An investigation of novel biomarkers of gastric mucosal preneoplasia

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Abstract

(i) Novel biomarkers of gastric preneoplasia following infection with *Helicobacter pylori*

Despite steady decline in incidence, gastric cancer remains a major global cause of morbidity and mortality and is responsible for over 700,000 deaths worldwide per annum. The commonest form arises following infection with the bacterium *Helicobacter pylori*. For reasons which remain poorly understood, a small proportion of infected individuals develop gastric epithelial remodelling which follows a well-defined sequence of changes culminating in cancer development. Our group and others have described the role of various gastric mucosal proteins in carcinogenesis following *H. pylori* infection. We hypothesised that these proteins might be upregulated in gastric preneoplasia and might in turn be used as biomarkers of the same.

We recruited patients attending hospital for diagnostic upper gastrointestinal endoscopy. Gastric biopsies were taken to determine the presence and extent of preneoplastic lesions by histology; *H. pylori* status; and the abundance of mRNA for putative biomarkers by real-time polymerase chain reaction. Blood was drawn to determine the serum concentrations of *H. pylori* IgG antibodies, fasting gastrin and pepsinogens 1 and 2. Genomic DNA was extracted from blood samples. For each subject, we performed genotyping for nine single nucleotide polymorphisms in the *MMP-7* gene to determine whether these polymorphisms might increase the expression of mucosal MMP-7 or increase the risk of developing gastric preneoplasia.

In our study population, the ratio of pepsinogens 1 and 2 (PG1/2 ratio) performed well as a diagnostic test for gastric mucosal preneoplasia. In combination with fasting serum gastrin concentration and *H. pylori* serology, the diagnostic accuracy was improved suggesting a role for these markers in clinical practice.

We have demonstrated that the gene transcript abundance of one mucosal protein – matrix metalloproteinase-7 (MMP-7) – was significantly increased in the presence of gastric preneoplasia. This effect was greater for disease phenotypes associated with higher cancer risk. These novel findings confirm the increased expression of *MMP-7* in gastric epithilial preneoplasia and offer insight into areas of development for the future including the use of MMP-7 as a noninvasive biomarker of gastric preneoplasia. We also found that gastric preneoplasia was significantly more common in minor allele homozygotes for SNP rs17352054 than for major allele homozygotes and that carriage of the minor allele of SNP rs11225297 is associated with gastric preneoplasia amongst *H. pylori* seropositive individuals. We have also shown a significant influence on mucosal MMP-7 relative mRNA abundance of SNP genotypes for six of the polymorphisms examined.

ii) A pilot study of the novel gastrin antagonist netazepide (YF476) for the treatment of type 1 gastric neuroendocrine tumours

Patients with autoimmune chronic atrophic gastritis develop hypergastrinaemia as a result of gastric hypochlorhydria. Hypergastrinaemia can induce hyperplasia of enterochromaffin-like (ECL) cells in the gastric mucosa. In a small proportion of patients, this progresses to dysplasia and the development of type 1 gastric neuroendocrine tumours (NETs). The majority of these tumours behave indolently but a small proportion exhibit rapid growth and metastasise. The current treatment for larger (>1cm) tumours is surgical antrectomy which obviates the source of hypergastrinaemia and causes tumour regression in many cases. We hypothesised that pharmacological inhibition of the gastrin/CCK2 receptor would have a similar effect.

We conducted a phase 2, open-label pilot study in 8 patients with chronic atrophic gastritis and type 1 gastric NETs. This was conducted in two stages. In the first, subjects received 50mg netazepide daily for 12 weeks. We performed a baseline gastroscopy to measure and count visible NETs and to take gastric mucosal biopsies. We also measured baseline serum chromogranin A (CgA) and fasting plasma gastrin concentrations. We repeated gastroscopy and biopsy after 6 and 12 weeks of treatment, and serum CgA and plasma gastrin every 3 weeks. We measured abundances of CgA and histidine decarboxylase (HDC) mRNA in gastric biopsy samples by reverse transcription polymerase chain reaction (RT-PCR). We assessed drug safety and tolerability by monitoring clinical and blood parameters. In the second stage, all 8 patients were treated with netazepide for 52 weeks in the same manner as the first stage. Study visits were performed as in the first stage but at 3 monthly intervals and endoscopy was performed at baseline and after 24 and 52 weeks of treatment.

In stage 1, 7 of 8 patients exhibited a decrease in the size and/or number of NETs but none showed complete tumour regression. Serum CgA concentrations decreased in all subjects during treatment, but fasting gastrin concentrations were unchanged. The abundances of CgA and histidine decarboxylase mRNA in gastric mucosal biopsies also decreased significantly whilst on treatment. In stage 2, after 12 months of netazepide treatment, we observed a further decrease in largest tumour size and tumour number. Serum CgA concentrations also decreased after restarting treatment. Netazepide was well tolerated in both stages in all subjects and no serious adverse effects were reported.

The present study suggests that netazepide is a promising, well-tolerated new medical treatment for type 1 NETs. Further trials involving longer treatment regimens and more patients are warranted.

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1 Introduction

1.1 The Human Stomach

The human stomach is a hollow, dilated segment of the upper gastrointestinal (GI) tract. It extends from the oesophagus at its proximal margin to the duodenum distally and has three main digestive functions: a) the short-term storage of ingested food, b) the mechanical disruption of food to be mixed with gastric secretions (including digestive enzymes) to form semifluid chyme and c) control of the rate of delivery of chyme into the proximal small intestine in order to facilitate its efficient digestion and subsequent absorption.

1.1.1 Gross anatomy

The organ lies in the left hypochondrial, epigastric and umbilical regions of the abdominal cavity and is typically described as “J-shaped”. The “lesser curve” forms the right-most border and is suspended from the liver by the lesser omentum and the “greater curve” comprises the left border. The greater omentum forms the gastrosplenic ligament superiorly and extends to the spleen whilst extending inferiorly to the transverse colon.

As in the rest of the GI tract, the gastric epithelium is superficial to a supportive layer of connective tissue, immune cells and blood and lymph vessels – the lamina propria. Both are separated from the submucosa by the muscularis mucosa, a thin layer of smooth muscle. Beneath the connective tissue of the submucosa lies the muscularis externa layer. In the stomach, this layer differs from elsewhere in the GI tract in that there is an additional layer of smooth muscle – the inner oblique layer.

The stomach is anatomically divided into four distinct regions (Figure 1-1): the cardia, the corpus, the fundus and the pyloric antrum.

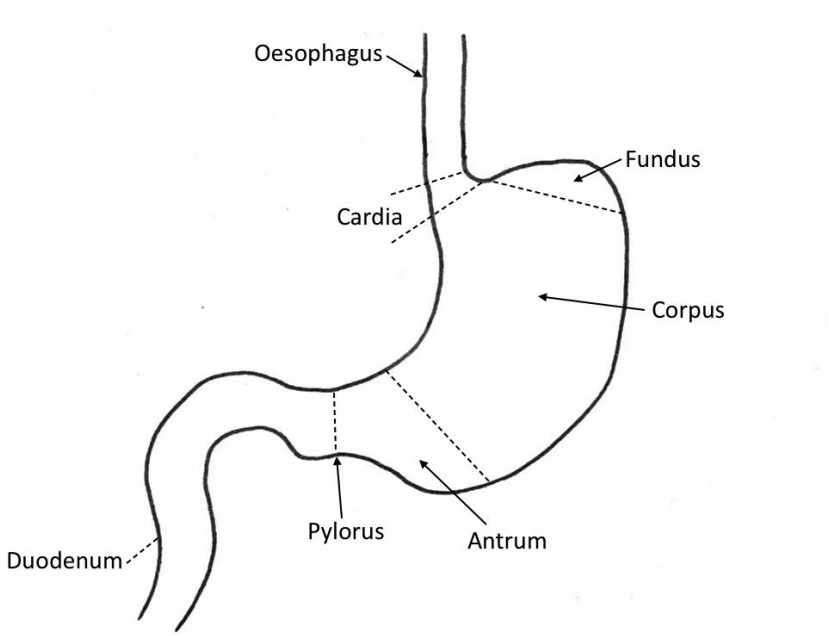

Figure 1-1 Topographical anatomy of the human stomach.

1.1.2 Neural anatomy

The enteric nervous system innervates the stomach by extension of the autonomic nervous system and comprises intrinsic neurons as well as processes of extrinsic neurons, both efferent and afferent. Sympathetic innervation is via preganglionic spinal nerve fibres and postganglionic coeliac plexus fibres. Parasympathetic innervation is derived from the vagus nerve whose gastric branches synapse with postganglionic fibres in the submucosal and myenteric plexuses. These fibres are distributed to secretory apparatus.

1.1.3 Mucosal structure & histology

The gastric mucosa is thick, vascular and rugose. Its epithelial layer is comprised of five main cell types: mucous cells, acid-producing parietal cells, enzyme-producing chief cells, enteroendocrine cells and undifferentiated stem cells.

Throughout the stomach, the surface epithelium is made up of “surface” mucous cells. These are tall (20 to 40µm) and columnar with basal nuclei. Invaginations of this layer – gastric pits – represent the luminal orifice of the gastric glands. These discrete functional units are lined with specialised cells, the nature of which varies depending upon their position in the gland and the anatomical location of the gland in the gastric epithelium.

