and -2. uPA positive patients had more lymph node metastases, tumour infiltration and peritoneal dissemination and had a poorer prognosis. The

PAI-1 negative group were also more likely to have liver metastases and poorer prognosis. No significant associations were noted with PAI-2 expression (Ito et al., 1996).

1.7.2.6 uPA and gastric preneoplasia:

In a study of 104 patients, Beyer et al. studied the expression of the uPA system in biopsy specimens of gastric carcinoma and also gastric intestinal metaplasia (Beyer et al., 2006). Increased expression of uPAR and PAI-1 was seen by immunohistochemistry in specimens with intestinal metaplasia and this was significantly associated with more advanced tumour stage and lymph node involvement. This suggests that uPAR and PAI-1 may potentially be useful as predictors of disease staging.

In another study, Farinati et al. analysed specimens from 92 patients - 12 with gastric adenocarcinoma, 33 with chronic atrophic gastritis (all exhibiting intestinal metaplasia and 12 with dysplasia) and 47 controls for the expression of uPA, PAI-1, CATB and CATL (Farinati et al., 1996). There was significantly greater expression of uPA, PAI-1, cathepsins B (CATB) and cathepsin L (CATL) in patients with chronic atrophic gastritis than controls and a similar difference was seen in patients with cancer. CATB and uPA were significantly increased in patients with chronic atrophic gastritis and dysplasia compared to those with chronic atrophic gastritis without dysplasia. This suggests that the uPA system may play a significant role not only in cancer invasion, but also in the progression of the preneoplastic changes to cancer.

H. pylori infection itself also results in the stimulation of the uPA system in the gastric corpus (Kenny et al., 2008). In this study, uPA induced hyperproliferation of gastric epithelial cells was demonstrated to be inhibited by PAI-1. PAI-2 has also been shown to be directly induced by *H. pylori* infection and the inhibitory effect of PAI-2 on cell invasion and apoptosis increases the risk of progression to gastric adenocarcinoma (Varro et al., 2004).

1.7.3 Matrix Metalloproteinases (MMPs)

1.7.3.1 MMP family

MMPs are a family of zinc-dependent enzymes that are secreted as pro-enzymes prior to activation by cleavage of the propeptide. Approximately 25 MMPs have been identified and they are classified according to their substrate specificity into the following groups:

- Collagenases: MMP-1, 8 and 13. These cleave interstitial collagen and breakdown several extracellular matrix (ECM) related molecules.
- Gelatinases: MMP-2 and 9. These as the name suggests digest and degrade denatured collagen and gelatin.
- Stromelysins: MMP-3 and 10. These activate a number of the MMPs and also digest several ECM components.
- Matrilysins: MMP-7 and 26. These are involved in processing cell surface molecules.

- Membrane type (MT): MMP-14, 15, 16, 17, 24, 25. These activate pro-MMP-2 and digest ECM-molecules.
- Uncategorised: these are said to process a variety of non-matrix substrates and are involved in the cleavage of numerous growth and angiogenic factors and also are involved in controlling cell migration.

1.7.3.2 Role played by MMPs in cancers

The breakdown of the extracellular matrix (ECM) in a timely manner is essential for embryogenesis, tissue resorption and remodelling. The matrix metalloproteinases (MMP) or matrixins play major roles in the regulation of these processes. Liotta et al identified proteolysis as an important step in tumour invasion and described the role of type IV collagenase in melanoma invasion and metastasis (Liotta et al., 1980). It was initially assumed that the MMPs were produced by tumour cells, however there is now more evidence to suggest that stromal cells respond to tumour cells by induction of MMPs and this concept of stromal production of MMPs was brought to the forefront by identification of stromelysin-3 as a stromal MMP in association with breast cancer (Basset et al., 1990). In situ hybridisation techniques have further confirmed the stromal origin of several of the MMPs and this is generally now though to be more common than tumourous origin. There is a general correlation between the level of MMP expression and tumour stage – the higher the MMP expression, the more advanced and invasive is the associated

tumour. Malignant tumours have been found to not only have increased expression of MMPs, but also to express a wider variety of MMPs compared to more benign tumours. For example, in colonic adenocarcinoma the expressions of stromelysin -1,-3, matrilysin and gelatinase A are all increased, whereas in adenomatous colonic polyps, matrilysin (MMP-7) was the only MMP that was found to be elevated (Newell et al., 1994).

It has also been shown that MMPs could have roles in tumour prognosis or diagnosis. For example, some MMPs such as stromelysin-3 are expressed only in breast adenocarcinomas and not by the normal breast or by fibroadenomas.

1.7.3.3 MMP-7 (Matrilysin)

MMP-7 belongs to the group of matrilysins and is unusual in that it is secreted by epithelial cells (malignant or normal) rather than stromal cells. MMP-7 is secreted as a 28-kD proenzyme and is enzymatically activated by endoproteinases, trypsin and plasmin (Wilson and Matrisian, 1996). Increased MMP-7 expression has been described in several malignancies.

1.7.3.4 MMP-7 and Gastric cancer

In a study of 47 gastric cancer patients, Honda et al. measured MMP-7 mRNA expression in tumour vs. normal tissues (Honda et al.,

1996). They demonstrated increased MMP-7 mRNA expression in 87% of tumour samples compared to corresponding normal tissues. Also, a tumour/normal MMP-7 expression ratio of more than 2:1 was associated with deeper invasion and more frequent lymphatic or vascular invasion than a tumour/normal ratio of less than 2:1. Subsequent immunohistochemistry confirmed that MMP-7 expression was localised in cancer cells, with only weak expression being observed in normal epithelial cells and no expression was seen in surrounding stromal tissue, confirming that the predominant source of MMP-7 is from epithelial cells (in this instance the malignant cells). This suggests that over expression of MMP-7 may play an important role in tumour progression in gastric cancer and also suggests that it may be an important marker for predicting the behaviour of tumours.

MMP-7 has also been demonstrated as being an epithelial derived signal for IGF-II, which in turn stimulates proliferation of both stromal and epithelial cells in the stomach (McCaig et al., 2006). MMP-7 is upregulated in *H. pylori* infection and therefore plays an important role in the hyperproliferation induced by the presence of this bacterium.

1.7.3.5 MMP-2

Also called gelatinase A and collagenase IV, MMP-2 is a 72-kD member of the MMP family which is capable of degrading gelatin and type IV collagen. This suggests that it probably plays a role in tumour invasion and metastasis. MMP-2, like other MMPs is secreted as an inactive

proenzyme and is activated by proteolytic cleavage. The activity of MMP-2 is regulated by transcriptional factors as well as by the tissue inhibitors of MMPs (TIMPs).

However, in contrast to other MMPs, MMP-2 is expressed by a large number of cell types and is also over expressed in a wide variety of tumour cells.

1.7.3.6 MMP-2 and gastric cancer

Following on from an earlier study of MMPs and gastric cancer, Kubben et al. highlighted the association between MMP-2 and gastric cancer (Kubben et al., 2006). In this study involving 81 gastric cancer patients, the investigators assessed expression of MMP-2, MMP-9, MMP-7, MMP-8, TIMP-1 and -2. All MMPs studied were found to be significantly increased in gastric cancer tissues compared to normal gastric mucosa. Increased MMP-2 levels were significantly associated with survival rates, whereas no correlation was seen with clinical and pathological parameters including the TNM stage of disease and Lauren/WHO classification of the tumour. However the other MMPs (-7,-8,-9) and the TIMPs (-1 and -2) showed some correlation with clinical and pathological parameters, but not with patient survival.

In another study of 74 gastric cancer specimens, Murray et al. demonstrated that MMP-2 was consistently over expressed by tumour cells, but was not detected in the adjacent normal gastric epithelium. This study therefore demonstrated the important role played by MMP-2 in gastric cancer invasion and metastasis (Murray et al., 1998).

In a recent study of 116 gastric adenocarcinoma specimens, Alakus et al. demonstrated that increased expression of MMP-2 was significantly association with more advanced tumour stage and poorer survival (Alakus et al., 2008).

MMP-2 has thus been shown in several studies to be of prognostic value – higher levels of expression seem to be associated with a poorer prognosis in keeping with the protein's role in regulating cancer invasion and metastasis.

In a study by Bergin et. al, increased MMP-2 expression was also observed in patients with *H. pylori* infection compared to controls. MMP-9 expression was also shown to be significantly elevated, suggesting that several members of the MMP family are involved in regulating *H. pylori* associated gastric inflammation (Bergin et al., 2004).

1.7.4 Tissue Inhibitors of matrix metalloproteinases (TIMPs)

TIMPS are the natural inhibitors of MMPs. TIMPS 1-4 have been identified and are secreted proteins which form complexes with MMPs and thereby regulate their activation and activity. The expression of TIMPs, like MMPs, is also regulated during tissue remodelling.

TIMP-1 is a general inhibitor of MMPs whereas TIMP-2 is usually associated with MMP-2. TIMPs have also been found to influence apoptosis and proliferation by mechanisms other than regulation of MMPs.

TIMP-3 has been shown to have unique features – it is the only TIMP that binds firmly to ECM and is associated with a specific disease -

Sorsby fundus dystrophy, characterised by early blindness due to excessive accumulation of TIMP-3 in Bruch's membrane. This occurs due to mutations of cysteine residues to serine (Felbor et al., 1995). TIMP-3 has been also shown to initiate apoptosis whereas TIMPs-1and -2 inhibits apoptosis.

TIMP-4 is the latest TIMP to be identified and is the largest. It shares many similarities with other TIMPs, in particular with TIMP-2. It does not activate, but inhibits MMP-2. TIMP-4 expression has been shown to be increased in tumours of the breast, ovary, cervix, endometrium, prostate, colon and papillary renal tumours and to be down regulated in pancreatic and some renal tumours (Bister et al., 2007; Lizarraga et al., 2005).

Bodger et al. demonstrated significantly elevated concentrations of specific TIMPs (TIMP-1, 3 and 4) in the glandular corpus mucosa of *H. pylori* infected humans compared to uninfected controls (Bodger et al., 2008). This suggests that TIMPs may play an important role in regulating the gastric inflammation associated that is associated with *H. pylori* infection.

Increased expression of TIMP-2 was also observed more frequently in gastric adenocarcinoma and this was associated with a poorer overall survival (Kubben et al., 2006). In a study of 74 gastric adenocarcinoma patients, increased expression of TIMP-1 and -2 was demonstrated in the tumour tissue (no expression in normal gastric tissue) and this was associated with advanced tumour stage (Murray et al., 1998).

Potential biomarker	Family group	Expression in			
		Hp	**Gastric preneoplasia**	**Gastric adenocarcinoma**	**Other cancers**
IGF-1	IGF	↓	↑	↑	↑
IGF-2	IGF	↓	↑	↑	↑
IGFBP-2	IGF	↑	↑	↑	↑
IGFBP-3	IGF	↓	↓	↓	NK
uPA	uPA	↑	↑	↑	↑
uPAR	uPA	↑	↑	↑	↑
PAI-1	uPA	↑	↑	↑	↑
PAI-2	uPA	↑	↓	↓	NK
MMP-2	MMP	↑	↑	↑	↑
MMP-7	MMP	↑	↑	↑	↑
TIMP 1,3,4	TIMP	↑	NK	↑	↑

Table 1-6: Summary of potential gastric tumour biomarkers and their relation to H. pylori infection, the presence of gastric preneoplastic pathology and gastric cancer. (NK=not known).

1.8 Aims and Objectives:

1. To determine in a population undergoing diagnostic gastroscopy, factors associated with the presence of gastric pre-neoplastic and neoplastic changes following *Helicobacter pylori* infection.
2. To evaluate the association between gastrin and gastric pre-neoplastic changes and the association between *cag*A status of infecting *Helicobacter pylori* and gastric cancer development.
3. To determine the association between factors and the presence of type-1 gastric carcinoid tumours in patients with autoimmune atrophic body gastritis.
4. To assess the role of the ‘octreotide suppression test’ in predicting whether patients with type-1 gastric carcinoid tumours benefit from antrectomy.

Chapter 2

2 Materials and Methods

2.1 Study design

A cross-sectional study (described in chapters 3 to 6) recruiting symptomatic patients attending for diagnostic gastropscopy at a single University Hospital funded by and funded by the National Institute of Health Research (NIHR) Biomedical Research Centre in Microbial diseases at Royal Liverpool and Broadgreen University Hospitals NHS Trust, Liverpool, UK.

2.2 Patient recruitment

Local adult ethics committee approval and informed patient consent was obtained from participating patients who were attending for a diagnostic gastroscopy for clinical indications. The study was sponsored by Royal Liverpool and Broadgreen University Hospitals NHS Trust (R&D Number 3592) and the University of Liverpool. REC reference number 08/H1005/37).

Exclusions to recruitment into this study included:

- patients with a bleeding diathesis or on anticoagulants
- hepatits B or C infection, HIV infection
- pregnancy

- active bleeding or hemodynamic instability at the time of gastroscopy
- inability to obtain/give informed consent.

Patients with known Barrett's oesophagus attending for gastroscopy as part of a Barrett's or oesophageal adenocarcinoma surveillance were also excluded, although some patients who were found to have a new diagnosis of Barrett's were recruited.

All patients were interviewed and consented by Senthil Murugesan and their height and weight were recorded. A clinical questionnaire was completed for each patient (see appendix 1). Symptoms resulting in referral for gastroscopy, history of previous gastric surgery, relevant family history and other co-existing illnesses were documented. Smoking status, alcohol consumption and drug treatment, in particular the dosage of PPIs and the time elapsed since the last doses of PPI were recorded. Previous *H. pylori* status and eradication therapies were also recorded in the clinical questionnaire.

2.3 Samples

20mls of blood was obtained by venepuncture (fasting) at the time of recruitment prior to gastroscopy. 5mls were stored at -20°C in an EDTA containing vacuette for future DNA analysis for various genetic polymorphisms (of which 1 ml was also stored on protein saver cards). 10mls of blood was centrifuged and the serum obtained was subsequently stored at -20°C. This was utilised to analyse fasting serum gastrin

concentrations and the *cag*A status of the infecting *Helicobacter pylori*. A further 5 mls was used for serological analysis for *Helicobacter* infection (anti-IgG antibodies to *Helicobacter pylori*; performed by the Microbiology department at the Royal Liverpool and Broadgreen University Hospital NHS Trust).

At gastroscopy, two biopsies (each ~ 2mm size) were obtained from the antrum and two from the corpus using standard biopsy forceps. These were formalin fixed and paraffin embedded for subsequent histological analysis by a GI histopathologist, Dr. Laszlo Tiszlavicz, University of Szeged. The histological analysis was performed following staining with Giemsa, immunohistochemistry, H&E (haematoxin and eosin) and PAS (Periodic Acid Shiff). The gastric biopsy report (see appendix 2) included the Modified Sydney classification for inflammation (Dixon et al., 1996), the Padova (Rugge et al., 2000) and the modified Vienna classifications for dysplasia scoring (Schlemper et al., 2000) and also the presence of *Helicobacter pylori*. A rapid urease test (PRONTO Dry®, MIC France) was also performed using two gastric biopsies (two biopsies from the antrum in those patients not on concurrent PPI therapy or one biopsy each from the antrum and corpus from those on concurrent PPI) in all patients in order to obtain a rapid diagnosis of *Helicobacter pylori* infection. In this study, a diagnois of current *Helicobacter pylori* infection was made in the presence of a positive histology for this organism (with or without positive serology/rapid urease test) in keeping with currently accepted 'gold standard' method for the diagnosis of this organism (Malfertheiner et al., 2012). 8 additional corpus biopsies were obtained and stored in RNA-

Later® at 4˚C for RNA extraction and subsequent analysis of mRNA abundance of various potential biomarkers of gastric carcinogenesis.

2.4 Gastrin analysis by Radioimmunoassay

The technique of radioimmunoassay for the measurement of serum concentrations of hormones was first described by Berson and Yalow (Yalow and Berson, 1960). This is based on the principle that unlabelled hormone (in serum) competitively binds to a specific antibody and thus inhibits the binding of labelled hormone. Therefore as the concentration of unlabelled hormone increases, the ratio of bound to free labelled hormone decreases. The concentration of the hormone can therefore be measured by comparing the inhibition observed with that produced by standard solutions containing known concentrations of the hormone being measured (Berson and Yalow, 2006).

The antibody utilised in this technique (L2 antibody) of radioimmunoassay was directed against the α-amidated C-terminal portion of gastrin molecules, therefore binding all bioactive gastrins irrespective of N-terminal peptide length and degree of sulfation. This antibody however does not bind to the immediate precursors of amidated gastrin namely progastrin or glycine extended peptides (Dockray, 1980; Varro and Ardill, 2003; Rehfeld, 2007). There are currently no assays that reliably measure total gastrin concentrations.

Serum gastrin concentrations were measured for all the patients recruited to the study by radioimmunoassay. Very high concentrations

were subsequently analysed by serial dilutions. Quality controls (Q_c; serum containing known concentrations of serum gastrin) were used in each assay to detect inter assay variations.

2.5 Determination of *cag*A status of infecting *Helicobacter pylori* by Enzyme Linked Immunoabsorbant Assay (ELISA)

As described in section 4.4.1, the presence of the *cag*A gene has been found to be associated with increased pathogenicity of *H. pylori* (leading to an increased incidence of peptic ulcer disease and gastric adenocarcinoma). Infection with cagA+ve *H. pylori* strains results in a serological response from the host to the cagA subunit (cagA antibodies).

The *cag*A status of the *Helicobacter pylori* in our study population was determined using a commercially available Enzyme Linked Immunosorbant Assay (ELISA) (Genesis Diagnostics Ltd, Product code GD33, cagA IgG ELISA) kit. This technique is based on the following principle.

Serum samples (diluted) were incubated in microtitre wells with recombinant cagA protein (immobilised on the titre wells). Rabbit anti-human IgG conjugated to horseradish peroxidase was subsequently added to the wells after washing out the unbound serum. This IgG antibody bound to the surface bound antibodies during the second incubation. Unbound conjugate was removed by washing and a solution containing 3, 3', 5, 5'- tetramethylbenzidine (TMB) and enzyme substrate was added to trace specific antibody binding. The reaction was terminated

by the addition of STOP solution (0.25M sulphuric acid), which provided the appropriate pH for the development of colour. The optical densities of the standards, controls and samples were then read at 450nm using a microplate reader. Optical density (OD) was directly proportional to the antibody activity in the sample. The use of quality controls (positive and negative controls and water blanks) in every assay helped to monitor for any reagent failure.

Sera obtained from all those patients in our cohort who were deemed *H. pylori* positive were analysed for the presence of cagA antibodies using the ELISA technique. Each assay included positive and negative controls and water blanks to assess inter-assay variation. All the samples were analysed in duplicate and the mean optical density was measured. The calculated mean optical densities for the standards were plotted against their known concentrations on a graph and the values of the samples (unknowns) were determined from this curve.

The sensitivity and specificity of this assay (compared to Western Blot assay) is reported to be 96% and 97% with a <12% interassay coefficient of variation. The interassay variation was however found to be <10% in our analyses.

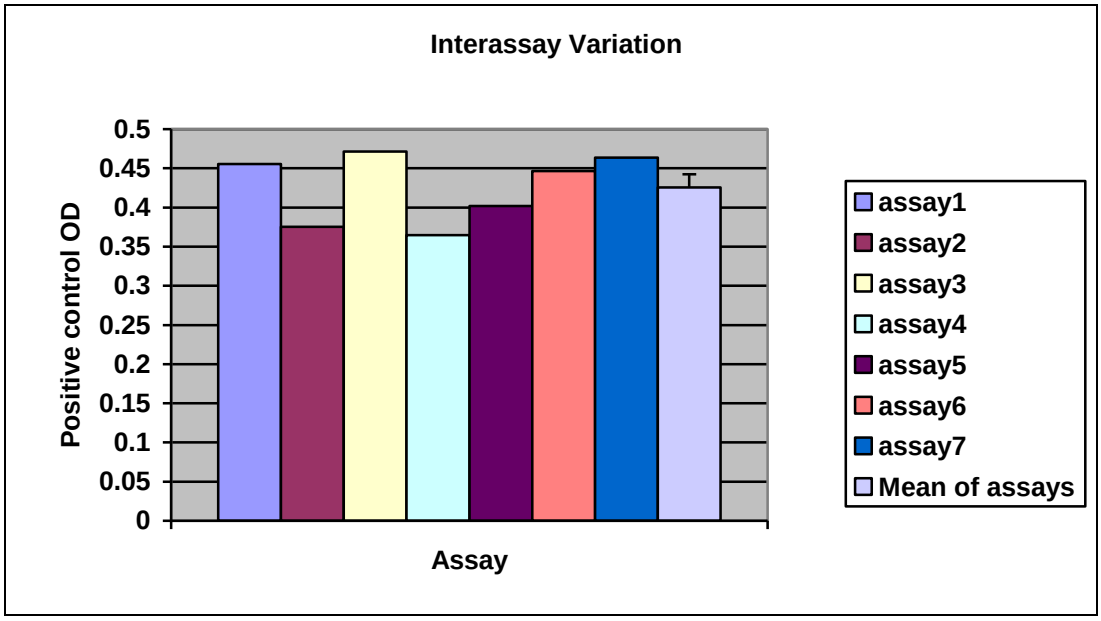


Figure 2-1: Mean optical density for positive controls per assay and the overall mean OD.

2.6 Determination of cagA status of infecting *Helicobacter pylori* by polymerase chain reaction (PCR)

In order to determine accurately and validate the ELISA technique for determination of the cagA status of the infecting *Helicobacter pylori*, we developed a specific PCR technique using primer sets (two sets for cagA and atp A) based on the Hp 700392 genome with the following sequences:

cagA set A: 198F + 470 R

cagA set B: 13F + 397R

atpA set C: 166F + 603R

atpA set D 759F + 1153R

and used as a positive control, Hp 700392 genomic DNA, at a concentration of 10ng/ml.

The initial development of this technique was performed by Prof. Rod Dimaline. Subsequent optimisation of the technique and performance of PCR of biopsy samples was performed by Senthil Murugesan.

Optimisation of the PCR technique was performed to identify:

1. the minimum required concentration of genomic DNA
2. template concentration
3. optimal annealing temperature
4. optimal magnesium concentration
5. optimal required quantity of Taq polymerase enzyme

The final total reaction volume used in the PCR assay was 25µl (master mix of 23µl and 1µl each of template i.e. sample or genomic *Hp* DNA). The master mix contained 3.5mM magnesium, 0.25µl of Taq polymerase enzyme, 2.5 µl STD Neb 10x buffer, 10mM dNTPs and 15.75µl distilled water. The reaction was performed using an annealing temperature of 55˚C.

Following optimisation of the technique, initial PCR reactions were performed on gastric corpus biopsy samples that were known to be *H. pylori* positive. Agarose gel electrophoresis of the PCR products was however unsuccessful in identifying *H. pylori* or cagA. This was felt to be secondary to the low prevailing concentrations of *H. pylori* DNA that was available in the gastric biopsy samples for the PCR technique to amplify.

Due to the consistency of the ELISA technique and its low inter-assay variation, the PCR technique was therefore not pursued further.

Chapter 3

3 Demographics, co-morbidities and endoscopic findings of study patients

3.1 Cohort demographics

A total of 1017 patients were recruited to the study. 576 (56.6%) were female with a median age of 59 years (interquartile range 47-69). 99.3% (1010 patients) were Caucasian reflecting the ethnic distribution of the local population. Of the remaining 7 patients, 4 were Asian, 1 Chinese, 1 African and 1 Middle Eastern in origin. The median BMI (Body Mass Index) of the cohort was 26.8 (range 13.5-54.8) with more than half the cohort (651 patients, 64.5%) exceeding a BMI of 25.

The BMI status of patients recruited to our current study is listed in table 3-1. BMI was not calculated in 8 patients and the reason for these included patients who were unable to stand/weight bear or who had had bilateral lower limb amputations.

BMI range	Definition	Total no. of patients (%)
<18.5	Underweight	21 (2%)
18.5 to <25	Normal	337 (33.4%)
25 to <30	Overweight	368 (36.5%)
30 to <40	Obese	257 (25.5%)
>40	Morbidly obese	26 (2.5%)

Table 3-1: BMI range and total number of patients.

3.2 Co-morbidities and other illnesses

A total of 527 (51.8%) patients reported the following co-morbidities listed in table 3-2.

Co-Existing Medical Illness	Total no. of patients (%)
Single comorbidity	
Diabetes mellitus (types-1 & 2)	35 (3.4%)
Ischaemic heart disease	20 (2.0%)
Cerebrovascular disease	9 (0.9%)
COPD	26 (2.6%)
Asthma	57 (5.6%)
Hypertension	93 (9.1%)
Coeliac disease	2 (0.2%)
Autoimmune thyroid disease (hypothyroidism/hyperthyroidism)	11 (1.0%)
Arthritis (osteoarthritis/rheumatoid/ psoriatic)	17 (1.7%)
Inflammatory bowel disease	13 (1.3%)
Hypercholestrolemia	10 (1%)
Liver and pancreatic diseases	6 (0.6%)
Multiple sclerosis	2 (0.2%)
Nephrotic syndrome	2 (0.2%)
Systemic lupus erythematosus	3 (0.3%)
Epilepsy	4 (0.4%)
Osteoporosis	4 (0.4%)
Multiple co-morbidities	213 (21%)

Table 3-2: Comorbidities and total patient numbers

213 (21%) patients had more than one comorbidity and the remaining 485 (47.7%) of the 1017 patients recruited had no significant co-existing medical illness. 5 (0.5%) patients had Familial Adenomatous Polyposis (FAP).