The pits themselves are lined with superficial mucous cells – continuous with the superficial epithelium. These give way to the upper margin of the gland proper which is described in three sections: the isthmus, the neck and the base. The isthmus is narrow and lined with immature stem cells whose proliferation and subsequent migration maintains the population of mucous cells above and of the various secretory cells below. The glandular neck is characteristically composed largely of mucous cells similar to the superficial cells though smaller and of heterogeneous morphology. At the base of the gland, the cellular composition is dependent on the site of the gland. In the cardiac mucosa, mucous cells with occasional enteroendocrine and parietal cells are found with increasing frequency toward the corpus. Corpus glands (illustrated in Figure 1-2) contain parietal cells and – deeper in the base – chief cells with scattered enteroendocrine cells.

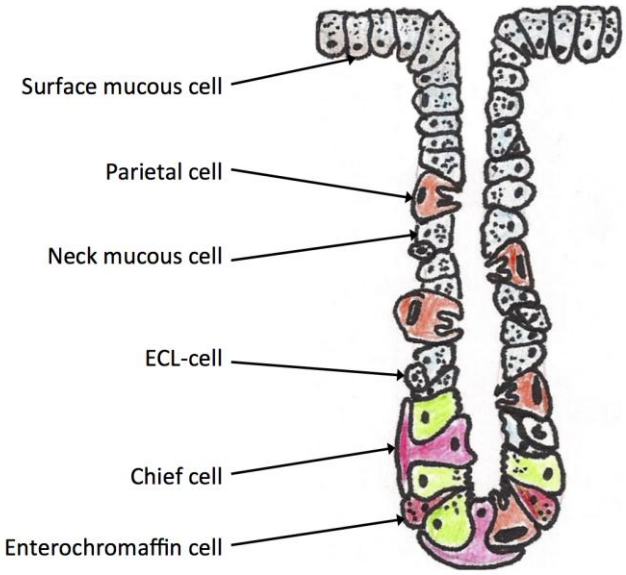

Figure 1-2 Cartoon depicting the cellular composition of a gastric oxyntic-type gland.

Antral glands (illustrated in Figure 1-3) exhibit a greater concentration of endocrine cells and increase in density closer to the pyloric sphincter.

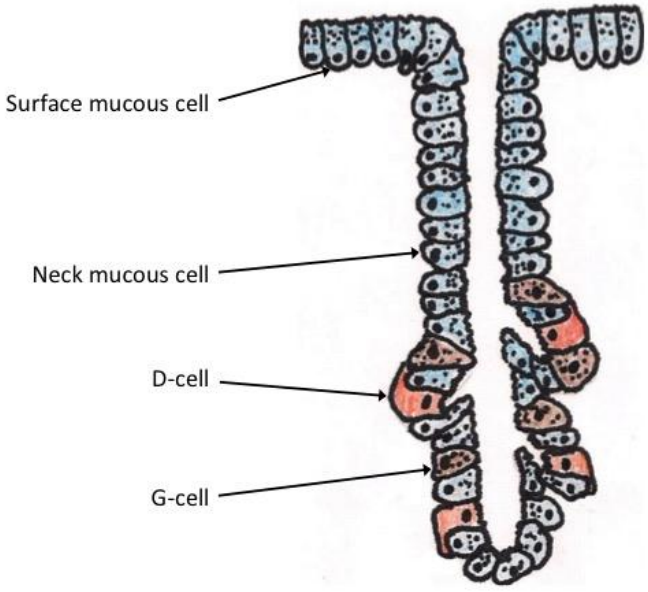

Figure 1-3 Cartoon depicting the cellular composition of a gastric pyloric-type gland.

The more proximally located cardiac glands are distinguishable by their lack of either parietal or chief cells. Aside from immature, undifferentiated cells, only mucous and endocrine cells are seen in these glands.

1.1.4 Mucus and bicarbonate secretion

Mucous cells form the surface of the glandular gastric mucosa. These cells are largely responsible for the protection of the gastric mucosa from the deleterious effects of ingested substances, secreted gastric acid, digestive enzymes and duodenal refluxate. This is achieved by their secretion of an adherent, viscous, gelatinous layer of mucus composed of mucin 5AC (MUC5AC). The secretion of mucin 6 (MUC6) predominantly by mucous cells within gastric glands adds a second, superficial and loosely adherent mucus layer(1). The mucus layer provides a barrier, which slows the diffusion of hydrogen ions from gastric acid towards the epithelial surface. In addition to this physical barrier, surface mucous cells are also thought to play an important role in the secretion of bicarbonate by means of a $Cl^-/HCO3^-$ exchange transporter on the cell membrane(2), setting up a pH gradient from the lumen (low pH) to the epithelium (higher pH). Not only does this prevent physical damage to the mucosal from gastric acid, the activity of the secreted enzyme pepsin is diminished as it diffuses slowly towards the epithelium and away from its optimal operating pH range.

Cosecreted with mucin molecules by mucus neck cells are trefoil factors (TFF). These small, enzyme-resistant proteins are found in two main forms in the mammalian stomach – TFF1 and TFF2. They act to increase mucous gel stability and viscosity and have been shown to contribute to epithelial repair, anti-apoptosis and tumour-suppression(3,4). They modulate immune responses to microbes and have generated interest for their potential use as biomarkers of gastric disease(5).

1.1.5 The Enterochromaffin-like Cell

Enterochromaffin-like (ECL) cells are the predominant enteroendocrine cell type of the mammalian stomach and constitute approximately one-third of oxyntic endocrine cells in humans. They are said to be “closed” endocrine cells as their secretory apparatus is not seen to open into the glandular lumen suggesting their paracrine and endocrine roles in gastric secretory regulation. Typically occupying the lower third of the gastric gland, ECL cells are commonly located in close contact with parietal and chief cells(6). Their nomenclature has its origins in the notion that in certain physiological states, they are structurally and histologically similar to enterochromaffin cells(7). The cells are spherical in shape with occasional digital extensions and multiple cytoplasmic vesicles(8). The ECL cells can be thought of as relay stations in the regulation of gastric acid secretion and their major product is histamine – the most important stimulus of parietal cell acid secretion humans.

Histamine is produced by decarboxylation of L-histidine by the enzyme histidine decarboxylase (HDC)(9). It is then stored in secretory vesicles by the action of vesicular monoamine transporter-2 (VMAT2)(10). The hormone gastrin binds to the CCK2-receptor (CCK2R), a G-protein coupled receptor (GPCR). Downstream signalling from CCK2R is mediated by $G\alpha_{q/11}$, leading to activation of phospholipase C with subsequent increases in intracellular calcium via inositol trisphosphate mobilisation and activation of protein kinase C via diacylglycerol(11) amongst other protein kinase pathways(12). Gastrin regulates the transcription and activity of HDC(13) and VMAT-2(10) as well as chromogranin A (CgA)(14). This matrix protein is expressed in neuronal cells of the central nervous system and neuroendocrine cells of the stomach, pancreas, intestine and adrenal glands. It is stored along with the other major cellular products in the secretory vesicles. CgA is thought to influence intracellular granule stability, and prohormone processing(15,16). Its cleavage products include pancreastatin and it participates in the regulation of peptide secretion and cellular adhesion(17,18).

Mammalian ECL cells also express PAC1 receptors whose major ligand is Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP), a neurotransmitter that stimulates histamine secretion by ECL cells via intracellular Ca^{2+} dependent pathways(19,20).

Both gastrin and PACAP exert trophic effects on ECL cells and have been shown to induce proliferative responses(20–25).

1.1.6 The Parietal Cell

The parietal cell is specialised to secrete gastric acid at pH 0.8 which serves to inhibit microbial growth and colonisation and to activate proteolytic enzymes(26). Typically pyramidal in shape, its limited luminal area is greatly increased by microvilli lined invaginations or cannaliculi. H^+K^+-ATPase – the primary gastric proton pump is the most abundantly expressed membrane protein in an extensive system of so called "tubulovesicles". This protein is an α,β heterodimer able to secrete hydrogen ions against a steep concentration gradient. H^+ ions are exchanged for K^+ ions and in the process, an intracellular hydroxide ion is generated. This is converted to bicarbonate by the carbonic anhydrase enzyme and exchanged at the basolateral membrane for chloride.
The basolateral membrane expresses receptors for stimulation via neural and endocrine pathways to stimulate acid secretion(27).

The parietal cell is also the source of intrinsic factor (IF), a glycoprotein required for binding of vitamin B12 and its subsequent absorption in the terminal ileum. The commonest cause of B12 deficiency is pernicious anaemia – a clinical syndrome comprising megaloblastic anaemia, low serum B12 concentration and the presence of autoantibodies to parietal cells and/or IF. This disease arises as a result of autoimmune destruction of parietal cells in the setting of autoimmune atrophic gastritis ("gastritis type A"), which is discussed in more detail in section 1.5.