Some patients were known to have co-existing cancer or had received previous cancer treatment and this is summarised in table 3-3.

Type of previous cancer	**Number of patients**
Oesophageal cancer	2 (past)
Gastric cancer	3 (past)
Breast cancer	15 (past)
Lung cancer	2 (1 past, 1 current)
Prostate cancer	5 (current)
Colorectal cancer	10 (past)
Throat cancer	1 (past)
Renal cancer	1(past)
Bladder cancer	1 (past)
Thyroid cancer	1 (past)
Non-hodgkins lymphoma	1 (past)
Nasopharyngeal MALTOMA	1(past)

Table 3-3: Previous cancer and number of patients

3.3 Concurrent drug therapy

482 patients (47.4%) were on a PPI at the time of recruitment, 28 (2.7%) on a H2-receptor antagonist (H2RA) and 5 (0.5%) on both PPI and H2RA. Patients who had discontinued PPI therapy at least 2 weeks prior to recruitment were considered to be 'off PPI'. 193 (18.9%) patients were on antiplatelet agents (172 on aspirin and 21 on clopidogrel) and 45 (4.4%) were taking a non-steroidal anti-inflammatory drug (NSAID) at the time of recruitment.

3.4 Smoking and alcohol history

484 (47.6%) of patients had never smoked, 276 (27.1%) were ex-smokers (those who had stopped smoking at the time of recruitment to the study) and 257 (25.3%) were current smokers. A total of 565 (55.7%) patients consumed alcohol, of whom 40 female patients and 50 male patients drank alcohol above the current recommended weekly upper limit (>14 units for women and >21 units for men; http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_075218). However these data may not reflect the true prevalence of excess alcohol intake in this cohort due to the high prevalence of underreporting and underestimation of alcohol intake by patients (Feunekes et al., 1999; Stockwell et al., 2004). Hence in the final analysis patients were classified as either consumers or non-consumers of alcohol.

3.5 Previous *H. pylori* eradication

118 (11.6%) patients reported receiving previous eradication treatment for *H. pylori* with varying intervals between receiving eradication therapy and recruitment into the study.

3.6 Indications for referral for gastroscopy

The following table (table 3-4) summarises the indication and symptoms prompting referral for gastroscopy. Row 1 includes patients with a single presenting symptom and row 2 those with multiple symptoms.

Symptoms	Total no. of patients	Reflux/ heartburn	Indigestion	Abdominal pain	Vomiting	Weight loss	Anaemia	Dysphagia	Haemetemesis/ malaena	Follow up gastric ulcer
One symptom	588	95	123	118	20	21	86	106	19	0
Mixed symptoms	429	154	179	143	54	76	63	80	23	14

Table 3-4: Indications for gastroscopy

3.6.1 Routes of referral for gastroscopy

386 patients (37.9%) were referred via the '2-week rule', 158 (15.8%) via GP open access endoscopy and 473 (46.5%) by a hospital clinician referral (outpatient clinics and inpatient referrals). The '2-week rule' referral, as stipulated by the Department of Health (DOH), is the chosen pathway for General Practitioners (GPs) to refer those patients with symptoms suggestive of cancer for urgent specialist management (http://www.dh.gov.uk/en/Publicationsandstatistics/Lettersandcirculars/Healthservicecirculars/DH_4004253).

3.7 Significant past upper gastrointestinal tract surgical history

Of the 1017 patients recruited, 54 (5.3%) had undergone previous gastric or oesophageal surgery (table 3-5) and an additional 14 (1.4%) had a history of previous peptic ulcer disease but without surgery.

Type of previous gastric surgery	Total number of patients
Antrectomy	3
Gastroenterostomy	7
Vagotomy	3
Partial and or distal gastrectomy	9
Billroth II gastrectomy	4
Oesophago partial gastrectomy	6
Oesophageal resection for cancer	4
Heller's cardiomyotomy for achalasia	1
Fundoplication	9
Details unknown (for symptoms of peptic ulcer disease)	7
Gastric banding	1

Table 3-5: Types of previous gastric or oesophageal surgery and total patient numbers

3.8 Family history of gastric cancer and peptic ulcer disease

5.5% (55) of patients reported a first degree relative with a history of gastric cancer and a further 2% (20) reported a second degree relative with a history of gastric cancer. 12.5% (125) also reported a family history of peptic ulcer disease (gastric or duodenal ulcer) (table 3-6).

Family history of gastric cancer			Family history of peptic ulcer disease (%)
Total number (%)	Affected first degree relative (%)	Affected second degree relative (%)	
75 (7.4%)	55 (5.4%)	20 (2%)	125 (12.3%)

Table 3-6: Total number of patients with a reported family history of gastric cancer (first degree or second degree relatives) and peptic ulcer disease (PUD)

3.9 Endoscopic diagnosis at gastroscopy

Table 3-7 is a summary of the most significant diagnosis made at gastroscopy. When more than one endoscopic diagnosis was observed (such as gastritis and gastric ulcer), the more significant pathology was taken into consideration (for example gastric ulcer would be recorded instead of any associated gastritis).

Diagnosis	Total number of patients (%)
Normal	246 (24.2%)
Hiatus hernia	80 (7.9%)
Post surgical	32 (3.1%)
Oesophageal dysmotility	12 (1.2%)
Vascular malformations	6 (0.6%)
Oesophagitis	49 (4.8%)
Gastritis and/or Duodenitis	477 (46.9%)
Peptic ulcer disease (Gastric and duodenal ulcer)	30 (3%)
Gastric polyps	45 (4.4%)
Gastric cancer	7 (0.7%)
Barrett's oesophagus	26 (2.5%)
Oesophageal cancer	7 (0.7%)

Table 3-7: Endoscopic diagnosis

3.10 Discussion

Several previous studies have addressed some of the individual risk factors that influence the development of gastric adenocarcinoma (detailed in section 1.5.2). However none of these studies have evaluated the interactions between environmental, bacterial and host factors in the development of gastric preneoplastic pathology and the subsequent

progression to gastric adenocarcinoma. This present study aims to evaluate the roles played by the above mentioned factors and the interactions between these factors during the development of gastric preneoplastic pathology.

There are several strengths to the current study. Patients have been recruited prospectively and the cohort reflects the local population being referred for endoscopy. The cohort if predominantly white Caucasian population which is unqiue compared with other studies. Patients with incomplete data sets have been excluded from the final analysis (n=13). Demographics and clinical details of all the recruited patients have been extensively recorded. *H. pylori* status of patients has been defined by stringent criteria and patients with previous eradication of this organism have also been clearly identified. Gastric biopsies have been reported based on internationally accepted classifications including the modified Sydney system, Vienna and Padova classification by a dedicated GI Histopathologist who was blinded to the endoscopy findings (section 2.2).

One of the main limitations of this study is that only those patients with symptoms have been recruited. Hence those patients who may have gastric preneoplastic pathology but who are otherwise asymptomatic and those who have not been referred for an endoscopy have not been represented in this study. Patients with previous or current known cancers have been included in this cohort and those patients with the known familial cancer syndrome ‘Familial Adenomatosis Polyposis’ (FAP) have not been excluded. The dosage and duration of therapy with proton pump inhibitors has not been reported in this study.

Patients with history of previous gastric surgery have not been excluded from the current study. Although previous gastric surgery (vagotomy etc.) maybe associated with increased fasting serum gastrin concentrations, this is usually modest (~100pM) (Murugesan et al., 2009). This group of patients were therefore not excluded from the study.

Chapter 4

4 Factors that determine the sensitivity of the rapid urease test (RUT) for the detection of *Helicobacter pylori* infection in adult patients

4.1 Introduction

H. pylori infection is not only a recognised cause of gastric and duodenal ulceration but also an important factor in the development of gastric adenocarcinoma as detailed in sections 1.4 and 1.5.2. It is also implicated in the development of gastric Mucosa Associated Lymphoid Tissue (MALT) lymphomas. Hence accurate diagnosis of the presence of this organism is essential for the optimal management of patients with dyspeptic symptoms (including the test and treat strategy (Chiba et al., 2002), peptic ulcer disease and gastric MALT lymphoma (Fischbach et al., 2004). Eradication of this organism has been associated with a reduction in the risk of developing gastric adenocarcinoma (Malfertheiner et al., 2005). Several methods have been developed to diagnose the presence of this organism and these include demonstration of the bacteria on histological samples (by conventional and or immunohistochemical staining), rapid urease testing, ^{13}C-urea breath testing, serology and stool antigen testing. Although histological examination of gastric biopsies currently remains the gold standard diagnostic test, none of the methods has a consistently high diagnostic accuracy (Malfertheiner et al., 2007).

Rapid urease tests (RUT) are currently routinely utilised to diagnose *H. pylori* infection as they are quick, cost effective and have been reported to have comparable sensitivity and specificity with histology, PCR and culture. As *H. pylori* prevalence is decreasing in Western countries, we hypothesised that the sensitivity of the rapid urease test may be impairing diagnosis and we have therefore evaluated whether the RUT is still useful in current clinical practice and have investigated the roles of various host, drug and bacterial factors which may affect the accuracy of RUT for detecting *H. pylori* infection.

4.2 Methods

RUT was performed using the commercially available PRONTO DRY® test as per protocol, using two biopsies from the antrum in patients who were not using acid suppressing drugs and one antral and one corpus biopsy if patients were taking a proton pump inhibitor (PPI). There was no defined unit policy regarding RUT testing on those patients on a concurrent H2 receptor antagonist and hence the RUT was performed using antral biopsies in such patients. The PRONTO test was considered positive for *H. pylori* if the reagent turned colour from yellow to red/pink after an hour's incubation as shown in figure 4-1.

Figure 4-1: Rapid urease test – PRONTO Dry. Top negative result and bottom positive with change in colour of the reagent well.

Gastric antral and corpus biopsies were analysed for the presence of *H. pylori* and any preneoplastic changes (atrophic gastritis, intestinal metaplasia or dysplasia) by a single experienced GI pathologist using haematoxylin and eosin (H&E) stained sections and Giemsa stains (section 2.2). *H. pylori* and *cag*A serology were determined by enzyme linked immunoassay (as described in detail in sections 2.2 and 2.4 respectively).

4.3 Results

Of the 1017 patients recruited, 985 patients (55.2% female; mean age 56.9 years) were included in this analysis in whom RUT and histology had both been performed. 469 (47.6%) were taking PPIs at the time of enrolment, 23 patients (2.3%) were on a H2 receptor antagonist (H2RA; 22

on ranitidine 150mg twice daily dose and one patient on famotidine 20mg once daily dose) and 5 were on both PPI and H2RA (one patient having discontinued both PPI and H2RA 7 days prior to endoscopy).

Table 4-1 shows the most significant pathology observed at gastroscopy in these 985 patients.

Diagnosis	Total number of patients (%)
Normal	237 (24.1%)
Hiatus hernia	80 (8.3%)
Post surgical	27 (2.5%)
Dysmotility	12 (1.2%)
Vascular malformations	5 (0.5%)
Oesophagitis	49 (4.9%)
Inflammatory (Gastritis, Duodenitis)	463 (47%)
Ulcer disease (Gastric and duodenal ulcer)	29 (2.9%)
Gastric polyps	44 (4.5%)
Gastric cancer	7 (0.7%)
Barrett's oesophagus	25 (2.5%)
Oesophageal cancer	7 (0.7%)

Table 4-1: Most significant pathology observed at gastroscopy

4.3.1 Sensitivity and specificity of RUT

218 (21.4%) patients showed histological evidence of *H. pylori* infection of whom 153 (15.1%) also had a positive RUT. 10 (1%) patients had a false positive RUT with no histological evidence of infection seen (but only 3 of these patients were seropositive for *H. pylori*; one patient had received previous *H. pylori* eradication therapy). 757 (74.4%) patients were negative for *H. pylori* infection by RUT and histology and 65 (6.4%) patients had a false negative RUT. The specificity of the RUT was therefore 98.9% and sensitivity 70% compared to histology. The probability of the presence of *H. pylori* infection with a positive RUT [positive predictive value (PPV) for the rapid urease test] was 90.5% and conversely the negative predictive value (NPV) was calculated to be 65.4% (Bayes' equation).

114 (11.2%) patients had received previous *H. pylori* eradication therapy. However 21 of this group of patients (18.4%) had histological evidence of persisting *H. pylori* infection (15 of whom were also RUT positive and 20 were serology positive; one patient did not have serology performed) suggesting failure of eradication therapy in these patients. Of the remaining 93 patients who had received previous *H. pylori* eradication therapy with no evidence of current *H. pylori* infection at histology, 48 had a positive *H. pylori* serology test and all had a negative RUT.

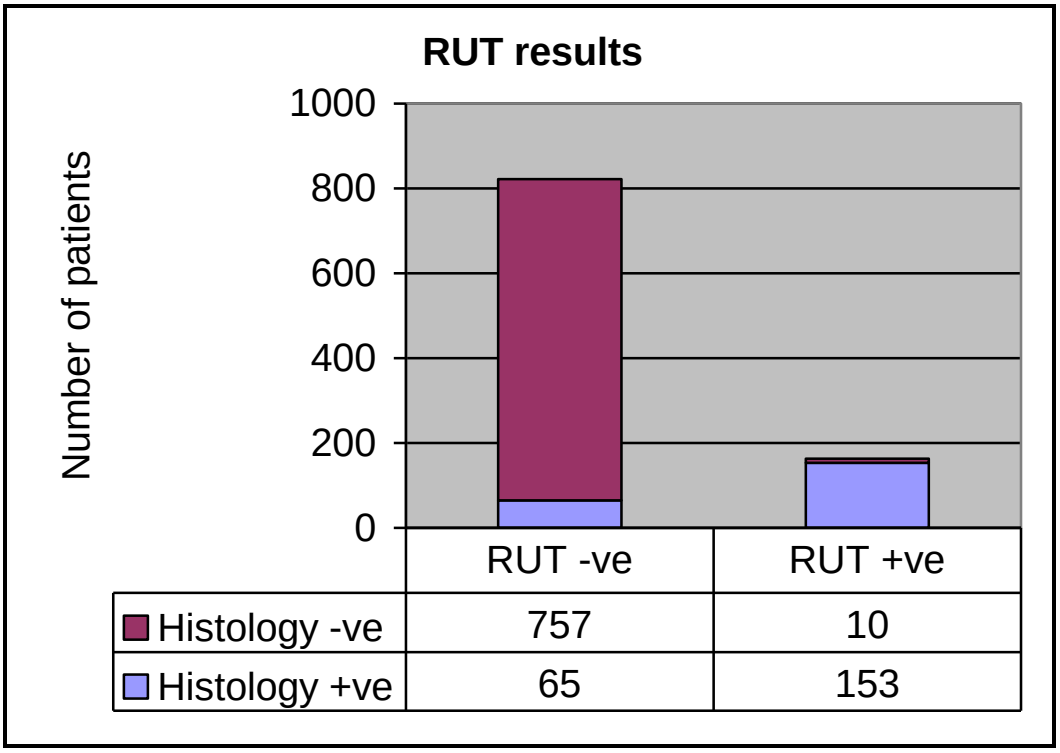


Figure 4-2: Rapid urease test vs. histology

4.3.2 The outcome of Rapid Urease Testing and the effect of concomitant PPI use, *H. pylori* serology, *cag*A status and gastric preneoplastic pathology

Of the 65 patients with a false negative RUT, 56 (86.2%) also had positive *H. pylori* serology (7 were negative and in 2 cases serology was not performed). 20 (30.8%) of the patients with a false negative RUT showed histological evidence of *H. pylori* infection in the gastric corpus only compared to only 2.6% of those patients who had a true positive RUT ($\chi 2$ $p<0.0001$).

A false negative RUT was also associated with concurrent PPI use (50.8% of patients with a false negative RUT were taking PPIs compared to only 23% of those with a true positive RUT (χ^2 $p<0.0001$)).

*Cag*A negative *H. pylori* serology was detected in 78.4% of the patients with a false negative RUT compared to 53.9% of those with a true positive RUT (χ^2 $p<0.001$).

The presence of preneoplastic gastric pathology (atrophic gastritis, intestinal metaplasia or dysplasia) (43% in patients with a false negative RUT vs. 36.6% in those patients with a true positive RUT) did not appear to affect the sensitivity of the RUT in this cohort of patients (table 4-2).

False –ve RUT vs. True +ve RUT			
	False –ve (%)	**True +ve (%)**	**p values (χ^2)**
Total Number	65	153	-
Concurrent PPI use	32 (49.2%)	35 (22.9%)	p<0.0001
Corpus only *H.pylori* infection	20 (30.8%)	4 (2.6%)	p<0.0001
*cag*A -ve *H.pylori* serology	46 (70.8%)	83 (54.2%)	p<0.001
Preneoplastic pathology	28 (43.1%)	56 (36.6%)	0.481

Table 4-2: False negative RUT vs. True Positive RUT.

4.4 Discussion

Helicobacter pylori infection of the stomach can be detected by several invasive and non-invasive methods as outlined in table 4-3. These methods have been reported to have varying sensitivities and specificities for the detection of *H. pylori* infection and it is known that some are influenced by factors including concurrent PPI therapy and the number of *H. pylori* bacteria in the biopsy samples. Low sensitivity and specificity has also been reported in patients with upper gastrointestinal tract bleeding and therefore rapid urease testing has not been recommended in this clinical setting (Schilling et al., 2003). False negative tests can also occur in patients with achlorhydria and in those on a concurrent PPI. It is thought that the high luminal pH resulting from the above two conditions can result in the destruction of the infecting *H. pylori* due to the action of its own urease enzyme (Basset et al., 2004; Schilling et al., 2003).

Diagnostic method	Sensitivity	Specificity	References
Invasive methods			
Histology	93%	99%	(Cutler et al., 1995)
Rapid Urease Test	85-95%	95-100%	(Tseng et al., 2005)
Culture of biopsy sample	80-90%	100%	(Ricci et al., 2007)
Serological testing	90-97%	50-96%	(Ekesbo et al., 2006)
Polymerase Chain Reaction (PCR)	85-96%	90-100%	(Li et al., 1996; Ashton-Key et al., 1996)
In-situ hybridisation	95%	100%	(Ruzsovics et al., 2004)
Non-invasive methods			
Urea Breath Test	90-98%	92-100%	(Goddard and Logan, 1997)
Stool antigen test	91%	93%	(Vaira et al., 2000)

Table 4-3: Various tests and their diagnostic accuracy for the detection of H. pylori infection.

RUT is routinely used for the immediate (rapid) diagnosis of *H. pylori* infection at gastroscopy. It is based on the ability of the urease enzyme produced by *H. pylori* to split the urea test reagent to form ammonia as discussed in section 1.4.3.3. This increases the pH which is detected by the indicator - phenol red. Commensal bacteria in the oral cavity also produce urease, however, the weaker enzyme produced by these bacteria

is rapidly denatured by the acidic pH of the stomach and therefore this is less likely to be associated with a false positive urease test.

Several different types of RUT have been developed and some of these are described in table (4-4).

Rapid Urease Test	Type	Sensitivity	Specificity	Time of assessment	References
CLO test	Gel-based	98%	97%	24 hours	(Weston et al., 1997)
Hp Fast	Gel-based	97.7%	87.7%	24 hours	(Laine et al., 1996; Yousfi et al., 1996)
PyloriTEK	Paper-based urea membrane test	96%	97%	One hour (positive and negative controls included)	(Laine et al., 1996)
ProntoDry	Paper-based	86.8%	97%	One hour	(Said et al., 2004)

Table 4-4: Rapid urease tests in the diagnosis of H. pylori infection – diagnostic accuracy and rapidity of test results.

It has previously been shown that the diagnostic accuracy of the various RUTs is decreased when the test is performed in patients on concurrent PPI therapy. We have established a similar significant association between false negative RUT and concurrent PPI use. This was in spite of obtaining samples for RUT from both the antrum and corpus in those patients who were on concurrent PPI therapy. Sampling error alone therefore cannot explain this association. It has been described that in the

presence of hypo or achlorhydria, such as that seen with PPI therapy, the usually antral predominant *H. pylori* infection colonises the corpus preferentially, however this cannot be the only explanation for a false positive RUT in those on a PPI. Other possible explanations include an associated decrease in the density of *H. pylori* infection, inactivation of the urease enzyme or indeed destruction of the bacteria above a certain gastric mucosal pH. A significantly greater proportion of patients with a false negative RUT also had histological evidence of infection in the gastric corpus only compared to those with a true positive RUT.

It has not previously been established whether other host factors (such as the presence of preneoplastic pathology) and the presence of bacterial virulence factors (*cag*A) also influence the diagnostic accuracy of the RUT in the diagnosis of *H. pylori* infection. In our analysis, *cag*A positive *H. pylori* infection was associated with a lower incidence of a false negative RUT. It is well known that the bacterial virulence factor, *cag*A, is associated with greater pathogenicity of the infecting bacteria. Hence this bacterial virulence factor should be considered as an important determinant influencing the rapid urease test result.

4.5 Conclusions

This analysis has shown that concomitant PPI use, corpus predominant *H. pylori* infection and less virulent (*cag*A negative) strains of *H. pylori* are more likely to lead to false negative RUT results. The RUT should therefore be used with caution as the sole determinant of *H. pylori*

infection in these settings, especially in areas of the world in which *H. pylori* prevalence is now decreasing.

Chapter 5

5 The associations between host, environmental and bacterial factors and the presence of gastric preneoplastic pathology

5.1 Introduction

As already discussed, *H.pylori* is a recognised gastric carcinogen (section 1.5.2.8). Several host factors such as age and sex, environmental factors such as smoking and alcohol consumption and bacterial virulence factors such as presence of the *cag*A gene have previously been suggested to increase the risk of developing gastric adenocarcinoma (detailed in section 1.5.2). However, it is not known how these factors interact to influence the development and progression of gastric preneoplastic pathology. Also it remains unclear whether factors such as Body Mass Index (BMI) and proton pump inhibitor therapy influence the development of gastric preneoplastic pathology. We have therefore investigated to what extent these factors are associated with the presence of gastric preneoplastic pathology (atrophic gastritis, intestinal metaplasia or dysplasia) in a large cohort of adult patients.

5.2 Methods

Patients enrolled in the study (described in section 2.1) funded by the National Institute of Health Research (NIHR) Biomedical Research centre in Microbial diseases at Royal Liverpool and Broadgreen University Hospitals NHS Trust, Liverpool, UK, were recruited in this analysis.

Clinical data recorded at recruitment, estimation of *H. pylori* and *cag*A serology, gastric antral and corpus biopsy analysis, measurements of fasting serum gastrin concentration are already discussed in detail in sections 2.1 to 2.3.

5.3 Statistical analysis

Statistical analysis was performed on those cases with no missing data (table 5.1 and table 5.2). Statistical analysis was performed using statistical software SAS v9.2 by statisticians Mr. Girvan Burnside (Lecturer in Medical Statistics, Department of Biostatistics, University of Liverpool) and Mr. Andrew McKay (Research Assistant, Clinical Trials Research Centre, University of Liverpool) working in liaison with the Biomedical Research Centre and the University of Liverpool. Power calculation for this study was realistically not possible due to the cross-sectional nature of the study. For some of the univariate analysis post hoc sample size estimations suggest that the sample size was adequate in detecting a true difference. For example a total of 199 patients (*H. pylori* positive with gastric preneoplastic pathology vs. *H. pylori* negative cases) would have been required in each group for a study powered at 80%, allowing for a type-1 error rate of 5% to demonstrate a 20% difference (background prevalance of gastric preneoplastic pathology estimated at 10%). However there does exist the possibility of type-2 error in some of the sub-grup analysis due to smaller sample size.

5.3.1 Univariate analysis

For each of the pathology outcomes, namely presence of preneoplastic pathology (atrophy, intestinal metaplasia, low grade/ high grade dysplasia or a combination of these pathologies), the relationship with individual variables including clinical and demographic features was assessed using univariate analysis (unadjusted association) without taking into account the influence of other factors. The associations are presented as odds ratios (compared to the reference category with an odds ratio of 1.0).

However for calculating the relationship between fasting serum gastrin concentrations and clinical and demographic features, a generalised logistic regression analysis was performed by calculating two odds ratios for each predictor, one ratio comparing serum gastrin concentrations between 40-100pM to serum gastrin concentration <40pM (upper limit of normal concentration) and the second odds ratio comparing serum gastrin concentrations >100pM to serum gastrin concentration of <40pM.

5.3.2 Multiple logistic regression analysis

In order to analyse the influence of more than one variable on the development of gastric preneoplastic pathology, multiple logistic regression analysis was performed. The odds ratio calculated has been adjusted for other variables in order to assess the effect of such individual variables on the outcomes.

5.4 Results

5.4.1 Demographics

As previously stated, a total of 1017 patients were recruited to the study. 576 (56.6%) were female with a median age of 59 years (interquartile range 47-69). The patient demographics for this cohort were described in detail in chapter 3.

5.4.2 Preneoplastic pathology

The following table is the summary statistic for the prevalence of gastric preneoplastic pathology and gastric adenocarcinoma in this cohort of patients (table 5-1). Although 1017 patients were recruited, a complete set of data was available in 1014 patients and these were therefore included in the final analysis.

Preneoplastic pathology	Total number of patients analysed	Total number (%)
Any gastric preneoplastic pathology	1014	246 (24.3%)
Atrophy (A)	1014	109 (10.7%)
Atrophy alone (without IM)	1014	43 (4.2%)
Intestinal Metaplasia (IM)	1014	187 (18.4%)
Intestinal Metaplasia alone (without atrophy)	1014	121 (11.9%)
IM + A	1014	66 (6.5%)
Low grade dysplasia (LGD)	1014	12 (0.2%)
High grade dysplasia (HGD)	1014	4 (0.4%)
Gastric adenocarcinoma	1014	8 (0.8%)

Table 5-1: Total number of preneoplastic pathology and gastric adenocarcinoma reported.

5.4.3 Summary of factors that are associated with the development of gastric preneoplastic pathology

The following table (5-2) summarises the key bacterial, environmental and host factors that are thought to be associated with the presence of gastric preneoplastic pathology.

Factors	Total number of patients analysed	Total number (%)
BACTERIAL FACTORS		
***H. pylori* infection**		
Total positive	1017	421 (41.4%)
Current positive (Histology +ve or RUT +serology positive)	1017	225 (22.1%)
Past positive (serology +ve)	1017	196 (19.3%)
Negative	1017	596 (58.6%)
***cag*A status of *H. pylori* infection**		
Current *H. pylori* infection	1008	94 (9.3%)
Past *H. pylori* infection	1008	60 (6.0%)
Overall *cag*A +ve *H. pylori* infection		154 (15.3%)
ENVIRONMENTAL FACTORS		
Acid suppression medication use		
Proton pump inhibitor use	1017	482 (47.4%)
H2RA use	1017	30 (2.9%)
Smoking status (ever smoked)	1015	533 (52.5%)
Alcohol consumers	1014	565 (55.7%)
HOST FACTORS		
Serum gastrin concentration (pM) categories Median serum gastrin concentration (pM): 32 (IQR: 19.2, 74).		
<40	1002	586 (58.5%)
40-100	1002	253 (25.2%)
>100	1002	163 (16.3%)
BMI		
<18.5	1009	21 (2.1%)
18.5-24.9		337 (33.4%)
25-29.9		368 (36.5%)
30-39.9		257 (25.5)
≥40		26 (2.5)
Family history of gastric cancer	1017	75 (7.4%)

Table 5-2: Bacterial, environmental and host factors – key summary.

5.4.4 Prevalence of *H. pylori* infection

A total of 421 (51.4%) patients had evidence of *H. pylori* infection. In this group, 225 (22.1%) patients had evidence of current infection with this organism as evidenced by the presence of *H. pylori* on histological analysis of either gastric antral or corpus biopsies or both rapid urease test and serology positive even if there was no histological evidence of this organism. 196 (19.3%) patients had evidence of previous infection with this organism (*H. pylori* serology only positive; histology negative) (table 5-2).

118 (11.6%) patients reported previous eradication treatment for *H. pylori* with varying intervals between receiving eradication therapy and recruitment into the study. However 22 (18.6%) of these patients had evidence of current *H. pylori* infection suggesting failure of previous eradication therapy. Of the 22 patients with previous failed eradication therapy, 21 were *H. pylori* serology positive and 16 had a positive rapid urease test. Of the remaining 96 patients who had received eradication therapy with no current evidence of *H. pylori* infection, 50 (42.4%) patients were *H. pylori* serology positive and only one patient had a positive rapid urease test at the time of recruitment into the study.

5.4.5 Prevalence of *cag*A positive *H. pylori* infection

Of the 421 patients who had evidence of infection with *H. pylori* (past or current infection), 154 (36.6%) were positive for *cag*A. In this group, 94

(22.3%) patients with current *H. pylori* infection and 60 (14.3%) patients with past *H. pylori* infection were positive for *cag*A (table 5-2).

5.4.6 Univariate associations between demographic and host characteristics and presence of preneoplastic pathology

The association between individual demographic variables namely gender, age and BMI and the presence of gastric preneoplastic pathology was assessed. This has been expressed as odds ratio with 95% confidence interval in table 5-3. Similarly the associations between environmental factors (such as smoking and alcohol consumption) and a family history of gastric cancer and the presence of preneoplastic gastric pathology are expressed as odds ratios with 95% confidence interval in table 5-4.

Univariate analysis therefore suggested that there was no increased incidence of preneoplastic pathology in male patients. However increasing age was significantly associated with the presence of gastric preneoplastic pathology (table 5-3).

Smoking was not associated with an increased incidence of gastric preneoplastic pathology in this cohort (table 5-5). Alcohol consumption on the other hand was associated with a lower incidence of gastric preneoplastic pathology (table 5-5). In the presence of a positive family history of gastric cancer, there was an associated significant incidence of gastric atrophy and intestinal metaplasia although no such association was

observed for all degrees of preneoplastic change combined (tables 5-5 and 5-6).

As there were insufficient numbers for a robust analysis in the low grade dysplasia, high grade dysplasia and gastric adenocarcinoma groups, odds ratios were not calculated for these pathology outcomes in any of the variables in tables 5-3 and 5-4.

Demographic variable	Atrophy			Atrophy (without IM)			IM (%)			IM (without atrophy)			A+IM (%)		
	n (%)	OR	(95% CI)	n (%)	OR	(95% CI)	n (%)	OR	(95% CI)	n (%)	OR	(95% CI)	n (%)	OR	(95% CI)
Gender															
Male	41 (9.3)	1.0	-	12 (2.7)	1.0	-	89 (20.2)	1.0	-	60 (13.6)	1.0	-	29 (6.6%)	1.0	-
Female	68 (11.9)	1.31	(0.87-1.97)	31 (5.4)	**2.04**	**(1.03-4.01)**	98 (17.1)	0.81	(0.59-1.12)	61 (10.6)	0.75	(0.51-1.10)	37 (6.5%)	0.98	(0.59-1.61)
Age															
< 50	15 (4.9)	1.0	-	10 (3.2)	1.0	-	34 (11.0)	1.0	-	29 (9.4)	1.0	-	5 (1.6)	1.0	-
50-70	59 (12.2)	**2.71**	**(1.51-4.88)**	21 (4.3)	1.35	(0.63-2.91)	86 (17.7)	**1.74**	**(1.14-2.67)**	48 (9.9)	1.06	(0.65-1.72)	38 (7.8%)	**5.17**	**(2.01-13.28)**
>70	35 (15.9)	**3.71**	**(1.97-6.98)**	12 (5.5)	1.73	(0.73-4.07)	67 (30.5)	**3.54**	**(2.24-5.6)**	44 (20.0)	**2.41**	**(1.46-4.00)**	23 (10.5%)	**7.1**	**(2.65-18.98)**
BMI															
<18	43 (12.8)	1.0	-	17 (5.0)	1.0	-	61 (18.1)	1.0	-	35 (10.4)	1.0	-	26 (7.7)	1.0	-
18 -<25	3 (14.3)	1.14	(0.32-4.03)	1 (4.8)	0.94	(0.12-7.44)	6 (28.6)	1.81	(0.68-4.85)	4 (19.1)	2.03	(0.67-6.37)	2 (9.5)	1.26	(0.28-5.71)
25 - <30	37 (10.1)	0.77	(0.48-3.78)	16 (4.4)	0.86	(0.43-1.73)	71 (19.4)	1.09	(0.74-1.59)	50 (13.6)	1.36	(0.86-2.16)	21 (5.7)	0.73	(0.40-1.32)
30 - <40	21 (8.2)	0.61	(0.48-1.22)	8 (3.1)	0.61	(0.26-1.44)	39 (15.3)	0.82	(0.53-1.27)	26 (10.2)	0.98	(0.57-1.67)	13 (5.1)	0.64	(0.32-1.28)
>40	4 (15.4)	1.24	(0.41-3.78)	1 (3.9)	0.75	(0.10-5.89)	8 (30.8)	2.01	(0.84-4.84)	5 (19.2)	2.05	(0.73-5.79)	3 (11.5)	1.56	(0.44-5.54)

Table 5-3: Relationship between the presence of gastric preneoplastic pathology and demographic variables – univariate analysis.

Demographic variable	LGD (%)	HGD (%)	All preneoplastic pathology (%)			Adenocarcinoma (%)
	n (%)	n (%)	n (%)	OR	(95% CI)	n (%)
Gender						
Male	7 (1.6%)	2 (0.5%)	104 (23.6%)	1.0	-	5 (1.1%)
Female	5 (0.9%)	2 (0.4%)	127 (22.1%)	0.92	(0.69-1.24)	3 (0.5%)
Age						
< 50	0 (0.0%)	0	45 (14.5%)	1.0	-	0
50-70	1 (0.2%)	1 (0.2%)	107 (22.15)	**1.67**	**(1.14-2.45)**	3 (0.6%)
>70	3 (1.4%)	3 (1.4%)	79 (35.8%)	**3.28**	**(2.16-4.98)**	5 (2.3%)
BMI						
<18	5 (1.5)	2 (0.6)	77 (22.9)	1.0	-	3 (0.9)
18 -<25	1 (4.8)	0 (0.0)	8 (38.1)	2.07	(0.83-5.18)	0 (0.0)
25 - <30	2 (0.5)	1 (0.3)	88 (24.0)	1.06	(0.74-1.51)	2 (0.5)
30 - <40	3 (1.2)	1 (0.4)	47 (18.3)	0.75	(0.50-1.13)	3 (1.2)
>40	1 (3.9)	0 (0.0)	9 (34.6)	1.78	(0.76-4.15)	0 (0.0)

Table 5-4: Relationship between the presence of gastric preneoplastic pathology and demographic variables – univariate analysis.

(NB: Bold text indicates significant changes)

Demographic variable	Atrophy			Atrophy (without IM)			IM (%)			IM (without atrophy)			A+IM (%)		
	n (%)	OR	(95% CI)	n (%)	OR	(95% CI)	n (%)	OR	(95% CI)	n (%)	OR	(95% CI)	n (%)	OR	(95% CI)
Smoking status															
Never smoked	52 (10.8)	1.0	-	20 (4.2)	1.0	-	77 (16.0)	1.0	-	45 (9.4)	1.0	-	32 (6.7%)	1.0	-
Ever smoked	57 (10.7)	0.99	(0.66-1.47)	23 (4.3)	1.04	(0.56-1.92)	110 (20.7)	1.36	(0.989 -1.88)	76 (14.3)	**1.61**	**(1.09-2.38)**	34 (6.4%)	0.96	(0.58-1.58)
Alcohol consumption															
No	62 (13.8)	1.0	-	26 (5.8)	1.0	-	95 (21.2)	1.0	-	59 (13.2)	1.0	-	36 (8.0%)	1.0	-
Yes	47 (8.4)	**0.57**	**(0.38-0.85)**	17 (3.0)	0.51	(0.27-0.94)	92 (16.3)	**0.73**	**(0.53-0.998)**	62 (11.0)	0.82	(0.56-1.19)	30 (5.3%)	0.64	(0.39-1.06)
Family history of gastric cancer															
No	96 (10.3)	1.0	-	41 (4.4)	1.0	-	167 (17.9)	1.0	-	112 (12.0)	1.0	-	55 (5.9%)	1.0	-
Yes	13 (16.3)	1.69	(0.90-3.17)	2 (2.5)	0.56	(0.13-2.53)	20 (25.0)	1.53	(0.90-2.60)	9 (11.3)	0.93	(0.45-1.91)	11 (13.8%)	**2.54**	**(1.27-5.08)**

Table 5-5: Relationship between the presence of gastric preneoplastic pathology and environmental factors such as smoking and alcohol consumption and also family history of gastric cancer – univariate analysis.

(NB: Bold text indicates significant changes)

Demographic variable	LGD (%)	HGD (%)	All preneoplastic pathology (%)			Adenocarcinoma (%)
	n (%)	n (%)	n (%)	OR	(95% CI)	n (%)
Smoking status						
Never smoked	4 (0.8%)	2 (0.4%)	97 (20.2%)	1.0	-	3 (0.6%)
Ever smoked	8 (1.5%)	2 (0.4%)	134 (25.2%)	1.33	(0.991-1.79)	5 (0.9%)
Alcohol consumption						
No	6 (1.3%)	4 (0.9%)	121 (27.1%)	1.0	-	7 (1.6%)
Yes	6 (1.1%)	0	110 (19.5%)	**0.65**	**(0.49-0.88)**	1 (0.2%)
Family history of gastric cancer						
No	9 (1.0%)	3 (0.3%)	210 (22.5%)	1.0	-	8 (0.9%)
Yes	3 (3.8%)	1 (1.3%)	21 (26.60	1.25	(0.74-2.10)	0

Table 5-6: Relationship between the presence of gastric preneoplastic pathology and environmental factors such as smoking and alcohol consumption and also family history of gastric cancer – univariate analysis.

(NB: Bold text indicates significant changes)

5.4.7 Univariate associations between bacterial virulence factors, concomitant PPI therapy and presence of preneoplastic pathology

H. pylori infection (past or current) was significantly associated with the presence of gastric preneoplastic pathology in this cohort (Table 5-7). Presence of atrophy, intestinal metaplasia and concurrent atrophy and intestinal metaplasia (A + IM) were associated with both past and current infection with this organism.

*Cag*A positive *H. pylori* infection was also significantly associated with the presence of gastric preneoplastic pathology. Past and current infection with *cag*A positive *H. pylori* strain was significantly associated with the presence of atrophy, intestinal metaplasia and concurrent atrophy and intestinal metaplasia (A + IM).

Fasting serum gastrin concentrations greater than 100pM were also significantly associated with the presence of gastric preneoplastic pathology (especially the presence of both atrophy and intestinal metaplasia) whereas fasting serum gastrin concentrations of less than 100pM showed no such association.

Concurrent PPI therapy was not found to be associated with the presence of gastric preneoplastic pathology in this cohort.

Variable	Atrophy			Atrophy (without IM)			IM (%)			IM (without atrophy)			A+IM (%)		
	n (%)	OR	(95% CI)	n (%)	OR	(95% CI)	n (%)	OR	(95% CI)	n (%)	OR	(95% CI)	n (%)	OR	(95% CI)
***H. pylori* infection**															
Negative	34 (5.7)	1.0	-	12 (2.0)	1.0	-	64 (10.8)	1.0	-	42 (7.1)	1.0	-	22 (3.7%)	1.0	-
Past	30 (15.3)	**2.97**	**(1.77-5.00)**	5 (2.6)	1.27	(0.44-3.64)	61 (31.1)	**1.32**	**(2.51-5.56)**	36 (18.4)	**2.95**	**(1.83-4.76)**	25 (12.8%)	**3.80**	**(2.09-6.90)**
Current	45 (20.0)	**4.11**	**(2.55-6.61)**	26 (11.6)	**6.33**	**(3.13-12.77)**	62 (27.6)	**1.15**	**(2.13-4.65)**	43 (19.1)	**3.10**	**(1.96-4.90)**	19 (8.4%0	**2.39**	**(1.27-4.51)**
***cag*A status**															
Negative for *H. pylori*	72 (8.5)	1.0	-	27 (3.2)	1.0	-	128 (15.0)	1.0	-	83 (9.8)	1.0	-	45 (5.3%0	1.0	-
Past	13 (21.7)	**2.99**	**(1.55-5.79)**	3 (5.0)	1.61	(0.47-5.46)	23 (38.3)	**3.51**	**(2.02-6.11)**	13 (21.7)	**2.56**	**(1.33-4.93)**	10 (16.7%0	**3.58**	**(1.71-7.53)**
Current	23 (24.5)	**3.51**	**(2.07-5.95)**	13 (13.8)	**4.90**	**(2.43-9.86)**	32 (34.0)	**2.92**	**(1.83-4.65)**	22 (23.4)	**2.83**	**(1.67-4.80)**	10 910.6%)	**2.13**	**(1.04-4.39)**
Concurrent PPI therapy															
No	61 (11.5)	1.0	-	26 (4.9)	1.0	-	94 (17.7)	1.0	-	59 (11.1)	1.0	-	35(6.6)	1.0	-
Yes	48 (10.0)	0.85	(0.57-1.27)	17 (3.5)	0.71	(0.38-1.33)	93 (19.3)	1.14	(0.81-1.53)	62 (12.9)	1.18	(0.81-1.73)	31(6.4)	0.98	(0.59-1.61)
Serum gastrin concentration (pM)															
<40	47 (8.1)	1.0	-	22 (3.8)	1.0	-	87 (14.9)	1.0	-	62 (10.6)	1.0	-	25 (4.3)	1.0	-
40-100	29 (11.5)	1.48	(0.91-2.41)	11 (4.4)	1.16	(0.55-2.43)	48 (19.0)	1.34	(0.91-1.97)	30 (11.9)	1.13	(0.71-1.80)	18 (7.1)	1.71	(0.92-3.2)
>100	28 (17.3)	**2.39**	**(1.44-3.96)**	8 (4.9)	1.33	(0.58-3.04)	46 (28.4)	**2.27**	**(1.50-3.42)**	26 (16.1)	1.61	(0.98-2.64)	20 (12.4)	**3.15**	**(1.70-5.83)**

Table 5-7: Relationship between the presence of gastric preneoplastic pathology and presence of H. pylori infection, cagA status of H. pylori, concurrent PPI therapy and fasting serum gastrin concentrations – univariate analysis.

Variable	LGD (%)	HGD (%)	All preneoplastic pathology (%)			Adenocarcinoma (%)
	n (%)	n (%)	n (%)	OR	(95% CI)	n (%)
***H. pylori* infection**						
Negative	6 (1.1%)	2 (0.3%)	76 (12.8%)	1.0	-	5 (0.8%)
Past	6 (3.1%)	2 (1.0%)	67 (34.2%)	**3.54**	**(2.52-5.18)**	1 (0.5%)
Current	0	0	88 (39.1%)	**4.38**	**(3.05-6.28)**	2 (0.9%)
***cag*A status**						
Negative for *H. pylori*	10 (1.2%)	3 (0.4%)	156 (18.3%)	1.0	-	6 (0.7%)
Past	2 (3.3%)	1 (1.7%)	26 (43.3%)	**3.41**	**(1.99-5.85)**	0
Current	0	0	45 (47.9%)	**4.1**	**(2.64-6.36)**	2 (2.1%)
Concurrent PPI therapy						
No	4 (0.8)	1 (0.2)	120(22.5)	1.0	-	2 (0.4)
Yes	8(1.7)	3 (0.6)	111(23.1)	1.04	(0.77-1.39)	6 (1.2)
Serum gastrin concentration (pM)						
<40	9 (1.5)	3(0.5)	111(18.9)	1.0	-	3 (0.5)
40-100	2 (0.8)	1(0.4)	59(23.3)	1.30	(0.91-1.86)	2 (0.8)
>100	1 (0.6)	0	55(33.7)	**2.18**	**(1.48-3.20)**	3 (1.9)

Table 5-8: Relationship between the presence of gastric preneoplastic pathology and presence of H. pylori infection, cagA status of H. pylori, concurrent PPI therapy and fasting serum gastrin concentrations – univariate analysis.

(NB: Bold text indicates significant changes).

5.4.8 Multiple logistic regression analysis for the presence of preneoplastic pathology

H. pylori infection (past and current) was also observed to be significantly associated with the presence of gastric preneoplastic pathology by multivariate analysis (table 5-9). Similarly *cag*A positive *H. pylori* infection was significantly associated with the presence of gastric preneoplastic pathology in this cohort, as was a fasting serum gastrin concentration greater that 100pM. However concurrent PPI therapy was not observed to be associated with the presence of gastric preneoplastic pathology.

Advancing age was associated with the presence of gastric preneoplastic pathology by multivariate analysis with no differences observed between the sexes. A positive family history of gastric cancer was also associated with the presence of gastric atrophy and intestinal metaplasia (Table 5-10). However smoking and alcohol consumption appeared to have no significant association with the presence of gastric preneoplastic pathology in this cohort by multivariate analysis. Results summarised in figure 5-3.

Variable	Atrophy		Atrophy (without IM)		IM (%)		IM (without atrophy)		A+IM (%)		All preneoplastic pathology (%)	
	OR	(95% CI)	OR	(95% CI)	OR	(95% CI)	OR	(95% CI)	OR	(95% CI)	OR	(95% CI)
***H. pylori* infection**												
Negative	1.0	-	1.0	-	1.0	-	1.0	-	1.0	-	1.0	-
Past	**1.94**	**(1.02-3.67)**	1.28	(0.44-3.69)	**2.71**	**(1.67-4.41)**	**2.85**	**(1.74-4.69)**	**3.22**	**(1.71-6.07)**	**2.48**	**(1.55-3.97)**
Current	**3.57**	**(1.95-6.54)**	**6.90**	**(3.39-14.04)**	**2.52**	**(1.49-4.24)**	**3.29**	**(2.05-5.29)**	**2.71**	**(1.38-5.33)**	**3.60**	**(2.25-5.77)**
***cag*A status**												
Negative for *H. pylori*	1.0	-	-	-	1.0	-	-	-	-	-	1.0	-
Past	2.25	(0.98-5.15)	-	-	1.82	(0.93-3.57)	-	-	**-**	**-**	**2.16**	**(1.11-3.87)**
Current	1.79	(0.90-3.57)	-	-	**2.08**	**(1.11-3.89)**	**-**	**-**	**-**	**-**	**2.2**	**(1.24-3.91)**
Concurrent PPI therapy (Current therapy vs. None)	-	-	-	-	-	-	-	-	-	-	-	-
Serum gastrin concentration (pM)												
<40	1.0	-	-	-	1.0	-	-	-	1.0	-	1.0	-
40-100	1.24	(0.74-2.08)	-	-	1.10	(0.72-1.68)	-	-	1.33	(0.69-2.54)	1.13	(0.75-1.68)
>100	**1.99**	**(1.17-3.40)**	**-**	**-**	**1.81**	**(1.16-2.82)**	**-**		**2.3**	**(1.21-4.39)**	**1.96**	**(1.27-3.01)**

Table 5-9: Multiple regression analysis: association between bacterial pathogenicity factors, fasting serum gastrin concentrations, PPI therapy and the presence of gastric preneoplastic pathology.

Demographic variable	Atrophy		Atrophy (without IM)		IM		IM (without atrophy)		A+IM		All preneoplastic pathology	
	OR	(95% CI)	OR	(95% CI)	OR	(95% CI)	OR	(95% CI)	OR	(95% CI)	OR	(95% CI)
Gender Female vs. Male	**-**	**-**	**2.17**	**(1.07, 4.39)**	-	-			-	-	-	-
Age												
< 50	1.0	-	1.0	-	1.0	-	1.0	-	1.0	-	1.0	-
50-70	**2.34**	**(1.26-4.33)**	**1.28**	**(0.44-3.69)**	1.31	(0.83-2.08)	**2.85**	**(1.74-4.69)**	**3.71**	**(1.41, 9.79)**	1.30	(0.85, 1.98)
>70	**3.45**	**(1.77-6.73)**	**6.90**	**(3.39-14.04)**	**2.68**	**(1.60-4.51)**	**3.29**	**(2.05-5.29)**	**4.91**	**(1.75,13.78)**	**2.74**	**(1.68, 4.48)**
BMI												
18-<25	-	-	-	-			-	-			1.0	-
<18			-	-			-	-			1.72	(0.62-4.75)
25 - <30			-	-			**-**	**-**			1.17	(0.79-1.72)
30 - <40			-	-			**-**	**-**			0.71	(0.45-1.12)
>40			-	-			**-**	**-**			1.68	(0.65-4.37)
Smoking status Ever compared to never	-	-	-	-	-	-	-	-	-	-	-	-
Alcohol consumption Any compared to none	0.65	(0.43-1.01)	0.59	(0.31, 1.13)	-	-	-	-	**-**	**-**	0.78	(0.56, 1.08)
Family history of gastric cancer Positive history compared to none	**2.24**	**(1.14-4.41)**	-	-	**1.82**	**(1.01-3.29)**	-	-	**2.90**	**(1.34, 6.25)**	-	-

Table 5-10: Multiple regression analysis: Effects of host, environmental and genetic factors (family history of gastric cancer) on the presence of gastric preneoplastic pathology. (NB: Bold text indicates significant changes).