1.1.7 Regulation of acid secretion

Gastric acid is required for the optimal digestion of proteins; the absorption of minerals such as iron and calcium; and inhibition of microbial growth. In order that acid secretion does not overwhelm the protective mechanisms of the gastric mucosa however, the process is under tight control by neuronal, paracrine and endocrine systems. Two intracellular signaling pathways are thought to participate in H^+K^+-ATPase activation: a) the Ca^{2+} pathway – induced by M3 muscarinic receptor stimulation by acetylcholine (Ach) (neuronal) and b) the adenylate cyclase pathway – induced by H2 receptor stimulation by histamine (paracrine). Parietal cells also express CCK2R though the significance on H^+K^+-ATPase recruitment of parietal stimulation by gastrin is subject to some debate(28). The most important regulator of acid secretion is the hormone gastrin, which works indirectly (via the intermediary ECL cell) (Figure 1-4). The principal inhibitor of acid secretion is somatostatin from gastric D cells, which acts in a paracrine fashion.

Stimulation of the G cells, ECL cells and parietal cells begins with the so-called cephalic phase of acid secretion during which stimuli arising in the brain (sight and smell of food etc.) culminate in efferent vagal stimulation. This induces the release of postganglionic neurotransmitters including ACh, PACAP, vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP). In the gastric mucosa, ACh exerts its stimulatory effect at the parietal ECL and G cells whilst at the same time inhibiting somatostatin secretion from D cells. GRP also acts at the G cell to stimulate gastrin secretion whilst the ECL cell is subject to PACAP stimulation. Direct stimulation of the parietal cell by histamine at the H2 receptor (H2R) is accompanied by inhibition of somatostatin secretion via the H3 receptor(29,30).

The gastric phase of acid secretion is characterised by the response of antral G cells to the stimuli associated with the ingestion of food. Luminal protein, amino acids and calcium ions induce gastrin secretion. Resulting endocrine stimulation of the ECL cell to produce histamine is the major regulatory step in acid secretion(28). Gastrin release is inhibited by somatostatin from gastric D cells in a paracrine negative

feedback fashion(26). Somatostatin secretion is itself stimulated by gastrin in a paracrine fashion but also via neuronal pathways in response to decreased gastric pH. The latter step is particularly relevant in conditions of impaired gastric acid secretion due to pharmacological intervention or parietal cell loss. In this situation, somatostatin secretion is inhibited, thus removing the brake on gastrin production and leading to hypergastrinaemia with all of its associated effects(28). Parietal cell inhibition by somatostatin is also mediated by direct, paracrine binding to parietal somatostatin receptors, the result of which is to abrogate intracellular cyclic adenosine monophosphate (cAMP) production(27).

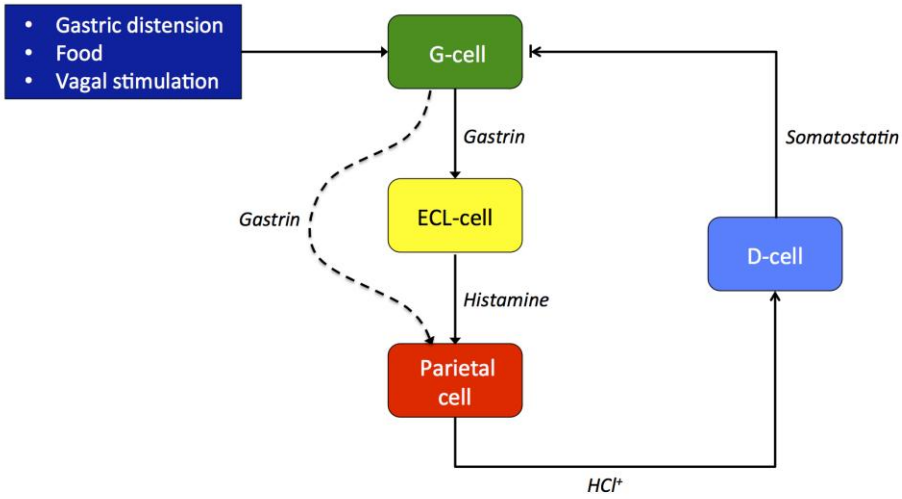

Figure 1-4 Gastrin mediated control of gastric acid secretion.

1.1.8 Pepsinogen secretion

Pepsinogens (PGs) are polypeptide zymogens secreted in the stomach and activated by gastric acid to form pepsins - the major group of gastric proteases. Pepsins themselves can activate PGs in an autocatalytic fashion. In humans, PGs are secreted in two major classes – PG1 (or PGI) and PG2 (or PGII). The former comprises 5 isoenzymes secreted only in the chief and mucous cells of oxyntic mucosa. On activation, PG1 forms pepsin 1 (or pepsin I). In contrast, PG2 (comprising 2 isoenzymes) is secreted by cardiac, oxyntic, pyloric and Brunner's glands(31) and is

activated to form pepsin 2 (or pepsin II). Both forms of pepsin are optimally active in acidic environs between pH 1.8 and 3.5. They are reversibly inactivated at pH 5.0 and denatured between pH 7.0 and 8.0.

1.2 Gastrin

In normal physiology, the antral hormone gastrin stimulates the secretion of acid and the proliferation of cells of the gastric epithelium. Excess gastrin production is associated with states in which the normal inhibitory mechanisms are lost or suppressed (such as with the use of proton-pump inhibitor drugs) or in which there is a pathological source of gastrin secretion (such as a secretory 'gastrinoma' in the Zollinger-Ellison syndrome). The 'classical' gastrins are those peptides whose carboxy terminus is amidated and they can also be sulphated at their single tyrosine residue. The various forms of classical gastrins exhibit similar activity at the CCK2 receptor though they vary in half-life.

1.2.1 Biosynthesis and processing

The gastrin hormone is encoded by a single gene located on the long arm of chromosome 17(32). It is primarily synthesised in G-cells of pyloric glands in the gastric antrum though G-cells are also found in duodenal Brunner's glands(33). The primary precursor – preprogastrin – is synthesised in the endoplasmic reticulum where the N-terminal sequence is cleaved to form progastrin for storage in exocytic vesicles (Figure 1-5). Here, progastrin is further modified by protease cleavage to generate COOH-terminal Gly-extended gastrins (G-Gly). These G-Gly peptides are then acted upon by peptidyl-α-amidating mono-oxygenase (PAM) to form COOH-terminal amidated gastrins, the two major forms of which are G17 and G34 with 17 and 34 amino acid residues respectively(34). Gastric acidity inhibits gastrin release via the secretion of somatostatin by antral D-cells in a negative-feedback fashion. G-cells release gastrin in response to stimuli associated with food ingestion. Gastric

nerves release gastrin-releasing peptide (GRP) and luminal amino acids and calcium ions act at luminal receptors on the G-cell.

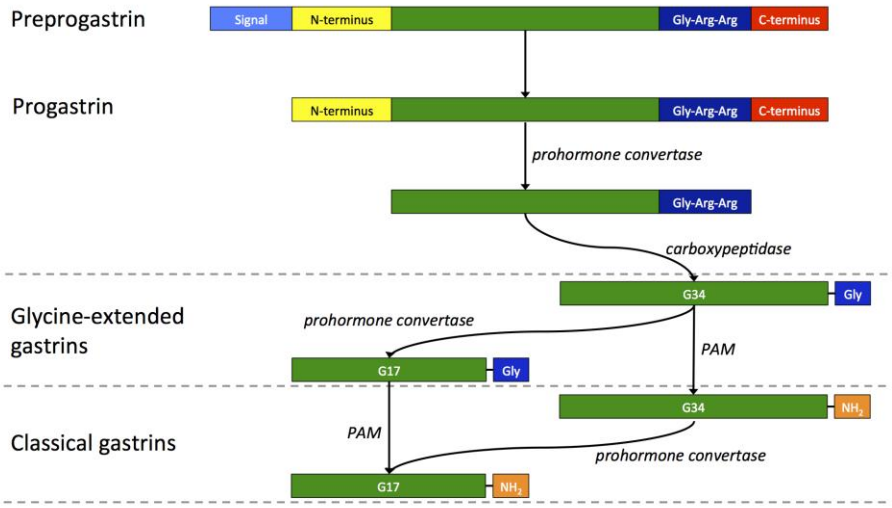

Figure 1-5 Biosynthesis and processing of gastrin.

Gastrins are said to belong to a family of peptide hormones that also includes cholecystokinin (CCK) as they both possess the COOH-terminal pentapeptide amide, which confers their biological activity. These peptides act at the CCK1- and CCK2 receptors and whilst CCK exhibits a high affinity for both receptors, gastrin binds to CCK2R with an affinity approximately 100-times that of CCK1(35). After ingestion of a meal, the circulating concentration of gastrin exceeds that of CCK by 5-10 times and so gastrin is thought to be the most important physiological agonist of CCK2R(36). In normal physiological states, gastrin is expressed on gastric parietal and ECL-cells as well as in the pancreas and brain.

1.2.2 Cellular effects

The role of gastrin in the control of gastric acid secretion has been summarised in section 1.1.7.

The other major role of amidated gastrins in normal physiology is in gastric epithelial cell proliferation – a phenomenon borne out through clinical observations and in numerous laboratory studies. As cellular proliferation is a key step in oncogenesis, this behaviour merits further discussion. Perhaps the best-characterised clinical effect of hypergastrinaemia is the gastric corpus hypertrophy with ECL-cell tumour formation and hyperacidity that is observed in patients with Zollinger-Ellison syndrome. In such patients, resection of the gastrin-secreting gastrinoma results in normalisation of gastric acid secretion and leads to reversal of parietal cell hyperplasia(37,38).