Discussion

Helicobacter pylori is a well recognised gastric carcinogen. Previous studies have established that c*ag*A positive strains of *H. pylori* are more likely to be associated with the development of gastric adenocarcinoma (as discussed in section 1.4). However only a minority of patients infected with this organism develop gastric preneoplastic pathology and progress to gastric adenocarcinoma. Therefore several other factors are thought to influence the development of gastric preneoplastic pathology and the subsequent progression to gastric adenocarcinoma. In the present study, the effects of several host (age, gender, BMI, fasting serum gastrin concentration and genetic factors), environmental (smoking, alcohol consumption and PPI therapy) and bacterial factors (presence of *H. pylori* infection and *cag*A status of infecting *H. pylori)* on the development of gastric preneoplastic pathology have been analysed using a large cohort of adult patients undergoing gastroscopy.

5.4.9 Host factors

Advancing age was observed to be significantly associated with the presence of gastric preneoplastic pathology. This observation is in keeping with previous studies (and national statistics) in which gastric

adenocarcinoma has been reported to be more prevalent in older people (> 65 years of age) (Cancer statistics registration, 2008). However, in the present study, the presence of gastric preneoplastic pathology (or gastric adenocarcinoma) was not particularly observed to be more prevalent in older men as previously described. Female patients on the other hand were observed to have a significantly greater prevalence of gastric atrophy. It is therefore possible that some of these patients had autoimmune rather than *H. pylori* associated atrophic gastritis.

A positive family history of gastric cancer (first or second degree relative with gastric cancer) was also significantly associated with the presence of gastric preneoplastic pathology in the current study. This raises the issue of possible genetic factors that may predispose patients to the development of gastric adenocarcinoma. None of the patients in this cohort with a positive family history of gastric adenocarcinoma had features to suggest Hereditary Diffuse Gastric Cancer syndrome, hence alternative genetic factors are likely to be involved.

Elevated BMI (>35) has also been reported in several epidemiological studies to be associated with an increased risk of gastric cardia adenocarcinoma (and oesophageal adenocarcinoma) (Abnet et al., 2008; O'Doherty et al., 2011). However no such increased risk has been demonstrated with distal or non cardia gastric adenocarcinoma in previous

studies. Similarly in this cohort of patients, elevated BMI was not found to be significantly associated with the presence of gastric preneoplastic pathology, although 64.5% of the cohort had an elevated BMI (>25).

5.4.10 Environmental factors

Smoking was not found to be associated with the presence of gastric preneoplastic pathology in this cohort, contrary to previous studies (Tredaniel et al., 1997). Among the group of patients who had ever smoked, distinction between current smokers and ex-smokers has not however been made during this analysis.

Previous studies assessing the effects of alcohol consumption have found no significant association between this habit and the development of gastric adenocarcinoma (Steevens et al., 2010). However in the present study, univariate analysis suggested that alcohol consumption may have a small beneficial effect (OR = 0.65; 95% CI = 0.49-0.88). No such effect was however demonstrated following multivariate analysis. One of the shortcomings of the current study is that data regarding self reporting of alcohol consumption may not be a true reflection of actual consumption (as has been discussed in chapter 3). Furthermore distinction between those who consumed alcohol within recommended limits and those who

consumed amounts above such limits has not been made. Differences in the various types of alcohol consumed (for example beer vs. wine vs. spirits) have also not been addressed.

Other factors such as diet including the consumption of fatty and highly processed foods and intake of fresh fruits and red meat have not been addressed in the current study. There is already some evidence to suggest that diet influences the development of gastric adenocarcinoma especially in susceptible individuals and this has been discussed in detail in section 1.5.2. Specific diets, especially adherence to a 'Mediterranean Diet' has been suggested in some studies to be beneficial in protecting against the development of gastric adenocarcinoma (Buckland et al., 2010). Processed meat, red meat and total meat intake have also been previously linked to an increased risk of developing gastric adenocarcinoma (Gonzalez et al., 2006; Ngoan et al., 2002).

5.4.11 Gastrin

The role of gastrin in the development of gastric adenocarcinoma has been described in several studies. Its influence on the progression of gastric preneoplastic pathology through its effects on angiogenesis, gastric epithelial cell mitosis, proliferation and migration has been previously

discussed (section 1.3.2.2). In the present study, elevated fasting serum gastrin concentrations (>100 pM) were significantly associated with the presence of gastric preneoplastic pathology (both atrophy and intestinal metaplasia). It has also been previously demonstrated in humans that moderate hypergastrinaemia (fasting serum gastric concentration >150pM) was associated with increased apoptosis of gastric epithelial cells in the presence of infection with *H. pylori* (Przemeck et al., 2008).

5.4.12 Proton pump inhibitors (PPI)

Concerns have previously been raised regarding the possible role played by acid suppressing drug therapy, especially proton pump inhibitors (PPI), in the development of gastric adenocarcinoma. It has been suggested that the increased fasting serum gastrin concentrations resulting from the achlorhydria or hypochlorhydria which are associated with PPI therapy could potentially increase the risk of developing gastric preneoplastic changes. However epidemiological studies have failed to demonstrate this association (Bateman et al., 2003; Garcia Rodriguez et al., 2006; Poulsen et al., 2009). Although both univariate and multivariate analyses found no significant association between the presence of gastric preneoplastic pathology and concurrent PPI therapy there are certain

limitations in this study. The effects of the dosage or the duration of treatment with proton pump inhibitors on gastric preneoplastic pathology was not analysed. Previous studies have reported an increased incidence of atrophic gastritis in patients treated with proton pump inhibitors in the presence of current *H. pylori* infection (Kuipers et al., 1996). Long term exposure to proton pump inhibitors (6 months) has been demonstrated to worsen atrophic gastritis leading to gastric adenocarcinoma development in Mongolian gerbils infected with *H. pylori* (Hagiwara et al., 2011). Although at the time of recruitment to the study it was possible to ascertain whether patients were on a current proton pump inhibitor therapy, further details regarding dose, duration of treatment (including change of proton pump inhibitor therapy due to perceived lack of efficacy) and compliance with therapy was not recorded.

5.4.13 *H. pylori* and *cag*A positive *H. pylori* infection

This study has confirmed the well described association between *H. pylori* infection and presence of gastric preneoplastic pathology. What this study also demonstrates is that this effect persists even after eradication or disappearance of this organism.

Similarly *cag*A positive *H. pylori* infection was observed to be significantly associated with the presence of gastric preneoplastic

pathology. Animal studies have previously demonstrated an association between infection with this strain of *H. pylori* and the occurrence of gastric atrophy (in Mongolian gerbils) (Rieder et al., 2005). Other studies in humans have demonstrated that the odds ratio of developing gastric adenocarcinoma was significantly higher in those patients with *cag*A positive *H. pylori* infection as compared with those with *cag*A negative *H. pylori* infection and especially in those patients presenting at a younger age and with more advanced disease (Blaser et al., 1995) as previously discussed in section 1.4.4.

5.5 Conclusions

This study has therefore confirmed the known association between *H. pylori* infection and the presence of gastric preneoplastic pathology. Host factors such as advancing age, bacterial virulence factor namely *cag*A and elevated fasting serum gastrin concentrations (>100pM) were also significantly associated with the presence of gastric preneoplastic pathology. Concurrent PPI therapy was not however associated with the presence of gastric preneoplastic pathology. Although no effects were observed with smoking, alcohol consumption may have a small beneficial effect. Further studies are therefore warranted to address the effects of

these exogenous factors on the development and progression of gastric preneoplastic pathology.

Chapter 6

6 Determinants of human fasting serum gastrin concentration – interaction between *H. pylori* infection, gastric preneoplastic pathology and proton pump inhibitor use

6.1 Introduction

The hormone gastrin plays an important role not only in the regulation of gastric acid secretion but also through its effects on cell proliferation, apoptosis and angiogenesis, it regulates gastric epithelial and ECL-cell growth. Long standing hypergastrinaemia in the setting of autoimmune atrophic gastritis results in ECL-cell hyperplasia and in some patients this can progress to type-1 gastric neuroendocrine tumours. Hypergastrinaemia can develop due to several factors and this has been previously discussed in detail in section 1.3. Fasting serum gastrin concentrations are often measured during the investigation of suspected gastrin secreting tumours such as Zollinger Ellison syndrome as well as during assessment of gastric neuroendocrine tumours. However interpretation of a fasting serum gastrin concentration is complicated because factors such as *Helicobacter pylori* infection, presence of gastric

preneoplastic pathology (especially atrophic gastritis) and concurrent proton pump inhibitor (PPI) therapy can all influence this parameter. Advancing age was previously considered to be associated with elevated fasting serum gastrin concentrations, but subsequent studies have demonstrated that this association was observed only in the presence of *H. pylori* infection or gastric atrophy (Katelaris et al., 1993; Jassel et al., 1999). Additionally it is also not known to what extent other factors such as gender, BMI and family history of gastric cancer influence fasting serum gastrin concentrations. Environmental factors including smoking and alcohol consumption may also have an additional effect on fasting serum gastrin concentrations. As the interactions between these various factors remain poorly understood, we have investigated how they influence fasting serum gastrin concentrations in our previously described cohort of prospectively recruited patients (described in section 2.1).

6.2 Methods

Patients enrolled in the study (described in section 2.1) funded by the National Institute of Health Research (NIHR) Biomedical Research Centre in Microbial diseases at Royal Liverpool and Broadgreen University Hospitals NHS Trust, Liverpool, UK, were recruited in this analysis.

Clinical data recorded at recruitment, estimation of *H. pylori* and *cag*A serology, gastric antral and corpus biopsy analysis, measurements of fasting serum gastrin concentration are already discussed in detail in sections 2.1 to 2.3.

6.3 Statistical analysis

Statistical analysis was performed on cases with no missing data for the variables that were being assessed. Statistical analysis was performed using statistical software SAS v9.2 by statisticians Mr. Girvan Burnside (Lecturer in Medical Statistics, Department of Biostatistics, University of Liverpool) and Mr. Andrew McKay (Research Assistant, Clinical Trials Research Centre, University of Liverpool) working in liaison with the Biomedical Research Centre and the University of Liverpool

6.4 Results:

Of the 1017 patients recruited to the study, 2 patients with an established diagnosis of pernicious anaemia and a further 13 patients who did not have complete data sets were excluded from the final analysis. Of the 1002 patients included in the analysis, 564 (56.3%) were female and 476 (47.5%) were on a concurrent proton pump inhibitor. 223 (22.3%) patients had evidence of current infection with *H. pylori* infection (as evidenced by the presence of this organism on histological analysis of

gastric antral and corpus biopsies or by the presence of a positive rapid urease test and positive serology) and 196 (19.6%) had evidence of previous infection with this organism (as evidenced by positive serology). 225 (22.4%) patients had evidence of preneoplastic pathology on histological examination of gastric biopsy samples (defined as the presence of one or more of atrophy, intestinal metaplasia and dysplasia).

Figure 6-1 shows the distribution of fasting serum gastrin concentrations in this cohort. 586 (58.5%) patients had fasting serum gastrin concentrations <40pM; 253 (25.2%) had fasting serum gastrin concentrations between 40 and 100pM, 145 (14.5%) had fasting serum gastrin concentrations between 100 and 400pM and the remaining 18 (1.8%) had serum fasting gastrin concentrations >400pM.

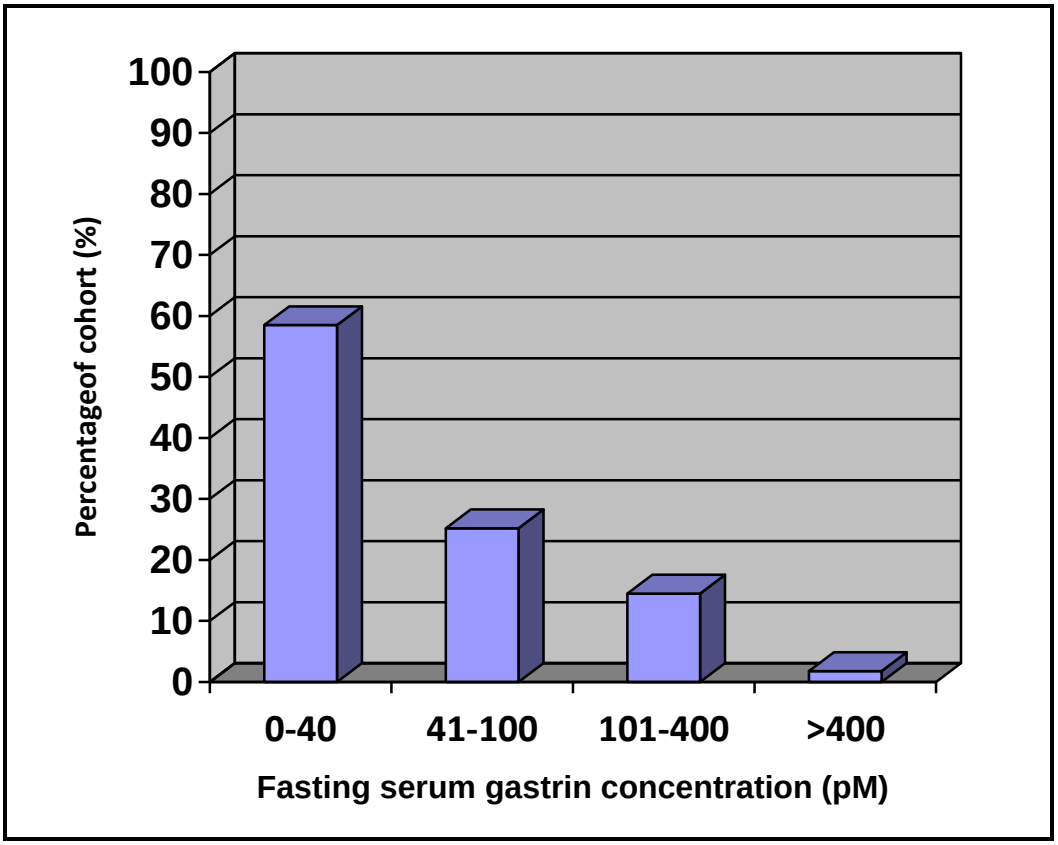


Figure 6-1: Graph depicting the percentage distribution of fasting serum gastrin concentrations in the cohort.

6.4.1 Fasting serum gastrin concentrations in patients without *H. pylori* infection, not on a concurrent PPI, with a normal gastroscopy and with no evidence of gastric preneoplastic pathology

In this cohort, 207 (20.6%) patients had a normal gastroscopy without evidence of current or past evidence of *H. pylori* infection (*H. pylori*

negative) and with no evidence of gastric preneoplastic pathology on histological analysis of gastric antral and corpus biopsies. This group of patients were not on concurrent proton pump inhibitors or H2 receptor antagonist therapy. The median fasting serum gastrin concentration in this group of patients was 19.9 pM (IQR 14.8, 29). The mean fasting serum gastrin concentration in this cohort was 38.4 pM (95% CI; 38.0-38.8) and this probably reflects the 'normal' range for fasting serum gastrin concentration in this cohort.

6.4.2 Does *H. pylori* infection influence fasting serum gastrin concentration?

In this cohort, 583 (58.2%) patients were negative for *H. pylori* infection, 223 (22.2%) had evidence of current infection and 196 (19.6%) had evidence of past infection with this organism (table: 6-1). Patients with *H. pylori* infection as a group (combining current and past infection) had significantly higher fasting serum gastrin concentrations ($p<0.001$, Kruskal Wallis analysis) compared to patients who had no evidence of infection with this organism. Patients with current evidence of *H. pylori* infection and with past only infection with this organism also had significantly elevated fasting serum gastrin concentrations ($p<0.001$, Kruskal Wallis analysis).

6.4.2.1 Does *cag*A positive *H. pylori* infection influence fasting serum gastrin concentration?

154 (36.7%) patients with evidence of *H. pylori* infection had infection with the *cag*A strain of *H. pylori* (94 patients had evidence of current infection and 60 patients had evidence of previous infection with this organism). Infection with a *cag*A positive strain of *H. pylori* was not found to be associated with significantly elevated fasting serum concentrations compared to infection with a *cag*A negative strain of *H. pylori* infection (table 6-2).

6.4.3 Does concurrent acid suppression affect serum gastrin concentration?

476 (47.5%) patients were on concurrent PPI therapy (table: 6-1). Patients on concurrent PPI therapy had significantly elevated fasting serum gastrin concentrations ($p<0.001$, Mann-Whitney test) compared to those not on PPI therapy.

6.4.4 Does the presence of gastric preneoplastic changes influence fasting serum gastrin concentration?

225 (22.4%) patients had evidence of gastric preneoplastic pathology (table: 6-1). Presence of gastric preneoplastic pathology was significantly associated with elevated fasting serum gastrin concentrations compared to patients with no evidence of gastric preneoplastic pathology ($p<0.001$, Mann-Whitney test).

Variable	Level	Total number of patients	Median fasting serum gastrin concentration (pM)	IQR	Range	p-value
H. pylori	Negative	583	30	(18,62)	1.2 to 900	<0.001 (Kruskal Wallis test)
	Current positive	223	30	(20,66)	3 to 795.8	
	Past positive	196	49	(22,120)	5.8 to 1256.6	
PPI	No	526	22.7	(16,38)	1.2 to 1256.6	<0.001 (Mann-Whitney test)
	Current	476	50.5	(28,96)	5.8 to 680	
Preneoplastic pathology	No	777	30	(18,64)	1.2 to 1256.6	<0.001 (Mann-Whitney test)
	Yes	225	40	(22,96)	6.4 to 680	

Table 6-1: The effect of *H. pylori* infection, concurrent PPI therapy and presence of gastric preneoplastic pathology on fasting serum gastrin concentrations.

***cag*A status**	**Fasting serum gastrin concentrations (pM)**						
	'<40'	**'40-100'**	**'>100'**	**odds of '<40' -> '40-100'**		**odds of '<40' -> '>100'**	
	n (%)	**n (%)**	**n (%)**	**OR1**	**(95% CI)**	**OR2**	**(95% CI)**
Overall *cag*A status							
*cag*A -ve infection	137 (32.7%)	69 (16.5%)	56 (13.4%)	1.0	-	1.0	-
*cag*A +ve infection	84 (20%)	41(9.8%)	29 (6.9%)	0.96	(0.60 - 1.55)	0.84	(0.50-1.42)
Current *H. pylori* infection							
*cag*A -ve infection	81 (19.3%)	25 (5.9%)	20 (4.8%)	1.0	-	1.0	-
*cag*A +ve infection	57 (13.6%)	26 (6.2%)	11 (2.6%)	1.47	(0.77 - 2.81)	0.78	(0.34 - 1.75)
Past *H. pylori* infection							
*cag*A -ve infection	56 (13.4%)	44 (10.5%)	36 (8.6%)	1.0	-	1.0	-
*cag*A +ve infection	27 (6.4%)	15 (3.6%)	18 (4.3%)	0.70	(0.33 - 1.48)	1.03	(0.50 - 2.14)

Table 6-2: The effect of cagA positive *H. pylori* infection on fasting serum gastrin concentrations.

6.4.5 How do *H. pylori* infection, concurrent proton pump inhibitor therapy and the presence of gastric preneoplastic pathology interact to influence fasting serum gastrin concentrations?

We analysed interactions of the three main factors namely presence of *H. pylori* infection, concurrent PPI therapy and presence of gastric preneoplastic pathology on fasting serum gastrin concentrations. Patients were differentiated into 12 different categories depending on the presence or absence of gastric preneoplastic pathology, concurrent PPI therapy and whether there was evidence of current or past *H. pylori* infection (as outlined in table 6-2).

	Categories	Number of patients	Median fasting serum gastrin concentration (pM)	IQR	Range
	All	1002	32	(19.2, 74)	1.2 to 1256.7
A	***H. pylori* negative / current PPI / preneoplastic pathology**	39	54	(33, 120)	11.2 to 628
B	***H. pylori* negative / current PPI / No preneoplastic pathology**	255	46	(30, 84)	8.8 to 680
C	***H. pylori* negative / No PPI / preneoplastic pathology**	33	30	(19.6, 160)	7.6 to 680
D	***H. pylori* negative / No PPI / No preneoplastic pathology**	256	19.9	(14.8, 29)	1.2 to 900
E	***H. pylori* current positive / current PPI / preneoplastic pathology**	33	66	(32, 120)	11.2 to 520
F	***H. pylori* current positive / current PPI / No preneoplastic pathology**	35	51	(28, 108)	11.6 to 172
G	***H. pylori* current positive / No PPI / preneoplastic pathology**	53	28	(19.2, 64)	10 to 629
H	***H. pylori* current positive / No PPI / No preneoplastic pathology**	102	26	(19.8, 40)	3 to 795.8
I	***H. pylori* past positive / current PPI / preneoplastic pathology**	37	40	(26, 86)	6.4 to 449.2
J	***H. pylori* past positive / current PPI / No preneoplastic pathology**	77	68	(32, 136)	5.8 to 400
K	***H. pylori* past positive / No PPI / preneoplastic pathology**	30	47	(24, 220)	13.6 564.2
L	***H. pylori* past positive / No PPI / No preneoplastic pathology**	52	25	(16.2, 86)	7.4 1256.7

Table 6-3: Pairwise differences in fasting serum gastrin concentrations between groups (Mann-Whitney U-tests). The pairs of interest were specified in advance (23 pairs), and the p-values were adjusted using a Bonferroni correction to account for the multiple comparisons.

In *H. pylori* negative patients, concurrent PPI therapy and the presence of gastric preneoplastic pathology were both individually associated with significantly elevated fasting serum gastrin concentrations (groups D vs. C; B vs. C – table 6-3). In the presence of both these factors (concurrent PPI therapy and presence of gastric preneoplastic pathology), *H. pylori* negative patients also had significantly elevated fasting serum gastrin concentrations compared to those without either of these factors (group A vs. D; table 6-3). In *H. pylori* negative patients on concurrent PPI therapy, the additional presence of gastric preneoplastic pathology did not significantly alter fasting serum gastrin concentrations (figure 6-2).

Current *H. pylori* infection caused significantly elevated fasting serum gastrin concentrations compared to *H. pylori* negative patients (in the absence of PPI use or preneoplastic pathology; group H vs. D) (figure 6-3). In those patients with current *H. pylori* infection compared to *H. pylori* negative patients, concurrent PPI therapy or presence of gastric preneoplastic pathology did not significantly alter fasting serum gastrin concentrations.

In patients with current *H. pylori* infection, concurrent PPI therapy was associated with significantly elevated fasting serum gastrin concentrations irrespective of the presence or absence of gastric preneoplastic pathology (groups E vs. H and F vs. H; table 6-3). Preneoplastic pathology however had no significant effect on fasting serum gastrin concentrations in the presence of current *H. pylori* infection (group G vs. H; table 6-3).

Past *H. pylori* infection was not associated with significantly elevated fasting serum gastrin concentrations compared to *H. pylori* negative patients (in the absence of preneoplastic pathology and in patients not on concurrent PPI; group L vs. D; table 6-3).). Preneoplastic pathology was also not associated with significantly elevated fasting serum gastrin concentrations in this group. However in those patients with evidence of past *H. pylori* infection, concurrent PPI therapy was again associated with significantly elevated fasting serum gastrin concentrations but only in the absence of preneoplastic pathology (p=0.034) (group J vs. L; table 6-3 and figure 6-4).

	A	B	C	D	E	F	G	H	I	J	K	L
A		1.000	1.000	**<0.001**	1.000				1.000			
B				**<0.001**		1.000				0.156		
C				**0.033**			1.000				1.000	
D								**0.001**				0.371
E						1.000	**0.035**	**0.001**				
F								**0.001**				
G								1.000				
H												
I										1.000	1.000	1.000
J												**0.034**
K												0.772
L												

Table 6-4: p-values from pre-specified pairwise comparisons of categories (Mann-Whitney U-test) adjusted using Bonferroni's method. Statistically significant differences ($p<0.05$) are shown in bold.

In addition to the above factors we also analysed the effect of host and environmental factors on fasting serum gastrin concentrations as detailed below.

6.4.6 Effect of age and gender of patients on fasting serum gastrin concentrations

Univariate analysis suggested that increasing age was associated with increased fasting serum gastrin concentrations (40-100pM and >100pM) (table 6-4), but patient gender did not appear to influence fasting serum gastrin concentrations in this cohort.

However, in the subgroup of those patients who were negative for infection with *H. pylori*, who did not demonstrate gastric preneoplastic pathology and were not on a concurrent PPI therapy, age did not influence fasting serum gastrin concentrations (table 6-5). Thus age is likely to be an indirect factor by its association with *H. pylori* infection and presence of preneoplastic pathology.

6.4.7 Effect of body mass index on fasting serum gastrin concentrations

Univariate analysis demonstrated a significant association between BMI of patients and increased fasting serum gastrin concentrations only at the extreme ends of the BMI spectrum (table 6-4). Patients who were underweight (BMI<18.5) and those who were morbidly obese (BMI>40)

were both found to have a significantly greater prevalance of fasting serum gastrin concentrations >100pM.

6.4.8 Effects of smoking and alcohol consumption on fasting serum gastrin concentrations

Smoking was associated with a greater frequency of mildly increased fasting serum gastrin concentration (40-100pM) in this cohort of patients by univariate analysis (table 6-5). Alcohol consumption was however found to be associated with lower frequencies of more markedly elevated fasting serum gastrin concentrations (>100pM).

6.4.9 Associations between positive family history of gastric cancer and fasting serum gastrin concentrations

Univariate analysis showed no significant association between fasting serum gastrin concentrations and a positive family history of gastric cancer (table 6-5).

6.4.10 Multiple logistic regression

Generalised multiple logistic regression analysis demonstrated increased prevalance of elevated fasting serum gastrin concentrations only in older patients and a decreased prevalance in those subjects who consumed alcohol. No effects were demonstrated with BMI, smoking, gender or a positive family history of gastric cancer (table 6-5).

Host factors	Fasting serum gastrin concentrations (pM)						
	'<40'	'40-100'	'>100'	odds of '<40' -> '40-100'		odds of '<40' -> '>100'	
	n (%)	n (%)	n (%)	OR1	(95% CI)	OR2	(95% CI)
Gender							
Male	267 (61.0)	108 (24.7)	63 (14.4)	1.0	-	1.0	-
Female	319 (56.6)	145 (25.7)	100 (17.7)	1.12	(0.84-1.51)	1.33	(0.93-1.89)
Age at baseline							
<50	225 (73.3)	52 (16.9)	30 (9.8)	1.0	-	1.0	-
50-70	251 (52.7)	140 (29.4)	85 (17.9)	**2.41**	**(1.67-3.48)**	**2.54**	**(1.61-4.00)**
>70	110 (50.2)	61 (27.9)	48 (21.9)	**2.40**	**(1.55-3.71)**	**3.27**	**(1.97-5.45)**
Body Mass Index (BMI): n (%)							
18.5-24.9: Normal (ref. cat.)	207 (62.5)	76 (23.0)	48 (14.5)	1.0	-	1.0	-
16.0-18.4: Underweight	10 (47.6)	4 (19.1)	7 (33.3)	1.09	(0.33-3.58)	**3.02**	**(1.09-8.33)**
25.0-29.9: Overweight	214 (58.8)	98 (26.9)	52 (14.3)	1.25	(0.87-1.78)	1.05	(0.68-1.62)
30.0-39.9: Obese	138 (54.6)	67 (26.5)	48 (19.0)	1.32	(0.89-1.96)	1.50	(0.95-2.36)
>=40.0: Morbidly obese	11 (44.0)	7 (28.0)	7 (28.0)	1.73	(0.65-4.63)	**2.74**	**(1.01-7.45)**

Table 6-5 Univariate associations between fasting serum gastrin concentrations and host factors

Host factors	Fasting serum gastrin concentrations (pM)						
	'<40'	'40-100'	'>100'	odds of '<40' -> '40-100'		odds of '<40' -> '>100'	
	n (%)	n (%)	n (%)	OR1	(95% CI)	OR2	(95% CI)
Age at baseline							
<50	92 (36.5)	8 (3.2)	4 (1.6)	1.0	-	1.0	-
50-70	90 (35.7)	9 (3.6)	5 (1.9)	1.15	(0.42-3.11)	1.27	(0.33-4.91)
>70	38 (15.1)	4 (1.6)	2 (0.8)	1.21	(0.34-4.26)	1.21	(0.21-6.88)

Table 6-6 Univariate association between age and fasting serum gastrin concentrations in *H. pylori* negative patients without gastric preneoplastic pathology and who were not on concurrent PPI therapy.

Environmental factors and family history	Fasting serum gastrin concentration (pM)						
				odds of '<40' -> '40-100'		odds of '<40' -> '>100'	
	'<40' n (%)	'40-100' n (%)	'>100' n (%)	OR1	(95% CI)	OR2	(95% CI)
Smoking status at baseline							
Never	298 (63.3)	104 (22.1)	69 (14.7)	1.0	-	1.0	-
Ever	287 (54.3)	149 (28.2)	93 (17.6)	**1.49**	**(1.10-2.01)**	1.40	(0.985-1.99)
Alcohol consumption							
None	238 (54.0)	113 (24.9)	90 (20.4)	1.0	-	1.0	-
Any	346 (62.0)	139 (24.9)	73 (13.1)	0.85	(0.63-1.14)	**0.56**	**(0.39-0.79)**
Family History of Gastric Cancer							
No	541 (58.7)	232 (25.2)	149 (16.2)	1.0	-	1.0	-
Yes	44 (56.4)	20 (25.6)	14 (18.0)	1.06	(0.61-1.84)	1.16	(0.62-2.17)

Table 6-7: Univariate associations between fasting serum gastrin concentrations and environmental factors and family history of gastric cancer

Demographic variable	Fasting serum gastrin concentration (pM)			
	Odds of '<40'; 40-100		Odds of <40; >100	
	OR1	**(95% CI)**	**OR2**	**(95%CI)**
Gender				
Female compared to male	-	-	-	-
Age at baseline				
< 50	1.0	-	1.0	-
50-70	**2.13**	**(1.43-3.18)**	**1.99**	**(1.23-3.23)**
>70	**2.20**	**(1.36-3.58)**	**2.17**	**(1.24-3.82)**
BMI				
<18				
18 -<25				
25 - <30				
30 - <40				
>40				
Smoking status at baseline: ever – compared to never				
Drinking alcohol: any – compared to none	0.95	(0.69-1.32)	**0.59**	**(0.40-0.86)**
Family History of Gastric Cancer – compared to none				

Table 6-8: Generalised multiple logistic regression analysis for fasting serum gastrin concentrations with demographic variables.

6.5 Discussion

Several factors have been demonstrated in previous studies to influence fasting serum gastrin concentrations in humans. These include

- Host factors such as age and the presence of gastric preneoplastic changes
- Bacterial factors, namely *H. pylori* infection
- Environmental factors including PPI therapy

However studies that have analysed the interactions between the above three factors and their effects on fasting serum gastrin concentrations have not been published to date. We have demonstrated in the current study several interactions between these factors and their effects on fasting serum gastrin concentrations.

H. pylori infection can cause mild to moderate elevations in fasting serum gastrin concentrations by two potential mechanisms. Firstly, antral predominant infection results in inhibition of somatostatin release which in turn causes hypergastrinaemia. The second mechanism involves the development of G-cell hyperplasia and hypergastrinaemia as result of achlorhydria due to chronic atrophic gastritis resulting from corpus predominant *H. pylori* infection (Murugesan et al., 2009). *cag*A positive *H. pylori* infection has been shown to be associated with a significantly higher incidence of peptic ulcers and gastric adenocarcinoma. This has been attributed to the higher inflammatory response evoked secondary to infection with this strain of *H. pylori*. It would therefore appear that similar

mechanisms may stimulate gastrin secretion. However studies have shown variable effects of *cag*A positive *H. pylori* infection on fasting serum gastrin concentrations. In a study of *cag*A positive *H. pylori* infection in children no significant changes in fasting serum gastrin concentrations were observed (Fukuda et al., 2003). Similarly, in the present study of adult patients, *cag*A positive *H. pylori* infection was not found to be significantly associated with elevated fasting serum gastrin concentrations.

PPI therapy has also been associated with mild to moderate hypergastrinaemia secondary to hypochlorhydria and G-cell hyperplasia (Murugesan et al., 2009).

In the present study, *H. pylori* infection, concurrent PPI therapy and presence of gastric preneoplastic pathology were all individually found to be associated with significant elevations in fasting serum gastrin concentrations.

Previous studies have also demonstrated the effect of concurrent PPI therapy in causing more pronounced elevation of fasting serum gastrin concentrations in patients with current *H. pylori* infection than that was observed with *H. pylori* infection alone (El-Nujumi et al., 1998; El-Omar et al., 1993). A similar interaction between current *H. pylori* infection and concurrent PPI therapy was observed in the present study. Moreover this effect was seen irrespective of the presence or absence of gastric preneoplastic pathology.

However in patients with past rather than current evidence of *H. pylori* infection, concurrent PPI therapy was significantly associated with

elevated fasting serum gastrin concentrations only in the absence of gastric preneoplastic pathology suggesting that this effect may be mediated mainly through PPI induced hypochlorhydria.

Older subjects have previously been reported to have higher fasting serum gastrin concentrations compared to younger subjects. However concentrations are often still within the normal range in the absence of other factors such as *H. pylori* infection or presence of gastric autoantibodies (Jassel et al., 1999). In the current study, we found a similar association between advancing age and elevated fasting serum gastrin concentrations. This is likely to be partly attributable to PPI use, *H. pylori* infection and gastric atrophy since no significant difference in fasting serum gastrin concentrations was observed with advancing age in the group of *H. pylori* negative patients who had no gastric preneoplastic pathology and were not on concurrent PPI therapy.

In addition, other host factors such as BMI have also been shown to influence fasting serum gastrin concentrations. Body mass index at either ends of the spectrum (BMI <18.5 and >40) were also found to be significantly associated with fasting serum gastrin concentrations >100pM in the current study. A positive association between elevated fasting serum gastrin concentrations and elevated BMI has been described in a previous human study (Lindstedt et al., 1985b) and in animal studies where hypergastrinaemia was demonstrated to be secondary to low overall gastric acid output (Morton et al., 1985). However an association between low BMI and elevated fasting serum gastrin concentrations has not been previously reported.

In the current study, cigarette smoking was found to be associated with elevated fasting serum gastrin concentrations during univariate analysis. Cigarette smoking has been demonstrated to be a significant risk factor for the development of gastric adenocarcinoma (Steevens et al., 2010). However the exact mechanisms that are involved are not entirely understood. Results from the current study suggest that smoking may possibly increase the risk of gastric adenocarcinoma through its effects on fasting serum gastrin concentrations.

Alcohol consumption on the other hand was found in this cohort to be associated with significantly lower fasting serum gastrin concentrations. Recent studies have found no significant associations between alcohol consumption and risk of developing gastric cancer (Steevens et al., 2010). However one of the limitations of the current study is that the differentiation between various amounts of alcohol consumed was not determined. Hence further studies are required to address the role played by alcohol in influencing fasting serum gastrin concentrations.

6.6 Conclusions

There are complex interactions between *H. pylori* infection, PPI use and the presence of gastric preneoplastic pathology in determining fasting serum gastrin concentrations. In addition, other host and environmental factors also appear to influence fasting serum gastrin concentrations. For example PPI use only appears to cause hypergastrinaemia in the absence of gastric preneoplastic pathology in patients with previous *H. pylori*

infection. With concurrent PPI therapy, current *H. pylori* infection is associated with significantly elevated fasting serum gastrin concentrations unrelated to the presence of absence of gastric preneoplastic pathology. Concurrent PPI therapy and the presence of gastric preneoplastic pathology (in those patients without evidence of *H. pylori* infection) were both significantly associated with elevated fasting serum gastrin concentrations. These factors should therefore be taken into consideration when interpreting fasting serum gastrin concentrations in individual patients.

Chapter 7

7 Investigation of factors that influence the progression of type-1 gastric neuroendocrine (NET) tumours

7.1 Introduction

Type-1 gastric neuroendocrine (carcinoid) tumours (NETs) are the most common gastric neuroendocrine tumours (Bordi, 1999). Achlorhydria, caused by loss of acid secreting parietal cells resulting from autoimmune atrophic body gastritis (pernicious anaemia) results in antral G cell hyperplasia and unopposed gastrin secretion (due to loss of negative feedback inhibition). The resulting hypergastrinaemia stimulates ECL-cell hyperplasia and in some patients type-1 gastric carcinoid tumour formation (Annibale et al., 2001a). This has been discussed in detail in sections 1.3.2.3 and 1.6. However not all patients with autoimmune atrophic gastritis develop type-1 gastric carcinoid tumours, with only about 5% of patients developing such tumours. Therefore factors in addition to achlorhydria and hypergastrinaemia are likely to influence the development of type-1 gastric carcinoid tumours in individual patients. Such factors however currently remain poorly understood. We therefore hypothesised that the following factors may influence the development of type-1 gastric carcinoid tumours in susceptible patients with autoimmune gastric atrophy:

1. The extent and characteristics of gastric atrophy as determined by the severity of the associated hypergastrinaemia, whether

there was associated vitamin B12 and/ or iron deficiency and the presence of autoantibodies namely anti-intrinsic factor and anti-gastric parietal cell antibodies

2. Evidence of previous or co-existent *H. pylori* infection
3. Presence of other autoimmune diseases such as hypothyroidism

We therefore analysed the importance of the above factors by assessing patients with chronic atrophic gastritis and varying degrees of ECL-cell hyperplasia and others with type-1 gastric NETs.

7.2 Methods

Local adult ethics committee approval and informed patient consent was obtained from participating patients who were attending for a diagnostic gastroscopy at the Royal Liverpool and Broadgreen University Hospital (RLBUHT) for clinical indications. Patients with histologically confirmed atrophic corpus gastritis and ECL-cell abnormalities were included in this study. Clinical and demographic data including age, sex, and concurrent drug use were recorded.

At gastroscopy the total number of visible carcinoid polyps/nodules was counted for each individual patient and the maximum diameter of polyps was measured by comparison with the open jaws of standard biopsy forceps which measures approximately 8mm. Two biopsies were taken from the gastric antrum and corpus. Patients were deemed to have

current *H. pylori* infection if this organism was seen by histological analysis of either antral or corpus biopsies.

Gastric corpus biopsies taken at the time of gastroscopy were assessed (by a Consultant Gastrointestinal Histopathologist at RLBUHT) for

- The presence of gastric atrophy
- Degree and extent of ECL-cell hyperplasia following chromogranin and or synaptophysin immunohistochemistry and
- Presence of *H. pylori* infection

Gastric corpus biopsies were analysed for the presence of ECL-cell hyperplasia and the extent of this hyperplasia was classified by an experienced GI histopathologist based on well defined criteria (Delle et al., 2005; Solcia et al., 1988) as:

- Simple (diffuse) ECL-cell hyperplasia (increase in number of ECL-cells by over 2 standard deviations over normal values)
- Linear ECL-cell hyperplasia (consisting of linear sequences of a minimum of 5 ECL-cells lying inside the basement membrane and at least two such chains seen per millimetre of the gastric mucosa)
- Micronodular ECL-cell hyperplasia (clusters of 5 or more cells of around 30-150 µm diameter in the lamina propria or within glands and at least one micronodule per millimetre of the gastric mucosa)

- Dysplastic ECL-cells (lesions between 150-500 µm located deep in the oxyntic mucosa with no evidence of deeper infiltration)
- Carcinoid tumours (greater than 0.5 mm diameter and confined to the mucosa and submucosa with neighbouring endocrine cells showing hyperplasia or dysplasia.

Fasting blood obtained at the time of gastroscopy was analysed for serum gastrin concentration by radioimmunoassay at the Physiological laboratory, University of Liverpool, as detailed in section 2.3. In some patients, fasting serum gastrin concentrations were measured using an in-house RIA method (using a rabbit polyclonal antibody) at the Royal Hammersmith Hospital, London instead. Serum concentrations of vitamin B12 and ferritin, presence of gastric parietal cell and intrinsic factor antibodies and thyroid function tests were also measured at the biochemistry and immunology laboratories at RLBUHT as part of standard clinical assessment. Serological analysis for *Helicobacter pylori* infection (using anti-IgG antibodies to *Helicobacter pylori*) was performed by the microbiology department at the Royal Liverpool and Broadgreen University Hospital NHS Trust.

Serum ferritin concentration was used as a marker of iron stores in the body and hence a low serum ferritin (below the lower limit of normal) or history of pre-existing iron deficiency anaemia was taken as a marker of iron deficiency anaemia and the presence of low serum vitamin B12

concentrations (or a history of vitamin B12 deficiency) as a marker of vitamin B12 deficiency in this cohort. Patients with a known history of hypothyroidism or with low serum total T4 concentration associated with an elevated thyroid stimulating hormone (TSH) concentration were considered to be hypothyroid. The biochemical reference ranges for the various parameters are described in table 7-1.

Parameter	**Normal Range (units)**
Vitamin B12	191-663 ng/L
Serum ferritin	30-400µg/L in males and 13-150 µg/L in females
TSH	0.3 to 6.0mU/l
Total T4	60 to 150nmol/l

Table 7-1: Normal reference ranges (Royal Liverpool University Hospitals NHS Trust, http://www.rlbuht.nhs.uk/jps/)

Intrinsic factor and gastric parietal cell antibodies were detected by enzyme linked immunoassay using human recombinant antigen (reference range < 20U/ml) and immunofluorescence microscopy using rodent tissue substrate (reference range negative) respectively. These were measured at the RLBUHT immunology laboratory.

7.3 Statistical analyses

7.3.1 ANOVA (one-way analysis of variance)

ANOVA was used to analyse the significance of means between the three groups of ECL-cell hyperplasia in sections 7.4.2 and 7.4.3. $p<0.05$ was considered to be statistically significant.

7.3.2 χ^2 test

Univariate analysis using χ^2 test was used in defining relationships between categorical variables (section 7.4.2). $p<0.05$ was considered to be statistically significant.

7.4 Results

7.4.1 Demographics of cohort

A total of 49 patients were recruited between 2003 and 2010. 34 (69.4%) were female with a median age of 63.7 years (range 20.8-86.1 years). All patients had histological evidence of atrophic gastritis on corpus biopsy and some degree of ECL-cell hyperplasia. 9 (18.4%) patients were on a concurrent PPI at the time of diagnosis. Only 3 (6.1%) patients had evidence of current *H. pylori* infection on histology. The extent or degree of ECL-cell hyperplasia, median age of patients and the number and size of polyps seen at gastroscopy is detailed in table 7-2.

ECL-cell hyperplasia	Number of patients (% female)	Median age years (IQR)	Mean number of nodules (range)	Mean maximum size of nodules mm (range)
Linear	6 (83.4)	62.2 (53.8, 69.7)	3 (0-12)	3.8 (0-15)
Nodular	31 (70.9)	63.2 (51.1, 69.3)	4.8 (0-20)	2.9 (0-12)
Carcinoid tumours	12 (58.3)	65.3 (60.6, 75.5)	9.3 (2-20)	12 (5-50)

Table 7-2: Demographics of patients with atrophic gastritis

Table 7-3 below summarises the prevalance of vitamin B12 and iron deficiency, presence of gastric parietal and intrinsic factor antibody, hypothyroidism, *H. pylori* infection and the mean serum gastrin concentrations in patients with linear, nodular and carcinoid ECL-cell hyperplasia.

ECL-cell hyperplasia (Total no.)	Vitamin B12 deficiency (%)	Iron deficiency (%)	+ve gastric parietal cell antibody (%)	+ve intrinsic factor antibody (%)	Hypothyroid (%)	Number of patients and range of fasting serum gastrin concentrations (%)			*H. pylori* histology (%)	*H. pylori* serology (%)
						<100 pM	100-400 pM	>400 pM		
Linear (6)	3 (50%)	3 (50%)	4 (66.7%)	1 (16.7%)	1 (16.7%)	0	3 (50)	3 (50)	0	1 (16.7%)
Nodular (31)	20 (64.5%)	14 (45.2%)	21 (67.7%)	4 (12.9%)	7 (22.6%)	0	10 (32.3)	21 (67.7)	3 (9.7%)	7 (22.6%)
Carcinoid (12)	9 (75%)	3 (25%)	11 (91.7%)	0	5 (41.7%)	0	7 (58.3)	5 (41.7)	0	2 (16.7%)

Table 7-3: Prevalence of vitamin B12, iron deficiency, presence of gastric parietal cell and intrinsic factor antibodies, concomitant hypothyroidism and H. pylori infection in patients with various degrees of ECL-cell hyperplasia or type-1gastric carcinoid tumours.

7.4.2 Age of patients and presence of ECL-cell hyperplasia

Patients with type-1 gastric carcinoid tumours had a higher median age compared to patients with linear ECL-cell hyperplasia however this difference did not reach statistical significance (p=0.342, ANOVA).

7.4.3 Polyp numbers and size

Patients with type-1 gastric neuroendocrine tumours had more polyps (p=0.067, ANOVA) and significantly larger lesions (p=0.0033, ANOVA) compared with patients with lesser degrees of ECL-cell hyperplasia.

7.4.4 Extent of gastric atrophy

7.4.4.1 Mean fasting serum gastrin concentrations

As a group, all patients had elevated fasting serum gastrin concentrations (all >100 pM). 20 patients (41%) had fasting serum gastrin concentrations between 100 and 400 pM and the remaining 29 patients (59%) had fasting serum gastrin concentrations greater than 400 pM (table 7-4). No significant differences were found with different degrees of ECL-cell hyperplasia.

Degree of ECL-cell hyperplasia (total number)	Total number of patients with fasting serum gastrin concentrations between 100-400 pM	Total number of patients with fasting serum gastrin concentrations >400 pM	p value, χ^2 test
Linear (6)	3	3	0.262
Nodular (31)	10	21	
Carcinoid (12)	7	5	

Table 7-4: Univariate analysis of fasting serum gastrin concentrations and varying degrees of ECL-cell hyperplasia

7.4.4.2 Vitamin B12 deficiency

Although a higher percentage of vitamin B12 deficiency (75%) was observed in patients with type-1 gastric carcinoid tumours as compared to patients with either nodular (64.5%) or linear (50%) ECL-cell hyperplasia, this association was again not statistically significant (p=0.82, χ^2 test).

7.4.4.3 Presence of antibodies (gastric parietal cell and intrinsic factor antibodies)

As with vitamin B12 deficiency, a higher percentage of patients with type-1 gastric carcinoid tumours were observed to have concurrent gastric parietal cell antibodies (91.7%) compared to patients with nodular (67.7%) or linear (66.7%) ECL-cell hyperplasia. However this association was not statistically significant (p=0.69, χ^2 test). In this cohort, none of the patients

with type-1 gastric carcinoid tumours had coexisting intrinsic factor antibodies.

7.4.4.4 Iron deficiency anaemia

No significant association was observed between the presence of iron deficiency anaemia and the degree of ECL-cell hyperplasia (p=0.60, χ^2 test).

7.4.4.5 Associated hypothyroidism

A higher percentage of patients with type-1 carcinoid tumours were observed to have associated hypothyroidism (41.7%) compared to patients with linear (16.7%) or nodular (22.6%) ECL-cell hyperplasia. However this association was not statistically significant (p=0.97, χ^2 test).

7.4.4.6 Concurrent *H. pylori* infection

Concurrent *H. pylori* infection was rare in this cohort of patients with ECL-cell hyperplasia. No correlation was observed between the presence of *H. pylori* infection (by histology or serology) and the degree of ECL-cell hyperplasia (p= 0.41, χ^2 test).

7.4.5 Discussion

In this study we have analysed the potential role played by several factors including age, extent and characteristics of gastric atrophy (as estimated by the severity of the associated hypergastrinaemia, associated vitamin B12 and iron deficiency and presence of anti-intrinsic factor and anti-gastric parietal cell antibodies), presence of previous or co-existent *H.*

pylori infection and the presence of another autoimmune disease namely hypothyroidism, that may influence the progression of ECL-cell hyperplasia to type-1 gastric NETs in some patients with chronic autoimmune atrophic gastritis.

All gastroscopies and polyp assessment were performed by the same endoscopist (Consultant Gastroenterologist) thereby reducing inter-observer error. All patients had two biopsies taken from the antrum and two biopsies taken from the gastric corpus to establish atrophic body gastritis as well as to assess ECL-cell hyperplasia. The number and site of gastric biopsies taken was deemed sufficient to demonstrate atrophy and ECL-cell hyperplasia as this is associated with autoimmune gastritis where there is a field change as opposed to sporadic NETs (Rindi et al., 1999a).

We have demonstrated in this cohort of 49 patients that patients with more advanced degrees of ECl-cell hyperplasia and those with type-1 gastric carcinoid tumours were older, but this difference was not statistically significant. The youngest patient to develop type-1 gastric NET was aged 43.9 years and the youngest to develop nodular hyperplasia was 20.8 years. Hence it is likely that the duration of hypergastrinaemia (in the presence of chronic atrophic gastritis) rather than the age of the patient appears to influence the progression to ECL-cell hyperplasia and subsequent development of type-1 gastric NETs in some patients with chronic atrophic gastritis. This confirms the previously proposed hypothesis that longstanding hypergastrinaemia confers a greater risk for the development and progression of ECL-cell hyperplasia and type-1 gastric NETs. Also, patients with the more advanced degrees of ECL-cell

hyperplasia and type-1 gastric NETS not only exhibited a higher number of nodules, but these nodules were also larger in size compared to patients with lesser degrees of ECL-cell hyperplasia.

This study has demonstrated that factors such as the presence of autoantibodies (gastric parietal cell or intrinsic factor antibodies), concurrent or past *H. pylori* infection or coexisting hypothyroidism were not significantly associated with the presence of more advanced degrees of ECL-cell hyperplasia or type-1 gastric carcinoid tumours. However although the associations did not reach statistical significance, a greater proportion of patients with more advanced degrees of ECL-cell hyperplasia had autoantibodies to gastric parietal cells, vitamin B12 deficiency and hypothyroidism. This suggests a possible role played by these factors in the progression of ECL-cell hyperplasia to type-1 gastric carcinoid tumours, but the cohort size was not large enough to confirm a statistically significant effect.