The trophic effect of gastrin was first described in animal models almost 45 years ago when the augmenting effects of pentagastrin on protein synthesis and parietal cell mass were demonstrated in rats (39,40). This has been studied extensively in other animal models. In mice in which the genes encoding either gastrin or its receptor (CCK2R) are deleted, there are reduced gastric populations of parietal and ECL cells accompanied by hypochlorhydria(41) suggesting that the effect of gastrin is to stimulate gastric stem cell proliferation and to influence stem cell differentiation towards a parietal or ECL-cell fate(42). In contrast, transgenic mice engineered to produce gastrin in pancreatic β-cells (INS-GAS) initially exhibit hyperproliferation of gastric epithelium, enhanced populations of parietal and ECL cells and hyperacidity(34). Interestingly, the same animals later lose parietal cell mass and develop foveolar hyperplasia in a histologically similar fashion to that seen in the human disease of chronic atrophic gastritis. The molecular mechanisms responsible for the proliferative influence of gastrin have also been explored *in vitro*. MKN-45 cells constitutively expressing CCK2R exhibit diminished proliferation when treated with a CCK2R antagonist(43). The corollary of this is seen with AGS-B cells stably transfected to express CCK2R and treated with gastrin. These cells exhibited more rapid proliferation associated with upregulation of cyclin D1(44).

The thesis that classical gastrin acting via CCK2R is responsible for gastric epithelial proliferation is however complicated by several observations. First, proliferating cells in normal gastric epithelium are not seen to express CCK2R with the exception of

ECL-cells and (to a lesser degree) parietal cells(45). Additionally, other cell-line studies have illustrated an inhibitory effect of gastrin on proliferation(46,47). Previous studies suggested a paracrine role for gastrin-induced ligands for the epidermal growth factor receptor (EGF-R) such as heparin-binding epidermal growth factor (HB-EGF)(48–50). Our own group examined this by first transfecting gastric cancer derived AGS cells with the CCK2R (AGS-G_R). Exposure to gastrin inhibited the proliferation of these cells but when the same cells were co-cultured with labelled AGS cells (AGS-GFP), exposure to gastrin induced proliferation of the latter, CCK2R deficient cells suggesting paracrine stimulation. The same study identified that the likely mechanism was via gastrin induced shedding of HB-EGF and that this was in-turn mediated by protein kinase C (PKC) dependent matrix metalloproteinase (MMP) activity(51). This mechanism is pertinent to the present study as *H. pylori* is known to induce hypergastrinaemia, MMP activity and the expression of growth factors including HB-EGF(48,52–56). Recent work has identified mitogen activated protein kinase 1 interacting protein 1 (MP1) as an essential partner in gastrin-induced phosphorylation of ERK1 and ERK2 and that this is responsible for gastrin-induced proliferation via the mitogen-activated protein kinase (MAP) pathway(57).

These paracrine pathways also appear to be important when considering the effect of gastrin stimulation on cellular migration and invasion – also key steps in oncogenesis and important when investigating preneoplastic epithelial remodelling. Using the same AGS-G_R/AGS-GFP co-culture methodology described above, gastrin stimulated cell migration both directly (for AGS-G_R) and in a paracrine fashion (for AGS-GFP). This phenomenon was found to be due to MAPK activity via HB-EGF(53). MAPK activity has also been implicated in the induction of MMP-9 expression and the gastrin-stimulated invasion of AGS-G_R cells through basement membrane *in vitro*(56).

The influence of gastrin on ECL-cell proliferation is one of direct stimulation. In rodents, it has been shown that ECL-cells are capable of self-replication(58) and that gastrin induces ECL cell proliferation in animals and *in vitro*(25,59). ECL-cell hypertrophy in response to hypergastrinaemia is seen in rats after a few days and

reaches a maximum plateau after 20 weeks(60). Pathological hypergastrinaemia in humans is considered as either a) primary - as seen in Zollinger-Ellison syndrome with excess production of gastrin by a secretory tumour or b) secondary – as seen in chronic autoimmune atrophic gastritis (AIG) with oxyntic atrophy, hypochlorhydria and loss of the negative feedback influence of gastric acid. The pathophysiology of the associated type 1 gastric neuroendocrine tumours is discussed in section 1.6.

1.3 Helicobacter pylori

1.3.1 Description

Bacterial colonisation of the human stomach was described and debated as early as the 19th century but until the first published description of *Helicobacter pylori* (*H. pylori*) culture in 1983, the prevailing assumption had been that the hostile, acidic environment of the gastric mucosa ensured its sterility(61,62). The organism originally labelled *Campylobacter pylori* by Marshall and Warren is a Gram-negative, helical, flagellate, microaerophilic bacillus. It grows slowly and requires highly regulated environmental conditions, making its culture *in vitro* somewhat challenging(63). Since its discovery, much of the research interest in *H. pylori* has focused on the factors that determine the outcome of infection. As discussed in section 1.3.2, the clinical consequences of gastric colonisation with *H. pylori* are dependent on a complex interaction between bacterial, host and environmental factors.

1.3.2 Outcomes of infection with *H. pylori*

It has been well established that *H. pylori* infection (of any strain) induces inflammation in the gastric mucosa in all infected hosts. Acute infection in adults can induce a syndrome comprising marked gastritis and achlorhydria accompanied by symptoms of abdominal discomfort and nausea(64,65). The subsequent clinical

sequelae of infection are, however, variable. There are three main groups as illustrated in Figure 1-6 (66): a) The benign pangastritis phenotype in which there is mild inflammation throughout the stomach without symptoms or serious complications such as ulceration; b) the antral predominant/peptic ulcer phenotype. In approximately 15% of infected subjects, the oxyntic mucosa is relatively spared whilst antral inflammation results in hypergastrinaemia and high parietal cell acid output. In these subjects, high acidity results in pyloric and duodenal ulceration; and c) the corpus predominant/gastric cancer phenotype. In contrast to the "duodenal ulcer" phenotype, these individuals exhibit corpus-predominant gastritis with sparing of the antrum. In around 1% of infected people, subsequent corpus atrophy results in loss of parietal cells and attendant hypochlorhydria. This can be followed by a sequence of cellular changes discussed in section 1.4 and which predisposes sufferers to the development of gastric adenocarcinoma. The causative link between *H. pylori* infection and the subsequent development of non-cardia gastric adenocarcinoma was subject to considerable debate but definitive studies published over a decade ago established the connection and illustrated the protective effect of eradicating *H. pylori* in infected subjects including those with established preneoplastic conditions(67–69). The International Agency for Research on Cancer has classified *H. pylori* as a type I carcinogen(70). It is estimated that *H. pylori* confers an increased risk of non-cardia gastric adenocarcinoma of between 6- and 8-fold compared to uninfected subjects(71,72).

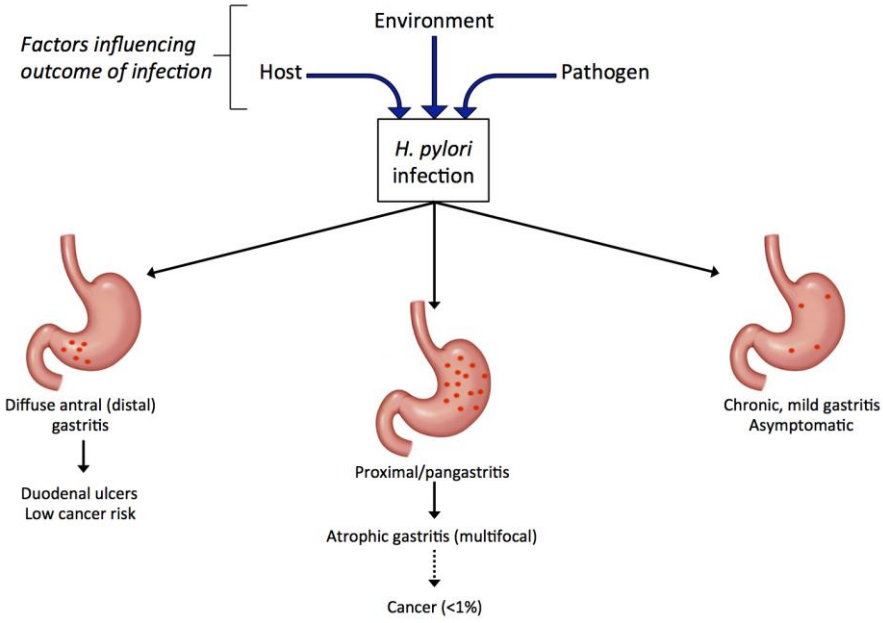

Figure 1-6 Outcomes of infection with *H. pylori*.

1.3.3 Epidemiology

Helicobacter pylori is a highly successful pathogen whose relationship with humans is thought to date back at least 58,000 years(73). A variety of *Helicobacter* species can be found in other mammals and it has been suggested that organisms of this genus are ancestral to mammals in general and that early humans may have been infected with *H. pylori* before evolving into *Homo sapiens*(74). Though falling in incidence in developed countries, the overall prevalence of *H. pylori* is estimated at greater than 50% of the global population(75) making it the world's commonest bacterial infection. Prevalence is considerably higher in developing than in developed countries(76) and in socially deprived populations(77).