Similar findings have recently been in another recent study of patients with chronic autoimmune atrophic gastritis (Vannella et al., 2011). In this study, patients with type-1 gastric carcinoid tumours were reported to have significantly higher mean fasting serum gastrin concentrations and a greater total number of polyps. However the presence of gastric parietal cell antibodies, anaemia or hypothyroidism were not found to be significantly associated with more advanced degrees of ECL-cell hyperplasia.

There are certain limitations to our study. Firstly we have utilised two different methods to measure fasting serum gastrin concentrations.

Most patients (67.3%) had their fasting serum gastrin concentrations measured at the University of Liverpool Physiological laboratories using the previously described radioimmunoassay (RIA) method of estimation of serum gastrin concentrations (section 2.3). However the remaining patients (32.7%) had their fasting serum gastrin concentrations measured at the Hammersmith Hospital, London, using an in-house RIA method (using a different rabbit polyclonal antibody). Although accurate, when fasting serum gastrin concentration was greater than 400pM, the serum of such patients measured at the Royal Hammersmith Hospital, was not subjected to further analysis for estimation of the actual serum gastrin concentration. On the other hand, if higher concentrations were observed whilst measuring serum gastrin concentrations at the Physiological Laboratories, University of Liverpool, further subsequent measurements were made following serial dilutions of patients' sera (using the RIA method as described in section 2.3). This enabled accurate estimation of the actual serum gastrin concentration. Hence in our analyses, the range of serum gastrin concentrations had to be used rather than comparing actual concentrations.

The other main limitation of the current study is the small sample size of only 49 patients. The study was therefore not adequately powered to achieve statistically significant outcomes in certain analyses. In addition there is a possibility of referral bias as only patients who are found to have nodules at gastroscopy are usually referred for further specialist evaluation. Patients without nodules may not have undergone biopsy for assessment of ECL-cell hyperplasia. Finally as older people with

pernicious anaemia secondary to autoimmune gastritis are not often referred for a gastroscopy but instead commenced on treatment with replacement vitamin B12 and this may have further affected our findings.

There are several other potential factors (both host and environmental) that have been proposed to influence the progression of ECL-cell hyperplasia and the development of type-1 gastric NETs. It is well known that hypergastrinaemia alone is probably insufficient to cause progression of ECL-cell hyperplasia and development of type-1 gastric NETs. For example, chronic proton pump inhibitor therapy and vagotomy, although associated with chronic hypergastrinaemia and hypochlorhydria, are rarely associated with the development of type-1 gastric NETs. It has therefore been suggested that factors including genetic, host response and exogenous environmental factors such as diet, smoking and alcohol could play an important role in the progression of ECL-cell hyperplasia and development of type-1 gastric NETs. These factors have not been addressed in the current study, but have been analysed in other studies.

Genetic factors that influence development of gastric NETs have been well characterised in animal models, especially in Mastomys, which has a genetic predisposition to develop gastric NETs (Nilsson et al., 1992). Approximately 40-60% of Mastomys develop gastric NETs by 2 years of age and this process is accelerated by the administration of acid suppressing drugs. ECL-cell hyperplasia develops within 8 weeks and gastric NETs between 12 and 16 weeks following treatment with acid suppressing drugs (Bilchik et al., 1989). However the genetic reasons for this increased susceptibility to the development of ECL-cell tumours are

still poorly understood. One explanation for this predisposition is explained by the finding that Mastomys have polymorphisms in the CCK-2 receptor gene, thereby conferring increased sensitivity to circulating gastrin (Reubi et al., 1992). Also gastric NETs in Mastomys have been demonstrated to exhibit increased numbers of CCK-2 receptors suggesting a gastrin stimulated growth of such tumours.

Studies in rats treated with acid suppressing drugs have also demonstrated an increase in the number of ECL-cells, which appears to be reversible if the hypergastrinaemia was short lived. However lifelong acid suppression eventually led to the development of gastric NETs. Changes in the endocrine cell population in the antrum have also been observed in rats with hypergastrinaemia secondary to acid suppression. An increase in G cells and a reciprocal decrease in D cells (with a overall increase in G:D ratio) has been noted in such rats with a subsequent loss of inhibitory effect of somatostatin on ECL-cell growth (Creutzfeldt et al., 1986).

Patients with multiple endocrine neoplasia type-1 syndrome (MEN-1) are at increased risk of developing type-2 gastric NETs and the underlying genetic defect is an autosomal dominant mutation in the MEN-1 gene coding for the MENIN protein. This MENIN protein is thought to act as a tumour suppressor protein through interactions with numerous nuclear and cytoplasmic factors that regulate cell and DNA repair and processing. A recent study of MEN-1 patients reported that 100% of the patients had abnormal ECL-cell distribution and 23% had gastric NETs (Berna et al., 2008).

Several growth factors have also been studied as potential factors that may influence the progression of ECL-cell hyperplasia and type-1 gastric NET formation. Reg peptide, initially described in 1984 in association with regenerating pancreatic tissue, has been shown to be secreted by human ECL and chief cells. Reg peptides have been demonstrated to be up regulated following gastric mucosal injury and also in rats with hypergastrinaemia induced by proton pump inhibitor drugs (Higham et al., 1999; Kinoshita et al., 2004).

There are likely to be other as yet unexplained factors that could also influence the progression of ECL-cell hyperplasia. It has been proposed that several non-*Helicobacter pylori* bacteria colonise the stomach in the setting of achlorhydria. Monstein et al. and Bik et al. have demonstrated several different bacterial species in the human stomach by DNA sequence analysis (Bik et al., 2006; Monstein et al., 2000). Similarly non-*Helicobacter pylori* bacteria were demonstrated in gastric biopsies of patients on long standing treatment with acid suppressing drugs (Sanduleanu et al., 2001). Such organisms which have been demonstrated to colonise the gastric mucosa may therefore play a role in the development and progression of ECL-cell hyperplasia through as yet unknown mechanisms. Also host responses and genetic predispositions which influence gastric epithelial cell apoptosis and cell proliferation may also play an important role in the progression of ECL-cell hyperplasia. It is therefore possible that as yet unidentified bacteria and host-bacterial responses could influence the progression of ECL-cell hyperplasia and subsequent development of type-1 gastric NETs.

7.4.6 Conclusions

Although our study has not demonstrated any statistically significant associations, there does appear to be some correlation between the presence of factors such as hypothyroidism, gastric parietal and intrinsic factor antibodies, iron deficiency and vitamin B12 deficiency and more advanced degrees of ECL-cell hyperplasia. We therefore recommend further prospective studies involving greater numbers of patients to verify these potential associations. Additional immunohistochemical analysis of gastric biopsies for G-cells and somatostatin may provide some information regarding the role played by somatostatin in ECL-cell hyperplasia and type-1 gastric NET development.

Chapter 8

8 Does the octreotide suppression test predict response to antrectomy in patients with type-1 gastric neuroendocrine tumours (NETs)

8.1 Introduction

Type-1 gastric neuroendocrine (carcinoid) tumours are thought to arise as a result of hypergastrinaemia. Small tumours (<1cm) rarely metastasise and hence are usually associated with an excellent prognosis. Such tumours are often managed by endoscopic surveillance or occasionally polypectomy (endoscopic mucosal resection). However larger tumours (>1 to 2 cms) have malignant potential and hence the management of such tumours remains controversial. This may involve endoscopic or surgical techniques (including resection of polyp, antrectomy or gastrectomy) or medical treatment with long acting somatostatin analogues.

Endoscopic resection has been recommended for large tumours (>1cm) that are deemed resectable at endoscopic ultrasound (no evidence of muscularis propria involvement) (Delle et al., 2012). Local surgical resection of polyps is adviced when such polyps are not amenable to endoscopic resection. Antrectomy (resulting in removal of G-cells) has been shown to be effective in reducing serum gastrin concentrations and has been reported to cause complete resolution of small type-1 gastric

NETs in a number of case series (Hirschowitz et al., 1992; Dakin et al., 2006).

Antrectomy (and therefore removal of gastrin secreting G-cells) may be an effective surgical treatment in those patients with type-1 gastric NETs greater than 1cm in size and also in those with a high polyp burden (> 5 polyps). Antrectomy has also been advocated in those patients with polyp recurrence following endoscopic polyp resection and in those patients undergoing endoscopic surveillance for type-1 gastric NETs who develop further gastric type-1 NETs or demonstrate an increase in size of existing type-1 NETs.

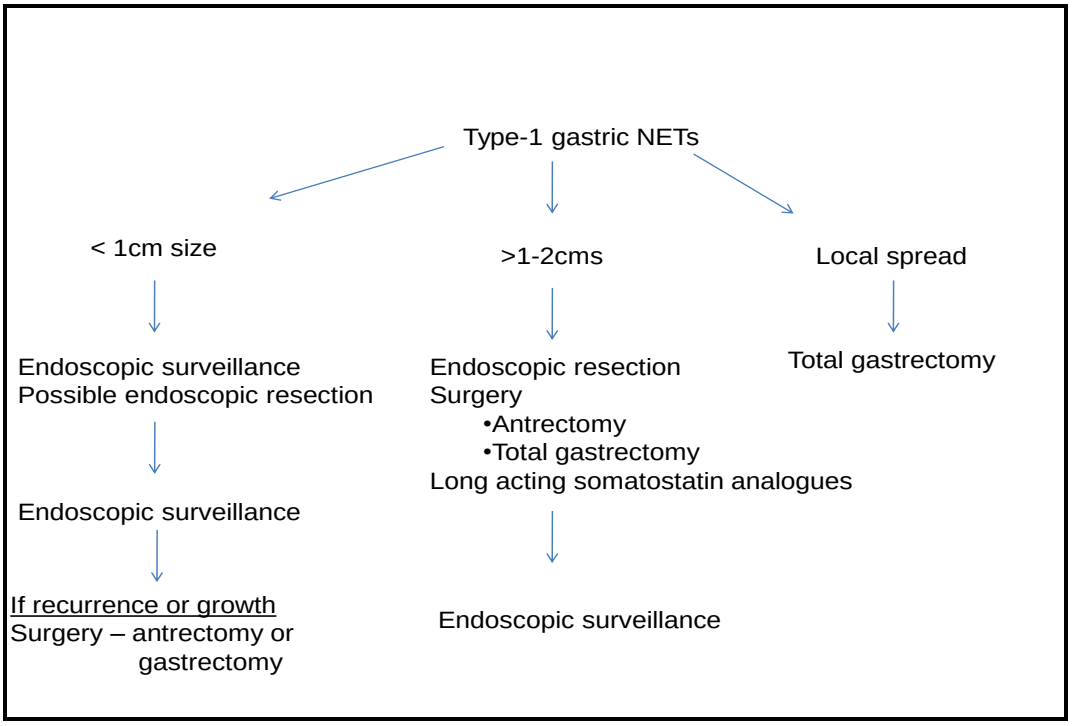


Figure 8-1: Proposed algorithm for management of type-1 gastric NETs (Plockinger et al., 2004).

Total gastrectomy rather than antrectomy has however been advocated for the management of larger type-1 gastric NETs, due to the observation that some tumours do not appear to regress following antrectomy (Wangberg et al., 1990).

Hence should antrectomy therefore only be considered in those type-1 gastric NETs which are gastrin-responsive? The role of an octreotide suppression test in determining those patients with type-1 gastric NET who may respond to antrectomy has previously been studied in a single case (Higham et al., 1998).

Octreotide administration has been shown to decrease serum gastrin concentrations, reduce ECL-cell and gastrin cell function and with long term use has also been shown to reduce the size of type-1 gastric NETs (Fykse et al., 2004; Jianu et al., 2011). Gastrin stimulates histamine release from ECL-cells by stimulation of the genes encoding the enzyme histidine decarboxylase (HDC). It has been demonstrated that elevated tissue HDC concentrations are observed in conditions associated with hypergastrinaemia such as gastrinomas. We have therefore utilised this enzyme as a surrogate marker for ECL-cell function and to determine the ECL-cell response to octreotide infusion. Octreotide mediated reduction in serum gastrin concentrations as well as a decrease in ECL-cell function can hence be potentially demonstrated by a fall in measured HDC mRNA levels in corpus and polyp/tumour biopsies. Such a response may suggest that these tumours are still gastrin responsive and hence removal of the source of gastrin may result in cessation of tumour growth and possibly even tumour regression.

8.2 Aim

To determine the role of the 'octreotide suppression test' in identifying those patients with type-1 gastric NETs in whom the tumours regress following antrectomy.

8.3 Methods

Local ethics committee approval and informed patient consent were obtained. Six patients with either >1cm and/or > 5 type-1 gastric NETs were studied (4 female, mean age 64.7 years). The initial diagnosis of type-1 gastric NETs was made either at diagnostic gastroscopy performed at Royal Liverpool and Broadgreen University Hospital NHS Trust (RLBUHT) or at other hospitals in the North West of England and subsequently referred to Prof. D.M.Pritchard at the RLBUHT and University of Liverpool for specialist management of their type-1 gastric NETs. Each patient's demographic data including concurrent medication use (proton pump inhibitors) was recorded.

Serological analysis for *Helicobacter* infection (anti-IgG antibodies to *Helicobacter pylori*) was performed by the microbiology department at RLBUHT. Serum concentrations of vitamin B12 and ferritin, presence of gastric parietal cell and intrinsic factor antibodies and thyroid function tests were also measured at the biochemistry and immunology laboratories at RLBUHT as part of initial clinical assessment. CT (computerised tomography) scan of the abdomen and a ^{111}In-octreotide scan

(somatostatin scintigraphy) were performed in order to rule out other gastrointestinal NETs and the presence of metastatic disease.

Patients were admitted to Royal Liverpool and Broadgreen University Hospital NHS Trust for the octreotide suppression test. On day 1 of the test, an initial diagnostic gastroscopy was performed. This was followed by a 72 hour intravenous octreotide infusion at a rate of 25mcg/hour. 10mls of blood was obtained before the commencement of the test (fasting) and subsequently at timed intervals during the 72 hour octreotide infusion as previously demonstrated in a previous single case study (Higham et al., 1998). Blood samples were centrifuged and the serum obtained was subsequently analysed for serum gastrin concentrations by radioimmunoassay (as detailed in section 2.3).

At gastroscopy, biopsies were obtained from the gastric antrum, the corpus and carcinoid polyps using standard biopsy forceps. Biopsies taken from the antrum, corpus and carcinoid polyp(s) were formalin fixed and paraffin embedded for subsequent histological analysis by a GI histopathologist. Histological analysis was performed following staining with H&E (haematoxylin and eosin and immunochemistry for chromogranin A and synaptophysin). Antral and corpus biopsies were analysed for the presence of ECL-cell hyperplasia and the extent of this hyperplasia was classified by an experienced GI histopathologist based on well defined criteria as outlined in section 5.2. These biopsies were also analysed for the presence of *Helicobacter pylori* infection. Biopsies obtained from the carcinoid polyp(s) and corpus were stored in RNA-Later® at 4˚C for subsequent RNA extraction.

A rapid urease test (PRONTO Dry®, MIC France) was also performed using two gastric antral biopsies in all patients in order to obtain a rapid diagnosis of *Helicobacter pylori* infection. Following the octreotide infusion, repeat corpus and carcinoid polyp biopsies were obtained and subsequently processed as described above.

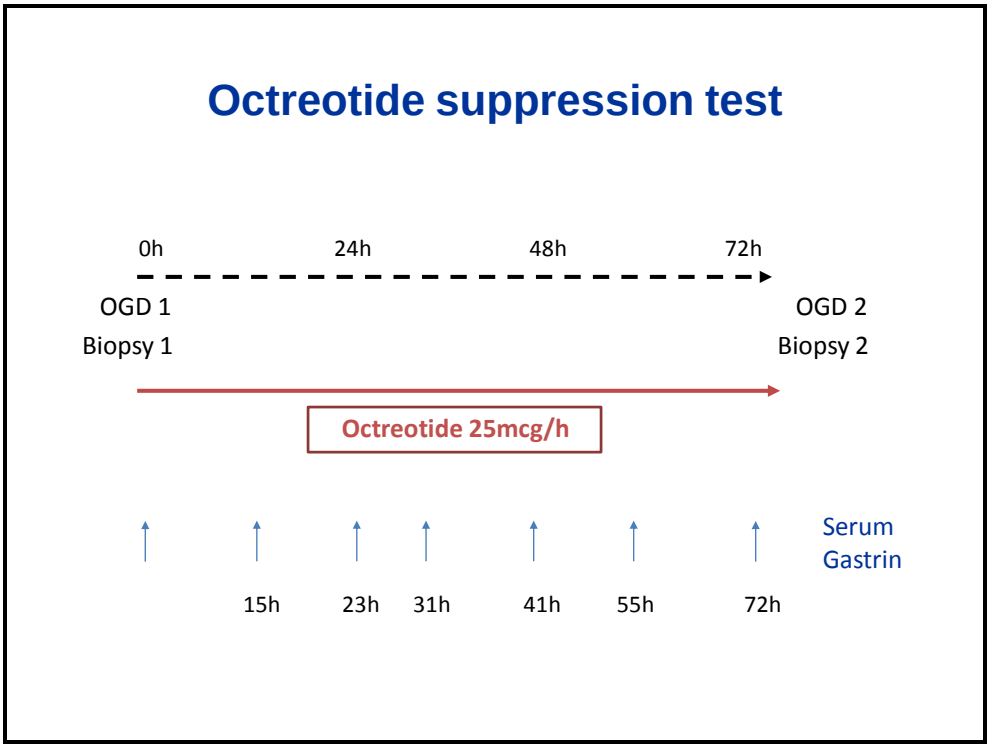


Figure 8-2: Schematic representation of the octreotide suppression test.

8.3.1.1 RNA extraction from gastric corpus and polyp biopsies

Corpus and polyp biopsies taken at endoscopy were initially stored in separate containers containing RNA-Later® at 4°C overnight and then at -20°C (after removal of RNA-Later®) if stored for longer. Extraction of RNA from the biopsy specimens was based on the 'single-step' method that is widely used for isolating RNA (Chomczynski and Sacchi, 2006). This is

based on the principle that RNA is separated from DNA after extraction with an acidic solution (containing guanidine thiocyanate, sodium acetate, phenol and chloroform) followed by centrifugation. Acidic conditions render the RNA in the upper aqueous phase of the supernatant, with the DNA in the middle (interphase) and other organic matter in the lower layer. RNA was subsequently recovered by precipitation with isopropanol.

8.3.1.2 Real time quantitative polymerised chain reaction

The technique of real time quantitative PCR (rt-qPCR) was utilised to analyse the abundance of HDC mRNA in the biopsy samples. 25µl of master mix/sample was analysed in triplicate using the following thermal profile (the whole process being completed in 90 minutes):

- Stage 1 – 50^{o}C for 2 minutes (1 repeat)
- Stage 2 – 95^{o}C for 10 minutes (1 repeat)
- Stage 3.1 – 95^{o}C for 15 seconds (40 repeats)
- Stage 3.2 – 60^{o}C for 1 minute

The master mix solution was titrated using the following components:

- Forward Primer - 2.2µl
- Reverse primer - 21µl
- Probe - 2.5µl
- Water - 20.3µl per reaction

The probes and primer used are as follows:

- Probe (hGAPDH): 71T CGT CGC CAG CCG AGC CAC A;

Forward primer (hGAPDH): 34F GCT CCT CCT GTT CGA CAG TCA and

Reverse primer (hGAPDH): 113R ACC TTC CCC ATG GTG TCT GA

- Probe (hHDC91): CTC TGT TAA ACT CTG GTT CGT GAT TCG GTCC

  Forward (hHDC-F91): CCC TGA GCC GAC GGT TT

  Reverse (hHDC-R91): GTA CCA TGT CTG ACA TGT GCT TGA

Both the RNA extraction and rt-qPCR were performed by Dr. Islay Steele.

8.4 Results

8.4.1.1 Demographics

The clinical features of the six patients studied are outlined in tables 8-1 to 8-3:

Patient Number	Sex	Age (years)	Findings at initial endoscopy		PPI	Antral Histology	Corpus Histology	Histology of polyps
			Total no. of polyps	Size of largest polyp (mm)				
Patient 1	F	67.4	20	7	No	Atrophic gastritis	Atrophic gastritis, intestinal metaplasia, Micronodular ECL-cell hyperplasia	Carcinoid tumour
Patient 2	M	43.5	4	10	No	Reactive gastritis	Atrophic gastritis, intestinal metaplasia, Linear ECL-cell hyperplasia	Carcinoid
Patient 3	F	65.3	18	10	No	Normal	Atrophic gastritis, intestinal metaplasia and Micronodular ECL-cell hyperplasia	Carcinoid
Patient 4	F	70.57	5	15	No	Normal	Atrophic gastritis, intestinal metaplasia and Micronodular ECL-cell hyperplasia	Carcinoid
Patient 5	M	75.7	4	16	No	Atrophic gastritis	Atrophic gastritis, intestinal metaplasia and Linear ECL-cell hyperplasia	Carcinoid
Patient 6	F	66.3	10	5	No	Reflux gastritis	Atrophic gastritis, intestinal metaplasia and Micronodular ECL-cell hyperplasia	Microcarcinoid

Table 8-1: Demographics and endoscopic findings of patients undergoing octreotide suppression test including concurrent PPI treatment and histology of antrum, corpus and polyp.

Patient Number	Fasting serum gastrin concentration (pM)	Fasting serum chromogranin A concentration (pM)	Rapid urease test (RUT)	*Hp* histology	*Hp* serology	CT abdomen	^{111}In-Octreotide Scan
Patient 1	2800	104	ND	Negative	ND	Normal	ND
Patient 2	680	50	Negative	Negative	Negative	Normal	Normal
Patient 3	190	45	Negative	Negative	Negative	Normal	Normal
Patient 4	838	33	Negative	Negative	Negative	Gastric carcinoid tumour	Normal
Patient 5	2116	60	Negative	Negative	Negative	Normal	Normal
Patient 6	340	11	Negative	Negative	Negative	Soft tissue mass in stomach	Normal

Table 8-2: Patients undergoing octreotide suppression test: fasting serum gastrin and chromogranin A concentrations, *H. pylori* status and CT and ^{111}In-octreotide scan findings. (ND=not done)

Patient Number	Vitamin B12 deficiency	Iron deficiency anaemia	Hypothyroidism	Anti gastric parietal cell antibody	Anti intrinsic factor antibody
Patient 1	Y	N	Y	Positive	Negative
Patient 2	Y	N	Y	Positive	Negative
Patient 3	Y	N	N	Negative	Negative
Patient 4	Y	N	N	Positive	Negative
Patient 5	Y	N	Y	Positive	Negative
Patient 6	Y	Y	N	NA*	NA*

Table 8-3: Patients undergoing octreotide suppression test: presence of vitamin B12 and iron deficiency, hypothyroidism and anti gastric parietal cell and intrinsic factor antibodies.

* Unable to accurately assess due to interfering anti-nuclear antibodies.

8.4.1.2 Response of serum gastrin concentration to octreotide infusion

Fasting serum was obtained prior to the first endoscopy of the octreotide suppression test. Immediately following the first endoscopy, patients were commenced on an intravenous infusion of octreotide at 25µg/hour and this was continued for a total of 72 hours. This was then followed by a second endoscopy. Serum was obtained for estimation of

gastrin concentrations at 15, 23, 31, 47, 55 and 72 hours of infusion. Serum gastrin concentrations were measured by radioimmunoassay as previously described in section 2.3 and the results are shown in Figure 8-3 and table 8-4.

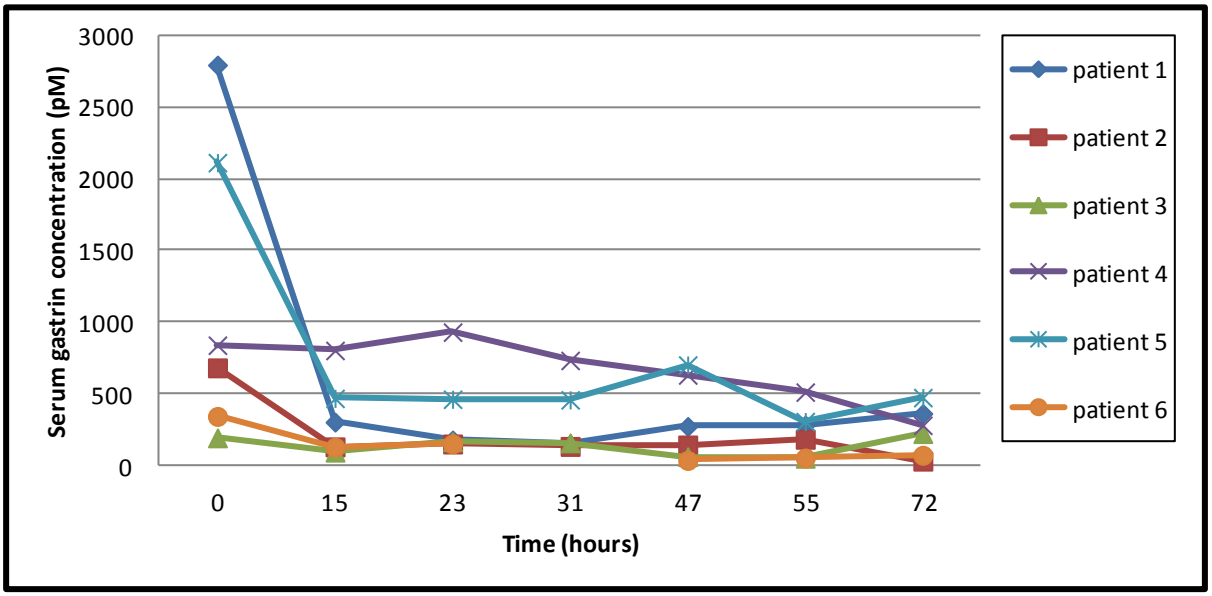


Figure 8-3: Change in serum gastrin concentrations during the 72 hour octreotide infusion.

time in hours	patient 1	patient 2	patient 3	patient 4	patient 5	patient 6	Mean Gastrin concentration (pM)
0	2800	680	190	838	2117	340	995
15	300	127	90	802	466	124	
23	175	147	163	930	460	148	
31	145	130	155	732	456	ND	
47	270	140	58	630	700	32	
55	280	180	47	510	304	49	
72	360	27	220	278	472	68	214

Table 8-4: Serum gastrin concentrations at timed intervals during intravenous octreotide infusion.

8.4.1.3 Change in Histidine Decarboxylase (HDC) mRNA in gastric corpus and polyp biopsies following octreotide infusion

Biopsies taken from the polyp and surrounding gastric corpus mucosa were analysed for the abundance of HDC mRNA by real time quantitative polymerase chain reaction (RT-qPCR) as previously described in section 8.3.1.2. There was a reduction in mRNA abundance in both the corpus and polyp in all patients following octreotide infusion and this is summarised in figures 8-3 and 8-4 and tables 8-3 and 8-4.

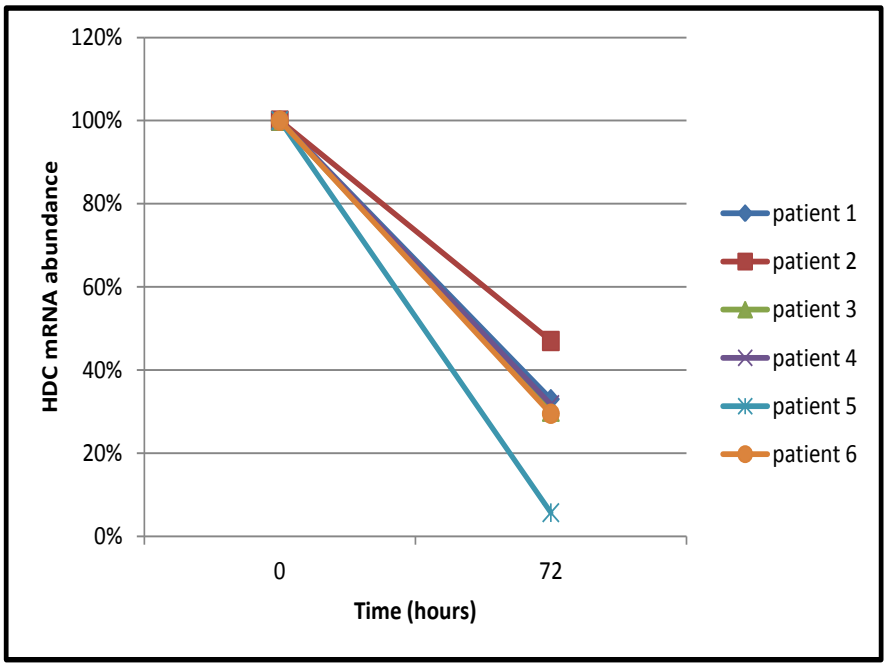


Figure 8-4: HDC mRNA abundance in the corpus and its response to intravenous octreotide infusion.

Patient	patient 1	patient 2	patient 3	patient 4	patient 5	patient 6
HDC mRNA abundance (% of pre octreotide abundance)	33%	47%	30%	31.7%	5.7%	29.5%

Table 8-5: Response of HDC mRNA abundance in corpus to intravenous octreotide infusion.

The mean mRNA HDC abundance was therefore 29% of basal abundance post octreotide infusion (range 5.7 – 47%) in the corpus biopsies.

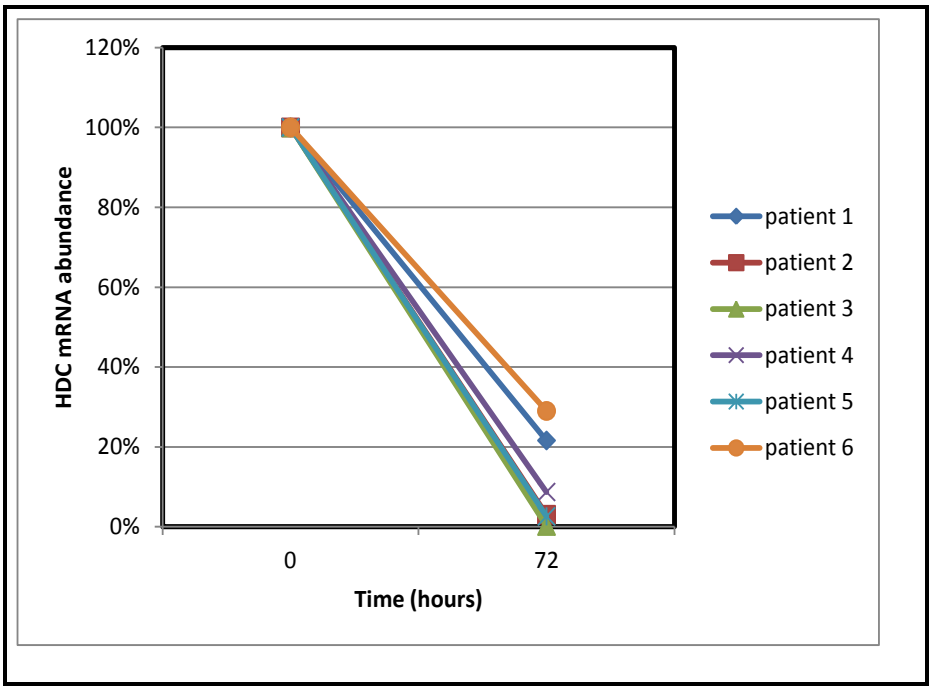


Figure 8-5: HDC mRNA abundance in the polyp/tumour and its response to intravenous octreotide infusion.

Patient	patient 1	patient 2	patient 3	patient 4	patient 5	patient 6
HDC mRNA abundance (% of pre octreotide abundance)	21.6%	3%	0.2%	8.7%	2.6%	29%

Table 8-6: Response of HDC mRNA abundance in polyp/tumour to intravenous octreotide infusion

The mean mRNA HDC abundance was 11% of basal abundance post octreotide infusion (range 2.6 – 29%) in the polyp biopsies.

8.5 Follow up

5 patients underwent a subsequent antrectomy and further surveillance gastroscopies, with follow up periods ranging from 2 to 10 years. The change in fasting serum gastrin concentration immediately post antrectomy is shown in figure 8-6. One patient (number 6) chose to have endoscopic surveillance in preference to antrectomy especially as no actual carcinoid histology was found and all polyps in this patient measured <10mm. At follow up gastroscopy, the number and size of carcinoid polyps and degree of ECl-cell hyperplasia were assessed. Serum was also analysed where possible for fasting gastrin concentration. The individual patient follow up is summarised in the following tables (8-7 to 8-12).

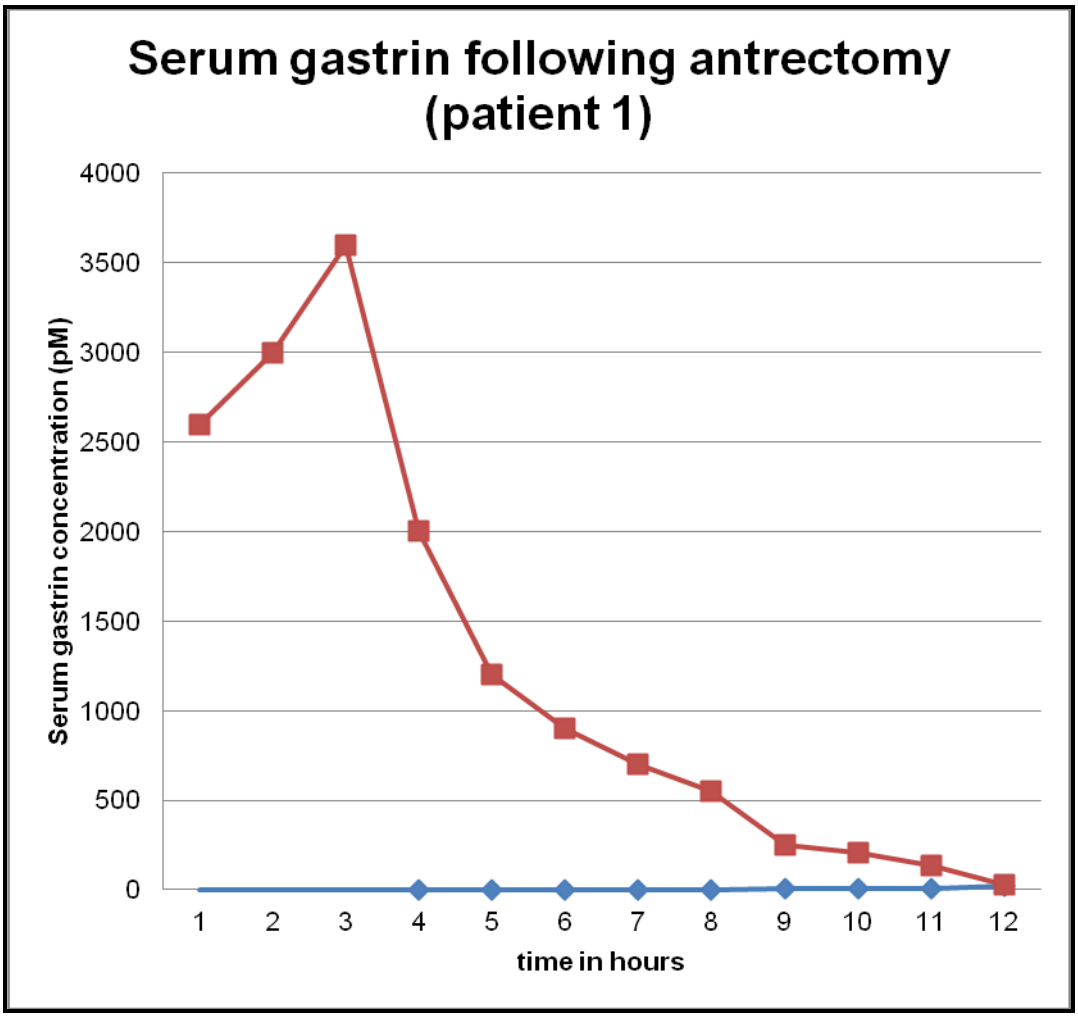


Figure 8-6: Graph demonstrating the change in fasting serum gastrin immediately following antrectomy in patient 1.

	Antrectomy- Date, Type of surgery (open/ laparoscopic)	Post antrectomy OGD	Findings at endoscopy		Corpus Histology			Polyp histology	Fasting serum gastrin concentration post antrectomy (pmol/L)
			total no. of polyps	size of largest polyp (mm)	Chronic atrophic gastritis	Intestinal metaplasia	ECl-cell hyperplasia L=Linear N= Nodular M=Microcarcinoid		
Patient 1	Open 2001	2004	2	3	+	+	M	M	112
		2005	2	3	+	+	M	M	42
		2007	2	3	+	+	N	L and M	104
		2012	0	0	+	-	N	L and M	23

Table 8-7: Patient 1- follow up gastroscopy, corpus and polyp histology and fasting serum gastrin concentration.

	Antrectomy- Date, Type of surgery (open/ laparoscopic)	Post antrectomy OGD	Findings at endoscopy (total no. of polyps, size of largest polyp in mm)	Corpus histology			Polyp histology	Fasting serum gastrin concentration post antrectomy (pmol/L)
				Chronic atrophic gastritis	Intestinal metaplasia	ECl-cell hyperplasia L=Linear N= Nodular M=Microcarcinoid		
Patient 2	2006 Laparoscopic	2006	No polyps	+	+	L	None	<10
		2007	No polyps	+	+	L	None	<5
		2008	No polyps	+	+	L	None	ND
		2010	No polyps	+	+	None	None	ND

Table 8-8: Patient 2- follow up gastroscopy, corpus and polyp histology and fasting serum gastrin concentration

	Antrectomy- Date, Type of surgery (open/ laparoscopic)	Post antrectomy OGD	Findings at endoscopy (total no. of polyps, size of largest polyp in mm)	Corpus histology			Polyp histology	Fasting serum gastrin concentration post antrectomy (pmol/L)
				Chronic atrophic gastritis	Intestinal metaplasia	ECl-cell hyperplasia L=Linear N= Nodular M=Microcarcinoid		
Patient 3	2008, Laparoscopic	2009	No polyps	+	+	L	None	7
		2010	No polyps	+	+	L+M	None	< 4

Table 8-9: Patient 3- follow up gastroscopy, corpus and polyp histology and fasting serum gastrin concentration.

	Antrectomy- Date, Type of surgery (open/ laparoscopic)	Post antrectomy OGD	Findings at endoscopy		Corpus Histology			Polyp histology	Fasting serum gastrin concentration post antrectomy (pmol/L)
			total no. of polyps	size of largest polyp (mm)	Chronic atrophic gastritis	Intestinal metaplasia	ECl-cell hyperplasia L=Linear N= Nodular M=Microcarcinoid		
Patient 4	2009, Laparoscopic	2010	3	10	+	+	L+M	Carcinoid	40.6
		2010	3	7	+	+	M	Carcinoid	ND
		2011	1	2	+	+	N	Carcinoid	23
		2012	1	2	+	-	N	L and M	35

Table 8-10: Patient 4- follow up gastroscopy, corpus and polyp histology and fasting serum gastrin concentration.

	Antrectomy- Date, Type of surgery (open/ laparoscopic)	Post antrectomy OGD	Findings at endoscopy		Corpus Histology			Polyp histology	Fasting serum gastrin concentration post antrectomy (pmol/L)
			total no. of polyps	size of largest polyp (mm)	Chronic atrophic gastritis	Intestinal metaplasia	ECl-cell hyperplasia L=Linear N= Nodular M=Microcarcinoid		
Patient 5	2009, Laparoscopic	2010	1	6	+	+	L+M	Carcinoid	17.6
		2011	1	6	+	+	L+M	Carcinoid	ND

Table 8-11: Patient 5- follow up gastroscopy, corpus and polyp histology and fasting serum gastrin concentration.

Following antrectomy in patient 5, although fasting serum gastrin concentrations were <40pM, the type-1 gastric NET did not appear to regress at follow up gastroscopy and biopsies confirmed persisting type-1 gastric NET. Endoscopic ultrasound did not show any evidence of local invasion and the patient was deemed suitable for an endoscopic mucosal resection of the polyp. However subsequent endoscopic mucosal resection of the carcinoid polyp was complicated by haemorrhage during the procedure requiring endoscopic haemostasis. This patient has now undergone a subtotal gastrectomy. Macroscopically the gastric NET was 15mmx10mm and reached the subserosa (but did not breach the serosal surface). Microscopically it was in keeping with a well differentiated gastric NET (grade 1) with a Ki 67 index of 3%.

In patient 4, all the type-1 gastric NETs that were visualised pre-antrectomy had regressed completely post-antrectomy. However this patient's latest surveillance endoscopy (three years since the antrectomy) demonstrated a small persistent 2mm polyp, but this was at a different site from the largest polyp pre-antrectomy and biopsies of this persisting poly showed no microscopic evidence of type-1 gastric NET.

Table 8-12 summarises the response of the number and size of gastric polyps, histology of the type-1 gastric NETs and fasting serum gastrin concentrations following antrectomy.

Patient No.	Age	Sex	Pre-antrectomy						Follow up post antrectomy (years)	No. of post antrectomy gastroscopies	Post antrectomy – latest surveillance gastroscopy findings			Latest fasting serum gastrin concentration (pM)
			No. of polyps	Maximum polyp size (mm)	Serum gastrin concentration (pM)	% reduction of serum gastrin (OST)	HDC mRNA abundance (% of pre octreotide abundance) (OST)	HDC mRNA abundance (% of pre octreotide abundance) (OST)			No. of polyps	Maximum polyp size (mm)	Carcinoid histology	
1	67	F	20	7	2800	95	21.6	33	6	3	2	3	Micronodular	104
2	43	M	4	10	680	96	3	47	4	4	0	0	None	<5
3	65	F	18	10	190	75	0.2	30	2	2	0	0	None	7
4	70	F	5	15	838	67	8.7	31.7	2	3	1	2	Carcinoid	23
5	75	M	4	16	2117	86	2.6	5.7	2	2	1	6	Carcinoid	18

Table 8-12: Summary of response of number and size of polyps, histology of gastric type-1 NETs and fasting serum gastrin concentration following antrectomy.

8.6 Discussion

Management of type-1 gastric NETs is currently largely determined by the size of the tumour (Plockinger et al., 2004; Ramage et al., 2005b). Small tumours (<1cm) are managed by endoscopic surveillance for tumour growth or endoscopic polypectomy. Large tumours (≥1cm) present a dilemma due to the uncertain nature of such tumours and the associated malignant and metastatic potential. Different treatment modalities have been advocated for the treatment of type-1 gastric NETs ≥1 cm in size and these include endoscopic polypectomy, antrectomy (Borch et al., 2005; Modlin et al., 2003b), total gastrectomy and long acting somatostatin analogues (Campana et al., 2008; Fykse et al., 2004; Grozinsky-Glasberg et al., 2008b).

Endoscopic polypectomy has been advocated for small tumours (<1cm) and for gastric NETs >1cm but with low tumour burden (less than 5 tumours in total) and in the absence of invasion of muscularis propria (Plockinger et al., 2004). The effectiveness of this modality of treatment has been demonstrated in several case series. Endoscopic treatment of gastric NETs is different to the conventional polypectomy techniques used for the removal of polyps elsewhere in the gastrointestinal tract due to the submucosal location of gastric NETs. In an initial study by Ichikawa et al., 5 patients with type-1 gastric carcinoid NETs showed no evidence of recurrence at follow up (ranging from 6 to 66 months) following endoscopic mucosal resection of these tumours (Ichikawa et al., 2003). In a subsequent study involving 8 patients with gastric NETs (7 with type-1

gastric NETs and 1 with type-3 gastric NET) endoscopic removal of the gastric polyps was achieved by endoscopic resection using a multi band mucosectomy device (Hopper et al., 2009). Although long term follow up of these patients has not been reported, endoscopic resection was reported in this study to be safe with complete removal of the gastric NETs. In a Japanese study, 8 patients with small type-1 gastric NETs underwent endoscopic resection and no recurrence was observed at follow up gastroscopy (follow up period of 30 months) (Higashino et al., 2004). Endoscopic tumour resection may however not be feasible with a high tumour burden or very large gastric NETs.

Therefore in patients with multiple large (>1cm) type-1 gastric NETs antrectomy has been shown to be an effective treatment modality. First described in a single patient in 1987 (Richards et al., 1987) and subsequently in two patients in 1988 (Eckhauser et al., 1988), it has since become the surgical treatment of choice in those patients with type-1 gastric NETs who have not responded to endoscopic therapy or in whom the tumours that have demonstrated an increase in size at endoscopic surveillance (Borch et al., 2005; Dakin et al., 2006). Hirschowitz et. al. demonstrated in a study of three patients with type-1 gastric NETs the effect of antrectomy on serum gastrin concentrations. Following antrectomy, all patients were observed to have normal serum gastrin concentrations and at follow up (30 months) neither ECL-cell hyperplasia nor type-1 gastric NETs were detected (Hirschowitz et al., 1992). In a study of 18 patients with type-1 gastric NETs, Dakin et al. have demonstrated that following antrectomy (10 patients), mean serum gastrin

concentrations decreased by 94% compared to 37.2% in medically treated patients (8 patients) (Dakin et al., 2006). However the effect of the different treatment strategies (antrectomy or medical) on existing type-1 gastric NETs (size, number) or on the development of new type-1 gastric NETs and progression of ECL-cell hyperplasia has not been studied. In a study by Borch et al., following antrectomy in 10 patients with type-1 gastric NETs, fasting serum gastrin concentrations normalised and 9 patients were tumour free after 65 months of follow up (Borch et al., 2005). Tumour progression was however observed in one patient. In studies by Hoshino et al (2 patients with type-1 gastric NETs) and Ozao-Choy (8 patients with type-1 gastric NETs), although antrectomy (laparoscopic) resulted in normalisation of fasting serum gastrin concentrations, it was not associated with regression of type-1 gastric NETs in all patients (Hoshino et al., 2010; Ozao-Choy et al., 2010).

Hence it would appear that not all patients with type-1 gastric NETs respond to antrectomy. Antrectomy, by removing the source of gastrin, is likely to be effective only in those patients with ‘gastrin responsive’ type-1 gastric NETs. No surrogate markers are currently available that would determine this gastrin responsiveness or indeed that would enable us to monitor such tumours and their growth.

A long acting somatostatin analogue (Octreotide) has also been used in the medical management of type-1 gastric NETs. The role of somatostatin in the regulation of acid secretion and gastrin synthesis has been described in section 1.2.3.2.2. In a study by Jianu et.al., 5 patients with type-1 gastric NET were initially treated with a long acting

somatostatin analogue (Sandostatin LAR, 20mg, administered intramuscularly monthly for a year) (Jianu et al., 2011). At the end of the first year of treatment, a reduction in serum gastrin (although fasting serum gastrin concentrations were still in the hypergastrinaemic range) and chromogranin A concentrations and a 50% reduction in the size of endoscopically visible nodules was observed. However at year 5 of follow up (following cessation after one year's treatment with long acting octreotide), there was an increase in the size and number of endoscopically visible polyps. There was no change in the serum gastrin concentrations at year 5 of follow up compared to the concentrations measured at the end of the first year of treatment. A similar trend was observed with serum chromogranin A concentrations. Based on the outcome of this study, it was therefore recommended that long acting somatostatin analogues once commenced should be continued long term in patients with type-1 gastric NETs (Jianu et al., 2011).

In our study, we have demonstrated that intravenous octreotide resulted not only in a decrease in serum gastrin concentrations but also a decrease in the abundance of HDC mRNA in corpus and polyp biopsies from all patients. This response, observed with the infusion of octreotide, was initially interpreted as suggesting that antrectomy, by eliminating the source of gastrin, should stop tumour growth and also possibly induce tumour regression. However not all patients studied were observed to have such an outcome. The time taken for regression of polyps varied and one patient has currently undergone surgery for removal of a type-1 gastric NET which has not regressed 2 years following antrectomy. This therefore

would suggest that following antrectomy, patients with type-1 gastric NETs should continue to undergo subsequent regular endoscopic follow up to assess tumour response. If tumours do not regress despite normalisation of serum gastrin concentrations, patients should be considered for evaluation by endoscopic ultrasound (EUS) for suitability for endoscopic resection of any persistent large type-1 gastric NETs (≥1 cms). EUS has been demonstrated in several case series to be highly accurate in assessing type 1 gastric NETs including tumour invasion of the muscularis propria layer of the gastric wall which would preclude endoscopic tumour resection (Varas et al., 2010; Yoshikane et al., 1998).

It is possible that octreotide, as used in our study, may have influenced ECL-cells by mechanisms independent of gastrin. It has been well established that octreotide itself has an inhibitory effect on ECL-cell function (Grozinsky-Glasberg et al., 2008a). This effect, coupled with a reduction in serum gastrin concentration may well account for the suppression of ECL-cell function observed in our study. It is also plausible that some type-1 gastric NETs may have progressed to grow autonomously in those patients who did not respond to antrectomy. If this was indeed the case then such autonomous tumours would not respond appropriately (by showing a decrease in serum gastrin and a decrease in ECL-cell function) to the octreotide infusion thereby suggesting that such tumours were still under the influence of gastrin. Response to a gastrin/CCK-2 receptor antagonist, by eliminating other nonspecific effects of octreotide may therefore be a more robust method to confirm tumour sensitivity to gastrin. We would therefore recommend further studies using

gastrin receptor antagonists to ascertain whether this would be a more reliable and consistent method to determine those patients with type-1 gastrin NETs who may respond to an antrectomy. Demonstration of CCK-2 receptor status/concentration on type-1 gastrin NETs by immunohistochemistry may also help identify those type-1 gastric NETs exhibiting autonomous growth.

In our study, the time taken for regression of type-1 gastric NETs in those patients who responded to antrectomy was variable with intervals ranging from 2 years to up to 5 years post antrectomy. However in all these patients microscopic evidence of gastric ECL-cell hyperplasia continued to persist post antrectomy.