1.3.4 Transmission

H. pylori infection is thought to occur predominantly in childhood with some populations exhibiting 90% carriage rates in children under 10 years old(78–80). Close, cohabiting family members are the main source, with the greatest risk factor

for infection being maternal *H. pylori* status(79). Though faecal-oral transmission is frequently cited, there are limited data supporting *H. pylori* survival following intestinal transit in healthy individuals. The organism can be cultured in faecal samples when diarrhoea is induced in otherwise healthy subjects and vomitus is a reliable source of viable organisms, raising the possibility that transmission occurs when individuals are exposed to family members with enteric illnesses(81,82). It is proposed that oral-oral transmission is the predominant route, though there are recent reports implicating maternal vaginal reservoirs of *H. pylori* colonies which might account for vertical transmission(66,83) and of sexually-transmitted *H. pylori* infection(84).

1.3.5 Colonisation

Although increasing understanding of the gastric microbiota has now superseded the paradigm of the "sterile stomach", the chemical and mechanical conditions of the gastric lumen represent a challenging niche for microbial survival and proliferation. *H. pylori* has evolved a series of highly specialised strategies for survival. *H. pylori* selectively colonises the mucous layer but in order to do so, first induces localised buffering of its cytoplasm and immediate surroundings by enzymatically degrading urea to generate ammonia and CO_2(85). The activity of the cytoplasmic and membranous urease enzyme is highly conserved across *H. pylori* spp. and forms the basis for rapid diagnostic tests including the urea breath test and urease gastric biopsy rapid assay(86). Components of the urease complex have also been shown to induce the upregulation of inducible nitric oxide synthase and the release of nitric oxide, prompting speculation that urease also plays a role in the mediation of epithelial inflammation(87).

Subsequent survival of *H. pylori* depends on migration to and colonisation of the mucous layer adjacent to the mucosal surface where the pH can be maintained at near-neutrality. To facilitate this, a *H. pylori* bacterium exhibits pH chemotaxis and employs its flagellate apparatus to travel to and maintain a position in close

proximity to the epithelial surface where critical interactions with host epithelial cells can take place(66). The long-held assumption that *H. pylori* bacteria are able to burrow through the viscous mucus-layer adjacent to the mucosa in a corkscrew fashion is probably over-simplistic. Recent studies have illustrated the immobility of *H. pylori* in porcine gastric mucus(88) suggesting that in humans, the organism is actually able to alter the rheology of gastric mucus. It has been shown to achieve this by altering its environmental pH (as described above) and by altering mucus composition and mucin production directly(88,89).

1.3.6 Adhesion and Host Interaction

1.3.6.1 Adhesins

A small proportion (estimated to be 1-20%) of colonising *H. pylori* organisms can be found adhered to gastric epithelial cells(90–92). The advantages of doing so are thought to be related to: a) resistance to mechanical removal and subsequent intestinal excretion, b) manipulation of the host cell response to attenuate the immune response and c) the acquisition of host cell nutrients(66,90,93). *H. pylori* have more than 30 genes responsible for the expression of outer membrane proteins (OMPs), some of which have been classified as adhesins(66). These protein complexes are regarded as bacterial virulence factors. The best-characterised group of adhesins belong to the Hop family of OMPs and include BabA, SabA, AlpA/B, HopQ/Z, and OipA(93). BabA is a membrane-bound adhesin molecule responsible for binding the blood-group antigen Lewis b (Le^b) on gastric epithelial cells. *H. pylori* strains carrying the babA2+ variant are associated with an increased risk for the development of mucosal preneoplastic conditions and subsequent adenocarcinoma(94). The sialic acid-binding adhesin SabA binds to epithelial sialyated carbohydrate complexes such as sialyl-dimeric-Lewis x(87,94). SabA is also implicated in pro-inflammatory neutrophil activation(95). OipA is an outer-membrane protein and infection with strains expressing this is associated with more

severe gastritis, duodenitis and ulceration and higher bacterial colonisation densities(87,96).

1.3.6.2 CagA and the cag Pathogenicity Island

Some strains of *H. pylori* express a genetic sequence known as the cag Pathogenicity Island (cag PAI). This 40kb region encodes cagA, a bacterial protein whose presence confers an enhanced risk of adverse outcomes including pronounced gastritis, peptic ulceration, preneoplastic changes and gastric adenocarcinoma(97). Other coding regions of the cag PAI encode components resembling those of a Type 4 secretion system (T4SS). *H. pylori* strains containing the cag PAI account for 60-70% of Western strains and almost 100% of those found in East Asia(97). It has been shown that *cagA* positive strains of *H. pylori* are able to inject CagA into host epithelial cells where it undergoes phosphorylation at EPIYA sites by two groups of cellular kinases: SRC and ABL. The resulting intracellular CagA products have been shown both to upregulate the expression of IL-8, IL-1β and TNF-α; and to activate NF-κB, thus enhancing the inflammatory response(66,93,98,99). A recent study has suggested that infection with *cagA* positive strains of *H. pylori* is associated with the increased production of H_2O_2 and a resulting legacy of oxidative DNA damage(100).

Whilst the precise mechanisms underpinning the effect of CagA phosphorylation on *H. pylori* pathogenicity are unclear, it has been shown that *cagA* positive strains confer a substantially increased risk of gastric cancer development following infection (OR=1.9) over other strains of *H. pylori*(101).

1.3.6.3 VacA

Another well-characterised *H. pylori* virulence factor is VacA. Unlike the cag PAI, VacA expression is highly conserved across strains of *H. pylori* though expression and effects vary depending upon genetic diversity. VacA is secreted via type 5 secretion systems whereupon a proportion of the secreted protein assembles at the host cell

membrane, inducing the formation of membranous pores(66). VacA has been shown to induce host cell apoptosis and disrupt epithelial tight junctions(102). The subsequent exposure of T cells to VacA in the lamina propria is thought to alter T cell function and inflammation by NF-κB activation and IL-8 upregulation(103). More recent studies have illustrated the role played by VacA in the control of gastric epithelial cell autophagy. In certain circumstances, VacA inhibits autophagy which results in the accumulation of reactive oxygen species(104). Paradoxically, a second study demonstrated the induction of gastric epithelial cell autophagy by VacA via binding to and activation of low-density lipoprotein receptor-related protein (LRP1)(105). This also leads to the accumulation of intracellular reactive oxygen species. Both scenarios might be expected to lead to oxidative DNA damage.

As in the case of *cagA*, the precise mechanisms of VacA dependent pathogenicity remain unclear but virulent variants of VacA (s1 type) have been shown to be associated with adverse outcomes including peptic ulceration and gastric adenocarcinoma(106).

1.3.7 Manipulation of the host immune system

Even for hosts infected with virulent strains of *H. pylori*, the progression from chronic gastric inflammation to cancer development is uncommon. Manipulation of the immune response to infection is essential for *H. pylori* persistence and the attenuated inflammatory response has been implicated in carcinogenesis(107). It has been suggested that epithelial inflammation is required for *H. pylori* to requisition nutrients, which would otherwise be inaccessible(108).

H. pylori has evolved several strategies to overcome and modify the host immune response – both innate and adaptive. For example, the bacteria preferentially induce a T-helper cell 1 (Th1) response, usually considered a requirement for immune destruction of intracellular organisms(109). *H. pylori* also exhibits molecular mimicry by expressing lipopolysaccharide (LPS) which is able to antagonise toll-like receptor

(TLR) 4 signalling, potentially facilitating evasion of the host's innate immune system(110,111).

1.3.8 Host factors influencing the outcome of infection with *H. pylori*

1.3.8.1 Matrix metalloproteinases (MMPs), their tissue inhibitors (TIMPs) and disintegrins and metalloproteinases (ADAMs)

Matrix metalloproteinases (MMPs) are a homologous protein family comprising 23 zinc-dependent endopeptidases that are classically defined by their role in degrading extracellular matrix and basement membrane. In this capacity, they are key participants in normal physiological processes including morphogenesis, angiogenesis and tissue repair(112). They are non-covalently inhibited by tissue inhibitors of metalloproteinases (TIMPs), a group containing 4 members(113). The disintegrins and metalloproteinases (ADAMs) are a related family of 24 membrane-bound peptides responsible for cell adhesion, cell migration, activation of signalling pathways and proteolysis(114).

MMPs exhibit proteolytic and non-proteolytic activity against a very broad variety of substrates and hence influence many biological processes. When their tight regulatory controls are disrupted during carcinogenesis, these peptides are readily co-opted to participate in cancer invasion and metastasis(115). Upregulation of MMP expression has been demonstrated for a wide variety of human malignancies and for some, can reflect tumour stage(116,117). Though MMPs exhibit several features which appear to confer suitability for therapeutic targeting, clinical trials of MMP inhibition with small molecule inhibitors have proved largely disappointing(116,118).

MMP-1 is thought to participate in *H. pylori* associated gastritis in a *cag* PAI dependent fashion via protein kinase C and is found, co-located with *H. pylori* at the site of adhesion in epithelial cells(119).

MMP-3 degrades several types of collagen, proteoglycans, fibronectin and laminin. Its role (along with MMP-9) in various disease states including inflammatory arthropathy, asthma, Alzheimer's dementia and delayed wound healing has been established(120). Though cell-line and murine models suggest an increase in MMP-3 expression associated with exposure to *Helicobacter* species, no difference is observed in humans(121). This may reflect the role of MMP-3 as predominantly an activator of other MMPs(120).