There may also have been the possible effect of the gastrin autocrine/paracrine pathway. It has been demonstrated that once malignant transformation of ECL-like cells occurs, activation of gastrin gene results in unregulated tumour production of precursor gastrins (progastrin and Gly-gastrin) and amidated gastrin (Smith et al., 1998). The proliferative effects of these peptides have been demonstrated to be mediated via as yet unkown receptors (unopposed by exogenously administered gastrin). Earlier studies in colonic tumour cells have also demonstrated the possible role played by the gastrin autocrine pathway in tumour growth and proliferation (Hoosein et al., 1988; Hoosein et al., 1990)

8.7 Conclusion

We conclude from this study that although the octreotide suppression test may help to identify type-1 gastric NETs that are gastrin responsive, it does not reliably identify those patients with type-1 gastric NETs who would benefit from an antrectomy. Further studies analysing the role of gastrin/CCK-2 receptor antagonists in predicting those patients with type-1 gastric NETs who would respond to an antrectomy are therefore warranted.

General Discussion

9 General Discussion

The human stomach acts not only as a reservoir for ingested food but also plays an important role in the digestion of food through the regulated synthesis and secretion of gastric acid and several gastrointestinal hormones. These hormones also regulate the growth of gastric epithelial and enterochromaffin like cells (ECL-cells). Gastrin is one such important gastrointestinal hormone which plays a major role in the regulation of not only gastric acid secretion, but also through its effects on cell proliferation, apoptosis and angiogenesis, it has an important role in the regulation of gastric epithelial and ECL-cell growth.

Several factors have previously been demonstrated to regulate fasting serum gastrin concentrations (chapter 1, section 1.3.2.4 and table 1:2) and hence interpretation of fasting serum gastrin concentrations is complicated in the presence of such factors. It is however not entirely known how these multiple factors interact to influence fasting serum gastrin concentrations. We have therefore investigated how multiple host, bacterial and environmental factors interact and influence fasting serum gastrin concentrations in a large cohort of adult patients (chapter 6). The bacterial factors analysed include the effects of *H. pylori* infection and infection with the cagA variant on fasting serum gastrin concentrations. Effects of host factors including age, gender, body mass index, positive family history of gastric cancer and the presence of gastric preneoplastic pathology on fasting serum gastrin concentrations were also analysed.

Finally the effects of environmental factors such as smoking, alcohol consumption and concurrent drug therapy with proton pump inhibitors on fasting serum gastrin concentrations were analysed in this cohort of adult patients.

Our analyses confirmed that *H. pylori* infection, concurrent proton pump inhibitor therapy and the presence of gastric preneoplastic pathology were all individually associated with significantly elevated fasting serum gastrin concentrations as demonstrated in previous studies (Orlando et al., 2007; Lamberts et al., 2001; Annibale et al., 2001b). Current *H. pylori* infection and concurrent therapy with proton pump inhibitor drugs were significantly associated with more pronounced elevations in fasting serum gastrin concentrations. This has also been demonstrated in previous studies (El-Nujumi et al., 1998). However, in our study, in those patients with past evidence of *H. pylori* infection only, concurrent acid suppression therapy was associated with significantly elevated fasting serum gastrin concentrations only in the absence of gastric preneoplastic pathology, suggesting that this may predominantly be a proton pump inhibitor driven hypergastrinaemia.

In our analysis, patients with advancing age without *H. pylori* infection or associated gastric preneoplastic pathology and not on a concurrent proton pump inhibitor therapy did not demonstrate significantly elevated fasting serum gastrin concentrations contrary to previous studies (Jassel et al., 1999). This therefore suggests that the previously observed association between advancing age and elevated fasting serum gastrin concentrations may be explained due to the presence of other confounding

factors such as concurrent *H. pylori* infection, presence of gastric preneoplastic pathology or therapy with acid suppressing drugs.

In addition to the observation of significantly elevated fasting serum gastrin concentrations in patients with a body mass index of over 40, in keeping with previous studies (Lindstedt et al., 1985a), we also observed that patients with a body mass index of less than 18.5 also had significantly elevated fasting serum gastrin concentrations.

Cigarette smoking was associated with significantly elevated fasting serum gastrin concentrations, but alcohol consumption was noted to be associated with significantly lower fasting serum gastrin concentrations.

These results therefore confirm our hypothesis that several host, bacterial and environmental factors interact to influence fasting serum gastrin concentrations. However these interactions remain complex and should be taken into consideration when interpreting individual patients' fasting serum gastrin concentrations.

Similarly several host, bacterial and environmental factors have also been described to influence the development and progression of gastric preneoplastic pathology. *H. pylori* infection has been demonstrated in several previous studies as a gastric carcinogen and infection with the cagA positive variant has been shown to be associated with increased pathogenicity of the infecting *H. pylori* bacteria (Nomura et al., 2002). Increasing age, male sex and cigarette smoking have also been observed to be associated with an increased risk of developing gastric adenocarcinoma (Blaser et al., 1995; Steevens et al., 2010). However it

remains unclear how these factors interact and influence the development and progression of gastric preneoplastic pathology. This was analysed in a large cohort of adult patients and the results from this analysis, detailed in chapter 5 of this thesis, confirm several of the earlier observations such as the significant association between advancing age and increased risk of gastric preneoplastic pathology. However host factors such as male gender and elevated body mass index were not found to be significantly associated with an increased risk of gastric preneoplastic pathology in this cohort. Similarly cigarette smoking was not found to be significantly associated with the presence of gastric preneoplastic pathology contrary to previous studies. However alcohol consumption was observed to have a small protective effect on univariate analysis alone in this study.

In chapter 5 we also analysed the possible role played by acid suppressing drug therapy (proton pump inhibitors), in the development of gastric preneoplastic pathology. No significant association with the presence of gastric preneoplastic pathology was observed, suggesting that although the long term use of acid suppressing medications is associated with hypergastrinaemia, it may not significantly influence the development or progression of gastric preneoplastic pathology.

We also assessed the efficacy of the rapid urease test (PRONTO®) in the detection of *H. pylori* infection and analysed those factors that may influence the outcome of this test (chapter 4). The sensitivity and specificity of the rapid urease test in diagnosing *H. pylori* infection has been quoted as between 85-95% and 95-100% respectively (Tseng et al., 2005). Concomitant therapy with proton pump inhibitors has previously

been reported to be associated with false negative rapid urease test results. However it is not known to what extent other factors such as the presence of gastric preneoplastic pathology and infection with cagA positive strains of *H. pylori* would influence the outcome of the rapid urease test. We have demonstrated the efficacy of the rapid urease test (sensitivity of 70% and specificity of 98.8%) compared to histology. Concomitant therapy with proton pump inhibitors, a predominantly corpus location of infection with *H. pylori* and infection with less virulent strains of *H. pylori* (cagA negative) were all observed to be associated with a more likely false negative rapid urease test result. Hence the rapid urease test should be used with caution as the sole method for diagnosing *H. pylori* infection, especially in those areas such as the UK which have a low or decreasing prevalance of infection with this organism.

There are several strengths to this study. Patients were recruited as part of the National Institute of Health Research (NIHR) funded study 'Identification of factors that influence development of gastric neoplastic pathology in adults' conducted by the Biomedical Research Centre in Microbial diseases at Royal Liverpool and Broadgreen University Hospitals NHS Trust, Liverpool, UK. All patients were prospectively recruited and were representative of the same population that hospital clinicians encounter in everyday clinical practice. This study recruited a predominantly ethinically homogenous population (Caucasian) compared to other studies. In addition to endoscopic and histological data, detailed demographic and clinical data were obtained in all patients. Fasting serum gastrin concentration was analysed by radioimmunoassay. The antibody

(L2 antibody) utilised in this technique of radioimmunoassay was directed against the α-amidated C-terminal portion of gastrin molecules, therefore binding all bioactive gastrins irrespective of N-terminal peptide length and degree of sulfation as explained in chapter 2. This therefore measures accurately the fasting serum gastrin concentrations. Very high fasting serum gastrin concentrations were subsequently analysed by serial dilutions. Quality controls were also used in each assay to detect inter assay variations. Hence this technique of measuring fasting serum gastrin concentration by radioimmunoassay remains both accurate and reliably reproducible.

Detection of the *cag*A status of the infecting *H. pylori* was performed using the ELISA technique. Although this was a commercially produced ELISA kit, it was demonstrated in this study to be accurate with low interassay variability (<10%). Furthermore, stringent criteria were established and adhered to for establishing the diagnosis of current and past infection with *H. pylori*. All histological specimens, after an initial analysis by a Consultant Gastrointestinal Histopathologist at the Royal Liverpool University Hospital as part of the patients' routine clinical management, were further analysed according to an established protocol by a specialist Gastrointestinal Histopathologist who remained blinded to endoscopic and clinical data. All histological samples were reported using a standardised histology report. This report was based on internationally accepted descriptions and classifications including the modified Sydney classification system for *H. Pylori*-associated inflammation, Padova classification and modified Vienna classification for dysplasia.

One of the weaknesses of the study is that there were incomplete data sets in some patients. However, those patients with incomplete data sets were subsequently excluded from the final analyses. Also, in hindsight, we realise that further in-depth data regarding alcohol consumption and cigarette smoking may have provided additional information regarding the role played by environmental factors in the development of gastric preneoplastic pathology. Similarly further information regarding the presence of a family history of gastric and duodenal ulcer disease (in addition to gastric adenocarcinoma) may also have provided further information regarding the role of familial risk of gastric preneoplastic pathology.

Studies in the past have mostly addressed the role played by individual factors (bacterial, host or environmental) in influencing the development of gastric preneoplastic pathology. This current study has not only addressed the role played by individual factors, but also the interaction between these factors in the development of gastric preneoplastic pathology. Similarly previous studies have focussed predominantly on individual factors which influence fasting serum gastrin concentrations. In our current study we have addressed the role played by individual factors and also the interaction between host, environmental and bacterial factors in influencing fasting serum gastrin concentration.

Overall our current study has helped address some of the important issues faced by clinicians in their everyday practice. The importance of a multi test strategy in establishing the diagnosis of *H. pylori* infection in low prevalance areas has been demonstrated in chapter 4. Factors that

influence the development of gastric preneoplastic pathology have been studied in detail in chapter 5 and this in conjunction with tumour marker studies will hopefully guide clinicians in developing risk prediction scores for gastric neoplasia development in susceptible patients. This would therefore enable risk stratification and development of appropriate surveillance strategies.

A further 400 patients have been subsequently recruited to the NIHR study and an additional analysis will take place at the end of this recruitment. As part of this NIHR study, potential tumour markers to identify risk of developing gastric neoplastic pathology are also being assessed in this cohort of patients. Hopefully this, as well as further analyses on the entire data set of 1400 patients, may hopefully provide further important information regarding development of gastric preneoplastic and neoplastic pathology. Linkage with the regional cancer registry will enable identification of those patients from this cohort who develop gastric adenocarcinoma in the future. This would potentially also enable risk stratification strategies along with the tumour marker studies.

Chronic hypergastrinaemia secondary to hypochlorhydria resulting from long standing autoimmune gastritis can lead to the development of ECL-cell hyperplasia. In some patients with long standing autoimmune gastritis this ECL-cell hyperplasia can progress to type-1 gastric neuroendocrine tumour formation. However factors which may influence this progression have not been entirely characterised. We hypothesised that factors such as the extent of gastric atrophy, presence of anti-gastric parietal cell antibodies and coexisting autoimmune conditions, such as

hypothyroidism, might influence this progression to type-1 gastric neuroendocrine tumours. In a prospective study of 49 patients with histologically confirmed atrophic body gastritis and ECL-cell abnormalities, we analysed the role of these factors in the development of type-1 gastric neuroendocrine tumours (chapter 7). We observed that the duration of hypergastrinaemia was definitely associated with significant ECL-cell abnormalities. However although the extent of gastric atrophy (as determined by the presence of vitamin B12 and iron deficiency anaemia), presence of anti-gastric parietal cell antibodies, and the presence of hypothyroidism, were more common in patients with the more advanced degrees of ECL-hyperplasia, this association did not reach statistical significance.

A similar association between these factors and advanced degrees of ECL-cell hyperplasia was observed in a recent study (Vannella et al., 2011). Several other factors including host factors (genetic and as yet unidentified growth factors), bacterial factors (non-*Helicobacter* species) and environmental factors (smoking, alcohol consumption and diet) may therefore play an important role in the progression of atrophic gastritis to type-1 gastric neuroendocrine tumours in some susceptible patients. It is possible that the small sample size of our study may have been a shortcoming and further studies with larger sample sizes may help to understand further the development of type-1 gastric neuroendocrine tumours. Also a longer duration of follow up of our patients may help provide further information regarding progression of ECL-cell hyperplasia and development of type-1 gastric neuroendocrine tumours.

The current consensus guidelines on the management of type-1 gastric neuroendocrine tumours are based on the size of these tumours (Plockinger et al., 2004). Small (<1cm) type-1 gastric neuroendocrine tumours have very low malignant potential and rarely metastasise whilst large (> 2cms) tumours have inherent malignant potential. Hence small, <1 cm, tumours can be managed by endoscopic surveillance alone. However if unsuitable for endoscopic surveillance, then endoscopic resection of such tumours is advocated (Ichikawa et al., 2003). If the type-1 gastric neuroendocrine tumours are large (>1-2cms) then gastrectomy, wedge resection or antrectomy and removal of the source of hypergastrinaemia have all been advocated as appropriate treatment strategies (Dakin et al., 2006). However not all patients with type-1 gastric neuroendocrine tumours who undergo antrectomy demonstrate subsequent complete resolution of type-1 gastric neuroendocrine tumours. Therefore should antrectomy be considered only in those patients with gastrin responsive type-1 gastric neuroendocrine tumours? The role of the octreotide suppression test in determining gastrin responsiveness of type-1 gastric neuroendocrine tumours and therefore response to antrectomy has been previously described in a single patient (Higham et al., 1999).

We assessed the role of the octreotide suppression test in determining gastrin responsiveness of type-1 gastric neuroendocrine tumours in 6 patients and subsequent response to antrectomy in 5 patients with type-1 gastric neuroendocrine tumours. All patients had at least one >1cm type-1 gastric neuroendocrine tumour and a minimum of at least 5 tumours overall. All 6 patients responded to a 72 hour infusion of

octreotide (‘octreotide suppression test’) with an appropriate decrease in serum gastrin concentration and also a corresponding decrease in ECL-cell function measured by a reduction in the abundance of mRNA for the enzyme histidine decarboxylase (HDC) in both the corpus and tumour biopsies. This response to octreotide therefore would suggest that these patients with gastrin responsive type-1 gastric neuroendocrine tumours would respond to antrectomy with regression of tumours. However not all of the 5 patients who subsequently underwent antrectomy (one patient elected to have endoscopic surveillance) were observed to have such a response to antrectomy. The time taken for regression of the type-1 gastric neuroendocrine tumours following antrectomy varied (between 2-5 years) except in the one patient in whom the tumour persisted. This patient has subsequently undergone a subtotal gastrectomy. In all 5 patients, fasting serum gastrin concentrations were within normal limits (<40 pM) following antrectomy. In some patients, ECL-cell hyperplasia persisted although no new type-1 gastric neuroendocrine tumours were observed at subsequent follow up surveillance endoscopies. From this study it would therefore appear that although the octreotide suppression test may be useful in determining the gastrin responsiveness of type-1 gastric neuroendocrine tumours, it does not appear to reliably identify all those patients with type-1 gastric neuroendocrine tumours who may benefit from an antrectomy. Octreotide may have had an effect on ECL-cell function through mechanisms independent of gastrin. Hence antagonists of the gastrin CCK-2 receptor may therefore eliminate such nonspecific effects of octreotide. Future studies assessing the role of gastrin/CCK-2 receptor

antagonists in determining gastrin responsiveness of type-1 gastric neuroendocrine tumours and to subsequent antrectomy are therefore advocated.

In conclusion, gastrin plays a major role not only in the regulation of gastric acid secretion, but also in the regulation of gastric epithelial and ECL-cell function. Several bacterial, host and environmental factors interact to influence fasting serum gastrin concentrations and these factors have to be taken into account when interpreting individual patient's fasting serum gastrin concentration. Similarly several host, bacterial and environmental factors influence the progression and development of gastric preneoplastic pathology. Rapid urease testing may not be the most reliable diagnostic test for infection with *H. pylori* in regions of low prevalance of infection with this organism due to the effects of several bacterial and environmental factors including concurrent acid suppression therapy. It would also appear that several factors may influence the progression of ECL-cell hyperplasia to type-1 gastric neuroendocrine tumours and further studies with sufficiently larger sample size may therefore be required. Determining gastrin responsiveness of type-1 gastric neuroendocrine tumours and therefore responsiveness to antrectomy remains elusive. Further studies using gastrin/CCK-2 receptor antagonists to assess the gastrin responsiveness of type-1 gastric neuroendocrine tumours are needed.

Appendix

Appendix 1

Clinical data sheet – page 1

Factors which affect the outcome of *Helicobacter pylori* infection in the stomach- CLINICAL DATA

Please affix patient label and BRC data no.

Demographics:

Age: Sex: M □ F□

Ethnicity:

Height (cms): Weight (Kg): BMI/comment:

Symptoms for which endoscopy performed:

Reflux/Heartburn:	Y□	N□
Indigestion:	Y□	N□
Abdominal Pain:	Y□	N□
Vomiting:	Y□	N□
Weight loss:	Y□	N□
Anaemia:	Y□	N□
Dysphagia:	Y□	N□
Haemetemesis/Malaena:	Y□	N□

Type of Referral:

2 week rule:	Y□	N□
Open Access Endoscopy:	Y□	N□
Hospital referral:	Y□	N□

Past Medical History:

Previous Gastric surgery: Y□ N□

If Y when:

Indication:

Type of surgery:

Diabetes: Y□ N□ **if Y:** Type I Y□ N□

Or Type II Y□ N□

Ischemic Heart Disease: Y□ N□

Cerebrovascular Disease: Y□ N□

Arthritis: Y□ N□

Respiratory Disease: Y□ N□…….

Others ……

Family History of Gastric disease: Y□ N□

Specify if Y:

Smoking history:

Smoker: Y□ N□ If Y: CPD: Years:

Non-smoker: Y□ N□

Exsmoker: Y□ N□ when stopped: Cpd: Years:

Alcohol: Y□ N□. If Y: ……units per week

Current drug use:

	PPI	H2RA	Aspirin	Clopidogrel	NSAID
Name					
Dose					
Last taken					

Endoscopic findings:

H pylori status:

a) H pylori test result:

	Positive	Negative	Indeterminate	Not Done
Rapid urease test (PRONTO/ CLO):				
Histology:				
Serology:				
^{13}C Urea breath test:				

b) Previous eradication therapy: Y□ N□ Unknown□

If Y:

i. Eradication therapy used:

	Name	Dose	Schedule	Duration
1				
2				
3				

ii. When/How long ago:

iii. Previous testing method:

Serology: Y□ N□
^{13}C Urea breath test: Y□ N□
RUT: Y□ N□
Histology: Y□ N□
Not Known: Y□ N□

Other relevant information:

Appendix 2

Gastric Biopsy Report

Gastric Biopsy report page-1

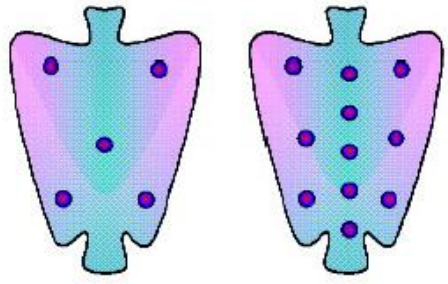
University of Szeged
Department of Pathology
Pathologiai Intézet
H-6720 **Szeged**, Állomás u. 2. (6701 Szeged Pf. 427.)
Hungary
Tel: 62-545 878 Fax: 62-545 868

Histology study code: ……

Gastric biopsy
Standard histopathological report

Patient identification
Name (given name / family name):……..Birth:……
Gender (M / F):….
Endoscopy pocedure:……..…….……
Clinical diagnosis:…….. ……
Serum sample available (Y / N)

Clinical data:……..
Basic sampling: (representative / not representative**)**



Updated sydney system | Italian group on gastric non-invasive neoplasia

Special stains: H&E, PAS-AK, Giemsa, Congo, other.:…….
Immunohistochemistry:……..

HISTOPATHOLOGY

Type of gastritis: A / B / C / other / other alterations:……
……

Helicobacter pylori-associated inflammation
(Modified Sydney classification: Dixon, DF. Am.J.Surg.Pathol.1996)

extension, localisation (antrum / body; superficial / transmucosal)

lymphocytes, monocytes :	0	1	2	3	4	5	6
activity (granulocytes)		0	1	2	3	4	5
6							
mucosal atrophy (body)		0	1	2	3	4	5
6							
Helicobacter pylori colonisation	0	1	2	3	4	5	6
Foveolar epithel damage	0	1	2	3	4	5	6
Intestinal metaplasie (type, extension)	0	1	2	3	4	5	6

Sydney score:
……/ 36

Others: MALT acquisition / MALT lymphoma / other lymphoma
Pancreas acinaris metaplasia (PAM)
Neuroendocrine hyperplasia
Foveolar hyperplasia
Chief cell / parietal cell hyperplasie / dilated glands
Blood capillar dilatation / lymphangiectasie
IEL / granuloma / eosinophilia / mastocytosis
Erosion / ulcer
Other microbas (f.e. *H. heilmanni*):……..
Other/s:……..

Gastric Biopsy Report

Gastric biopsy report page-2

Padova classification (1998)

1. Negative for dysplasia
- 1.0. normal
- 1.1 reactive foveolar hyperplasia
- 1.2. intestinale metaplasia
  - 1.2.1. IM complet (I.type)
  - 1.2.2. IM incomplet (II. and III. type)

2. Indefinitive for dysplasia
- 2.1. foveolar hyperproliferation
- 2.2. hyperproliferative IM

3. Non-invasive neoplasia („flat" or „elevated", synonyma.: adenoma)
- 3.1. LG
- 3.2. HG
  - 3.2.1. carcinoma suspicion without invasion
  - 3.2.2. carcinoma without invasion

4. Invasíve carcinoma suspicion

5. Invasíve adenocarcinoma

Modified Vienna classification (2000, 2002)

1. category: **0 (negativ for neoplasia / dysplasia)**

2. category: ***ANDD (indefinitive for neoplasia / dysplasia)***

3. category: ***LGD/LGA***

4. category: „intramucosal borderline neoplasia „
- ***4.1. HGD/HGA***
- ***4.2. intramucosalis carcinoma (pTis), well differentiated***

5. definite carcinoma
- ***5.1. Intramucosal carcinoma, moderately or poorly differenciated***
- ***5.2. Submucosal carcinoma or beyond***

Comment:……
……

Histological diagnosis:
……..

Szeged, ……..

1. Senior pathologist
Dr. Laszlo Tiszlavicz PhD
tiszlats@yahoo.com

2. Junior pathologist

Appendix 3

Published abstracts/papers

Published Papers

1. Nørsett KG, Steele I, Duval C, Sammut SJ, Murugesan SV, Kenny S, Rainbow L, Dimaline R, Dockray GJ, Pritchard DM, Varro A. 'Gastrin stimulates expression of plasminogen activator inhibitor (PAI) -1 in gastric epithelial cells.' American journal of Physiology. Gastroenterology and Liver Physiology. 2011 Sep; 301(3):G446-53.

2. Murugesan SV, Varro A, Pritchard DM. 'Strategies to determine whether hypergastrinemia is due to Zollinger Ellison Syndrome rather than a more common benign cause.' Aliment Pharmacol Ther. 2009; May 15; 2 9(10):1055-68.

Published abstracts

1. 'Determinants of human fasting serum gastrin concentration– interaction between H. pylori infection, gastric preneoplastic pathology and proton pump inhibitor use.' Poster of Distinction, DDW – 2011. Senthil V. Murugesan, László Tiszlavicz, Islay Steele, Tracey Farragher, Andrew R. Moore, Andrea Varro, David M. Pritchard.

2. 'The effects of host, environmental and bacterial factors on development of gastric preneoplastic pathology.' Poster presentation, DDW – 2011. Senthil V. Murugesan, László Tiszlavicz, Islay Steele, Tracey Farragher, Andrew R. Moore, Andrea Varro, David M. Pritchard.

3. 'Factors that determine the sensitivity of rapid urease tests for the detection of Helicobacter pylori infection.' Poster presentation, DDW – 2011. Senthil V. Murugesan, László Tiszlavicz, Islay Steele, Tracey Farragher, Andrew R. Moore, Andrea Varro, David M. Pritchard.

4. 'Octreotide suppression test predicts suitability for antrectomy in type-1 gastric carcinoid tumours'. Poster presentation, Gastro 2009 (UEGW/WGO). Senthil V. Murugesan, Islay Steele, Andrea Varro, David M. Pritchard.

5. 'Investigation of factors which affect the development of type-1 gastric carcinoid tumours'. Poster presentation, Gastro 2009 (UEGW/WGO). Senthil V. Murugesan, Islay Steele, Andrea Varro, David M. Pritchard.

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