MMP-9 degrades collagen type IV and is implicated in *H. pylori* associated gastritis and carcinogenesis. Its proteolytic activity is observed to increase in *H. pylori* infected gastric epithelia and to diminish following *H. pylori* eradication(122,123). Interleukin-21 (IL-21) is thought to be responsible for upregulation of MMP-2 and -9 in *H. pylori* associated gastritis and is found in high concentrations in gastric tissue from subjects with *H. pylori* gastritis. Unlike other MMPs, MMP-2 and -9 are secreted by AGS cells in response to stimulation with IL-21(124). Our own group demonstrated gastrin-stimulated MMP-9 expression and epithelial cell invasion in gastric cancer cells expressing CCK2R (AGS-G_R)(56).

MMP-7 is responsible for the degradation of proteoglycans, fibronectin, elastin and casein(112) and is produced in epithelial cells (unlike the majority of other MMPs). There has been considerable interest in MMP-7 and its participation in the response to *H. pylori* infection as it has been shown to mediate the inflammatory response to flagellated bacteria in other epithelial systems(125). Our group previously reported that MMP-7 expression is increased in gastric epithelia in response to *H. pylori*. In the same study, MMP-7 exhibited growth factor-like actions attributed to activation of other MMPs, MAP kinase and PI-3 kinase pathways. MMP-7 was localised to the advancing edge of spreading colonies of gastric epithelial cells (and in AGS gastric cancer cell lines) where the rates of migration were higher in *H. pylori* infected cultures compared with controls(53). Following on from this, the same group showed that MMP-7 acted via insulin-like growth factor-2 (IGF-2) release to enhance

epithelial cell and myofibroblast proliferation, suggesting that MMP-7 is important in mediating hyperproliferation of mucosal cells in response to *H. pylori* infection(126).

Several studies have examined the role of ADAM-10 and -17 in the epithelial response to *H. pylori* infection. In antral biopsies, both are increased in abundance in *H. pylori* infected tissue compared with controls(127). Both influence cell signalling via pathways known to be involved in gastric carcinogenesis including via tumour necrosis factor-α (TNFα), E-cadherin and the Notch signalling pathway(121).

1.3.8.2 The urokinase plasminogen activator (uPA) system

The urokinase plasminogen activator (uPA) system comprises the serine protease uPA, its receptor (uPAR) and a number of inhibitory protease peptides, most notably plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2). The primary role of uPA in normal physiology is the cleavage of its primary substrate - plasminogen - to its active form – plasmin. Plasmin is also a serine protease and has several recognised substrates. It is capable of promoting ECM degradation both by direct proteolysis of fibronectin, vitronectin and fibrin; and via the activation of several of the MMPs(128). In this manner, uPA bound to uPAR is thought to have a role in initiating the chain of proteolysis which facilitates cancer cell invasion and metastasis(129).

Expression of uPAR has been characterised in a number of human cancers and cell lines and for gastric cancer, this correlates with features of tumour 'aggressiveness' including invasiveness and capacity to metastasise(130,131). In an early series of gastric cancer patients, increases in both uPA and PAI-1 expression in tumour biopsies were shown to be independent prognostic parameters(131).

The evidence for an association between *H. pylori* infection and activation of the uPA system is increasingly clear. A study of gastric cancer cases found that in non-cancer gastric mucosa uPAR was more frequently expressed in the epithelial cells of *H. pylori* infected specimens than those free from infection(132). Our own group has

reported that *H. pylori* infection stimulated the expression of all three of uPA, uPAR and PAI-1 in gastric epithelial cells *in vitro* and that this was accompanied by uPA dependent cell proliferation via HB-EGF stimulation. In the same study, the authors reported increased uPA, uPAR and PAI-1 expression in the gastric corpus tissue of individuals with *H. pylori* infection compared to those without(133).

1.3.8.3 Insulin-like growth factors (IGFs)

Insulin-like growth factors-1 (IGF-1) and -2 (IGF-2) are predominantly synthesised in the liver. Growth-hormone (GH) stimulated IGF-1 is the major regulator of post-natal growth whilst GH independent IGF-2 is important for normal foetal development(134).

Expression of both IGFs and their receptors has been described for gastric cancer cell lines(135–137) and in human gastric cancer tissue(138). Gastric cancer cell growth *in vitro* is potentiated by the addition both of IGF-1 and IGF-2(135,136).

Our group has previously demonstrated that stromal-cell mediated inhibition of IGF-2 via transforming growth factor beta-induced (TGFB1) abrogated IGF-2 stimulated cancer cell migration and proliferation(139). A study published in 2004 set out to determine the effect on circulating IGFs in response to acute infection with *H. pylori*. The authors reported a significant decrease in the serum concentrations of both IGFs and this was attributed to an acute-phase response(140). This study would appear to have little bearing on the role of IGFs in *H. pylori* associated gastric preneoplasia and carcinogenesis. Another study reported reduced serum concentrations of the major circulating IGF binding protein, IGFBP-3 in patients with gastric preneoplasia than in controls. The same authors showed increased frequency in subjects with antral intestinal metaplastic preneoplasia of an *IGFBP-3* gene promoter polymorphism known to reduce IGFBP-3 production compared with controls. Finally, these authors also reported increased IGFBP-3 expression in gastric cancers of ‘well or moderately differentiated’ histology compared with tumours of the ‘poorly differentiated’ type.

They therefore concluded that IGFBP-3 might be protective against gastric cancer development(141), though their findings may have actually been reflective of a difference in phenotype of gastric preneoplasia and subsequent gastric cancer type. In a recently published study, 54 patients with *H. pylori* had serum concentrations of IGF-1 and IGFBP-3 measured before and after *H. pylori* eradication. The authors reported that there was a significant decrease in the serum concentration of IGF-1 following proven *H. pylori* eradication. In this small series, it was not possible to determine an association between IGF-1 and/or IGFBP-3 and gastric preneoplasia(142).

1.3.8.4 Host genetic factors

The host response to *H. pylori* infection is largely mediated via cytokine release. As well as the bacteria specific variables described above, the magnitude of cytokine release can also be affected by host genetic polymorphisms.

The best studied are polymorphisms in the interleukin-1β gene (*IL1B*) and in the interleukin-1 receptor antagonist gene (*IL1RN*). These are associated with increased mucosal IL1β and inflammation(143) following *H. pylori* infection. Individuals with the so-called 'pro-inflammatory' genotypes exhibit an increased propensity for the development of both preneoplastic lesions and non-cardia adenocarcinoma(66). As might be anticipated, the summative effect of 'pro-inflammatory' *IL1B* polymorphisms and bacterial virulence factors confer a markedly greater risk (as much as fifty-fold increase) for cancer development(144). Other polymorphisms associated with increased *H. pylori*-related inflammation and risk of non-cardia gastric cancer include those in the *TNFA* gene encoding TNFα and in genes encoding innate immune factors such as TLR4. It has been suggested that individuals carrying a compound genotype comprising 'pro-inflammatory' genotypes for several immune factors are prone to the development of the pangastric/corpus predominant, hypochlorhydric pattern of *H. pylori* gastritis most likely to proceed to preneoplasia and gastric cancer development(66).

1.4 Gastric Cancer

1.4.1 Epidemiology & classification

Despite several decades of declining incidence, gastric cancer remains the fifth commonest cancer and the second commonest cause (after pulmonary malignancy) of cancer mortality worldwide(145–147). Approximately 9% of cancer deaths globally are attributed to the disease with an estimated 952,000 new cases and 723,000 deaths in 2012(147). Gastric cancer incidence reports illustrate the skewed geographical distribution of the disease. Over 50% of cases occur in East Asia (mostly China). In contrast, European cases make up only 15% of the total(148). Incidence is also divided along socioeconomic lines with higher rates typically being observed in developing than in developed nations and in lower socioeconomic groups within individual nations and ethnic groups(148–150). Age-standardised rates for males are approximately double those for females(147). In the United Kingdom, gastric cancer is the thirteenth commonest malignancy (2.5% of all cancers) with an age-standardised incidence rate of 8.6 per 100,000 persons. There are almost 8,000 new cases of gastric cancer diagnosed annually and over 5,000 deaths(151). Global incidence and mortality rates have diminished steadily since the middle of the 20th century and the UK is no exception. Here, the mortality rate has fallen by 70% since the 1970s(152). This global decline is attributed to a number of environmental factors including improved hygiene, increased intake of fresh fruit and vegetables, diminished tobacco use, serendipitous *H. pylori* eradication with antibiotic use and decreased dietary intake of salt(153–157). Interestingly, against this background of globally diminishing rates of gastric cancer, there appears to be a subpopulation of younger people in whom non-cardia, intestinal-type gastric adenocarcinoma is increasing in incidence(158–160). This phenomenon has been characterised in several European countries and in North America. The mechanism responsible for this paradoxical observation is unclear. It has been suggested that the widespread use of antisecretory drugs including proton-pump inhibitors and H2-receptor antagonists might be partly responsible. Other authors have suggested increased

pathogenicity of *H. pylori*; either by the introduction of more virulent strains with population migration; or by a loss of diversity in the gastric microbiome due to antibiotic use and better sanitation which allows *H. pylori* to expand its niche in the absence of competition(161,162).

Perhaps unsurprisingly, gastric cancer predominantly affects older people. Fewer than 8% of cases are diagnosed in the under-55 year old age group and the rate rises steeply after the age of 60 years(151). The attendant comorbidities of this older patient population combined with the propensity of the condition to present later in its natural history result in poor survival rates. Despite steady improvement, the UK 5-year survival rate stands at just 15%(163).

By far the commonest form of gastric malignancy is adenocarcinoma. This accounts for approximately 95% of gastric neoplasms with the remainder comprised of lymphomas, neuroendocrine and stromal tumours(164,165). Gastric adenocarcinoma is most commonly categorised according to the Laurén classification. This recognises two histological types: *intestinal* and *diffuse*(166). Intestinal-type carcinoma is characterised by the presence of gland-like tubular structures, resembling intestinal glands, and is found more commonly in men (male/female ratio = 2:1) and older patients. It is this type which is more closely related to environmental risk factors and which has been seen to decline in frequency over the last five decades. In contrast, diffuse-type carcinoma has a near-uniform frequency across the world(165). This variant is characterised histologically by poorly differentiated, non-cohesive tumour cells and is more frequently located in the proximal stomach. There is a sub-type: *signet-ring cell adenocarcinoma* that is characterised by rounded cells with abundant intracellular mucin flattening the nuclei at the cell periphery. It is thought that the signet-ring cell variant of diffuse-type gastric cancer is increasing in incidence, accounting in part for the increase in incidence of proximal gastric cancer(167).

A second system for categorisation of gastric carcinoma is by topographical site. Though traditionally grouped together in registries and clinical trials, proximal gastric

(also referred to as *cardia* or *gastro-oesophageal junctional*) and distal gastric (also referred to as *non-cardia*) adenocarcinoma exhibit quite distinct clinical and molecular features. Adenocarcinomas of the distal stomach i.e. *non-cardia* cancers are primarily caused by infection with *H. pylori*. Proximal adenocarcinoma i.e. *cardia* cancer is a more heterogeneous disease. A proportion of these tumours exhibit the clinical, histological and molecular characteristics of non-cardia adenocarcinoma (intestinal or diffuse). The remainder more closely resemble oesophageal carcinoma and these are associated with gastro-oesophageal reflux disease (GORD) and obesity. Proximal gastric cancer has a more pronounced predisposition in males (5:1) and increases in frequency with socioeconomic status(165,168). Recently published work has identified the targeted sequencing of cancer-associated genes in cases of proximal gastric adenocarcinoma as being a potentially useful method of distinguishing between molecular phenotypes and potentially directing targeted therapies(168).

1.4.2 Risk factors

1.4.2.1 Genetic predisposition

Polymorphisms associated with increased risk of cancer development in response to *H. pylori* infection have been discussed in section 1.3.8.4.

Inherited susceptibility to the development of gastric cancer is implicated in 1-3% of cases and is more often seen in diffuse-type cancers than in the intestinal-type(169). The difference in familial aggregation is such that in first-degree relatives of patients with diffuse-type cancer, there is a 7-fold increase in risk as compared with a 1.4-fold increase for relatives of patients with the intestinal phenotype. Hereditary Diffuse Gastric Cancer (HDGC) is a rare syndrome most commonly associated with germline mutations in the E-cadherin gene (CDH1) and which predisposes carriers to early-onset (mean age at diagnosis 40 years) diffuse-type gastric cancer, often of the signet-ring subtype(170–172). Recently published consensus guidelines recommend

testing for CDH1 germline mutations in high-risk groups which include: a) individuals with diffuse-type gastric carcinoma under the age of 40 years; b) those with relevant family histories; and c) people with a personal or family history of diffuse-type gastric cancer and lobular breast cancer(169). As only 25-30% of families with HDGC carry a germline CDH1 mutation, those with normal genetic screening but high-risk family histories are advised to undergo annual surveillance gastroscopy. For CDH1 mutations, the penetrance is considered so high (>80%) that prophylactic gastrectomy is advised(169).

Several other genetic disorders are associated with an increased risk of developing of gastric adenocarcinoma. Lynch syndrome is one of the major autosomal dominant forms of inherited colorectal cancer and is caused by germline mutations in four mismatch repair genes. There is an established risk of gastric cancer in carriers of these mutations, and particularly in the genes *MLH1* or *MSH2*(173). Peutz-Jehgers syndrome is caused by a germline mutation in LKB1 inhibiting its function as a tumour suppressor gene. There are a number of reports of affected individuals developing gastric cancer at a young age(174,175).

Outside of these specific and uncommon examples, familial clusters of gastric cancer may be related to true genetic predisposition or to exposure to common environmental factors. In a study of 100 first-degree relatives of patients with proven gastric cancer, the prevalence of hypochlorhydria and gastric atrophy was increased but only amongst those with *H. pylori* infection(176). In a German case-control study, an association between *H. pylori* and family-history of gastric cancer was identified but it was also shown that family-history was an independent risk factor for gastric cancer development(177). Furthermore, a meta-analysis of studies examining the risk of gastric mucosal preneoplastic lesions in first-degree relatives of individuals diagnosed with gastric cancer found a significantly higher prevalence of *H. pylori* infection, gastric atrophy and intestinal metaplasia(178).

1.4.2.2 Helicobacter pylori

Bacterial factors associated with increased risk of gastric cancer development have been discussed in detail in section 1.3.

For gastric adenocarcinoma (as for gastric MALT lymphoma), *Helicobacter pylori* is the leading aetiological factor. Conservative estimates suggest that approximately 75% of cases are attributable to infection(179,180). Other authors have reported much higher rates of *H. pylori* involvement in gastric cancer development. The Eurogast-EPIC study (in a European population) suggested that 93% of cases were associated with *H. pylori* infection(181) and a recent Japanese study reported that fewer than 1% of cases were not associated with this bacterium(182). Generally speaking, areas of high non-cardia gastric adenocarcinoma prevalence correspond to areas of high *H. pylori* prevalence. There are exceptions to this observation including areas of sub-Saharan Africa and South Asia where the high *H. pylori* infection rates are not associated with high gastric cancer incidence rates. This is assumed to be a reflection of the complex interactions between host, environmental and bacterial factors(183,184).

1.4.2.3 Diet, lifestyle and gastric cancer risk

Epidemiological studies have shown that high dietary intake of salt is thought to increase the risk of cancer(185). The exact mechanisms remain unclear though a synergistic effect with *H. pylori* to enhance gastric inflammation perhaps mediated through altered epithelial mucous viscosity has been proposed(185). A recent meta-analysis of prospective studies determined that relative risk of gastric cancer increases with the magnitude of dietary salt intake and that this effect seemed to be greater in Japanese populations(186). The effect is seen for all histological variants and appears to be independent of other variables including *H. pylori* infection and virulence factors, and tobacco smoking(187).

The reverse effect is seen with dietary intake of fresh fruit and vegetables. A meta-analysis of cohort studies reported an inverse relationship between dietary intake of fruit and the incidence of (but not mortality from) gastric adenocarcinoma. The same study reported a similar, inverse relationship between vegetable intake and the incidence and mortality rates of gastric cancer(188). The World Cancer Research Fund systematic review also highlights limited evidence that suggest a protective effect from the consumption of legumes and selenium-containing foods whilst chilli, processed meat, and smoked/broiled foods may increase the risk of developing gastric cancer(189).

The major lifestyle factor implicated in gastric carcinogenesis is tobacco smoking, where the evidence for increased risk in unequivocal and dependent on the magnitude of tobacco consumption(190). In those with a long or heavy exposure to cigarette smoking, it is estimated that the risk of gastric cancer is almost doubled(189). No convincing epidemiological association between alcohol intake and gastric cancer has been identified(191). Similarly, and although established as a risk factor for oesophageal adenocarcinoma, no epidemiological association between obesity and non-cardia gastric cancer has been demonstrated(192). Interestingly, a recent meta-analysis has demonstrated a possible independent augmenting effect of pre-existing diabetes mellitus on the risk of gastric-cancer development. The authors report an increase in risk of approximately 19% but did not conduct subgroup analyses to examine the effect of obesity in this higher-risk cohort(193).

1.4.3 Gastric preneoplasia

As for several other epithelial malignancies, the pathway to developing intestinal-type gastric cancer has been well described. The multistep model - developed by Correa et al. – postulates that there is a sequence of pre-malignant mucosal changes culminating in the development of adenocarcinoma(194). The development of diffuse-type cancer is less well described and the links between this and the

precursor lesions of intestinal-type cancer are unclear. That said, it seems clear that both phenotypes share a close link to the index change in the gastric mucosa; that of *H. pylori* associated inflammation(154).

The multistep pathway from *H. pylori* associated gastritis to gastric adenocarcinoma formation (Correa's multistage cascade of gastric oncogenesis) emerged as a result of epidemiological studies performed in the 1970s in Colombia(194). It includes sequential stages of mucosal remodelling that precede or coexist with gastric adenocarcinoma (Figure 1-7). In this model, chronic inflammation leads to loss of gastric glands (*chronic atrophic gastritis* or *gastric atrophy*) and/or metaplastic transformation of the indigenous glandular structures (*intestinal metaplasia*). The subsequent step in the cascade is 'de-differentiation' of the metaplastic epithelium and acquisition of neoplastic cellular characteristics (*dysplasia* or *intra-epithelial neoplasia* (IEN)). Dysplasia leads on to stromal invasion and the development of invasive adenocarcinoma. A national cohort study in the Netherlands showed that the risk of gastric cancer development increased with each step in severity of the cascade. Chronic atrophic gastritis (CAG), intestinal metaplasia (IM), low-grade dysplasia and high-grade dysplasia conferred risks of developing gastric adenocarcinoma in a subsequent five year follow-up period of 0.1%, 0.25%, 0.6% and 6% respectively(195). A recent meta-analysis demonstrated regression of atrophy in the gastric corpus of patients following eradication of *H. pylori*. The same study found that gastric antral atrophy and gastric intestinal metaplasia (IM) did not improve following *H. pylori* eradication, suggesting that IM might represent a histopathological "point of no return"(196).

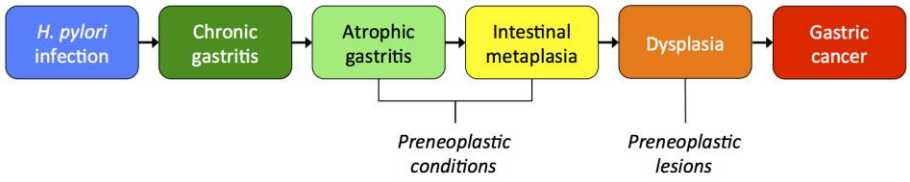

Figure 1-7 Correa's cascade of gastric oncogenesis

1.4.3.1 Chronic atrophic gastritis and gastric intestinal metaplasia

Recently published consensus guidelines on the management of gastric preneoplasia adhere to the convention observed for diseases elsewhere in the gastrointestinal tract in distinguishing between '*precancerous conditions*' and '*precancerous lesions'*(197). The eminent gastrointestinal pathologist Basil Morson defined the former as 'a clinical state associated with a significantly increased risk of cancer' and contrasted this with the definition of the latter as 'a histological abnormality in which cancer is more likely to occur'(198). CAG and IM are regarded as precancerous conditions whereas dysplasia/intraepithelial neoplasia of any grade is regarded as a precancerous lesion.

CAG is characterised histologically by a 'loss of appropriate glands'(199) and an accompanying loss of gastric secretory function. 'Loss of appropriate glands' encompasses two phenotypes of mucosal remodelling. Native glandular units might be lost and replaced by fibrous expansion of the lamina propria or glands might be replaced by metaplastic variants (i.e. not 'appropriate' glands). Hence this definition of gastric atrophy can include intestinal metaplasia. The distinction between these phenotypes is addressed in histopathological systems of nomenclature discussed below. So-called 'extensive' CAG involving the lesser curvature of the gastric corpus and fundus and resulting in achlorhydria is the specific variant associated with increased cancer risk(195,197,200).

Epithelial metaplasia is defined as a 'potentially reversible change from one fully differentiated cell type to another'(201) and its development implies cellular adaptation to environmental stimuli. Considered a step further in severity according to the Correa cascade, IM has been shown to confer a substantial risk of gastric cancer development which exceeds that of CAG alone(5,202). Metaplastic remodelling of the gastric epithelium is an inhomogeneous pathological process and several subtypes are recognised (by several systems of classification). Amongst the various types, *pseudopyloric* or *spasmolytic polypeptide-expressing metaplasia* (SPEM) and IM are associated with a higher risk of gastric cancer development with

the former considered a precursor for the latter(203). IM itself is subdivided by its histological characteristics. *Complete* IM describes gastric epithelium which exhibits the histological features of small intestinal epithelium, with classical enterocytes and goblet cells; whereas in *incomplete* IM, the features resemble those of the colonic epithelium. An additional system of nomenclature describes three types of IM with differing expression profiles of mucin. Type I IM (complete) expresses only sialomucins and type III IM (incomplete) only sulfomucins. Type II IM expresses both and is regarded as a mixed or indeterminate type. Several authors have reported higher rates of gastric cancer development in type III (or incomplete) IM(178,204) and a recent systematic review found that IM subtyping is a potentially important predictor of gastric cancer risk(205). IM is said to demonstrate a proclivity for developing first at the gastric incisura angularis before subsequent extension along the lesser curve to the corpus above and antrum below. The topographical extent of IM has been shown to correlate closely to its type i.e. extensive gastric IM comprises mostly type III (incomplete) IM and carries a significantly greater risk of carcinogenesis(206). For this reason, it has been suggested that topographical distribution of IM might serve as a surrogate for histopathological typing in estimating prognosis of gastric preneoplasia(202).

1.4.3.2 Dysplasia

There is disparity in nomenclature of dysplasia/intraepithelial neoplasia dependent on geographical location and area of observer interest but for the purposes of this thesis, we will adhere to the 'traditional' (World Health Organisation 2000) terminology of low- and high-grade dysplasia.

Dysplasia is regarded as a 'precancerous lesion' and was originally defined in terms of its histological phenotype(198). Molecular characterisation of gastric dysplastic lesions has reinforced the paradigm that such lesions are biologically contiguous with invasive cancer and that dysplastic cells derived from epithelia following their transformation to IM(207). The recognition of this close similarity between the morphological and genotypic characteristics of dysplastic lesions and invasive cancer essentially highlights the key distinguishing feature between them as being the ability of cancer epithelial cells to invade the stroma. Gastric dysplasia is characterised by epithelial atypia reflective of abnormal differentiation, loss of original glandular specialism and disorganised mucosal architecture (197,207).

Accurate diagnosis and grading of gastric dysplasia is regarded as important because these can be used to determine both the risk of cancer development in the lesion in question and the occurrence of metachronous cancer development(197). Reported rates of progression to cancer formation vary between 0% and 73%. Such variation presumably reflects both the heterogeneity of the disease and the wide variation in definitions and assessment of dysplasia(197,207,208).

1.4.4 Diagnosis and staging of gastric mucosal preneoplasia

The approach to the diagnosis and management of gastric preneoplasia is arguably dependent on the epidemiological features of the population in question. In some high-risk populations such as those seen in East Asian countries, diagnosis is frequently made as the result of population screening. In these endemic areas,

familiarity with diagnostic modalities and access to the requisite services for subsequent treatment and/or surveillance is likely to be well established. These measures are likely to be responsible for the high rates of early gastric cancer diagnosis and comparatively good survival rates seen in countries such as South Korea and Japan, where programmes for population screening for gastric cancer are well-established(209–211).

In low-risk populations however, population-based screening is unlikely to be cost-effective given the comparatively low burden of gastric cancer in these areas. Nevertheless, individuals in these populations still stand to benefit from strategies aimed at early cancer diagnosis and arguably from the diagnosis of premalignant conditions. For example, surveillance programmes for Barrett's oesophagus are well established in Western countries including the United Kingdom and the United States of America. Barrett's oesophagus is an interesting comparison as it also represents a condition that predisposes an individual to gastrointestinal epithelial adenocarcinoma development via an analogous inflammation-metaplasia-dysplasia-cancer pathway. Additionally, and as for gastric adenocarcinoma, survival rates for invasive adenocarcinoma at this site is very poor. The rates at which Barrett's oesophagus progresses to adenocarcinoma are unclear but two recent population-based studies conducted in Northern Ireland and Denmark, estimate the annual rates to be 0.38% and 0.12% respectively when restricted to those individuals with proven intestinal metaplasia(212,213). There are limited data regarding the rate of progression of gastric IM to cancer and published estimates vary considerably as a result of the wide variability in study design, nomenclature and population characteristics. One European study suggested an annual progression rate of 0.18%, which would suggest a similar magnitude of risk to that posed by Barrett's oesophagus(195). There would be considerable practical obstacles to introducing a surveillance protocol along the lines of Barrett's oesophagus however. In the first instance, securing an initial diagnosis is considerably more problematic (as discussed in the following sections). Not only this but the affected mucosal area for surveillance is much larger. It may therefore be more practical in these populations to consider more targeted screening (perhaps based on the presence of other risk

factors). The following sections outline how individual risk stratification might be undertaken in such populations.

1.4.4.1 Endoscopy

It is widely accepted that conventional white-light video endoscopy is of limited benefit in identifying either *H. pylori* gastritis or gastric preneoplastic lesions(197,214,215).

Emerging strategies to improve detection of these lesions (and others including early gastric cancer) seem to show promise. High-resolution video endoscopes(216), advanced imaging modalities(217–220) and magnification endoscopy with or without chromoendoscopy(221–223) result in improved accuracy in some studies but all are dependent on operator expertise and there are – as yet – no large multicentre studies supporting their widespread use(197).

1.4.4.2 Gastric biopsy sampling and histological assessment

Though gastric preneoplasia is sometimes referred to as a 'field change', implying involvement of the entire mucosal surface in precancerous remodelling, there is good evidence to support the assertion that these lesions are in fact multifocal and often patchy in their distribution(224,225). The most widely applied histopathological scoring system for gastric mucosal preneoplasia is the updated Sydney system(199). This recommends the sampling of five arbitrarily selected gastric sites – greater and lesser curve of the antrum, greater and lesser curve of the corpus and the angularis incisura. The recently published 'Management of precancerous conditions and lesions in the stomach (MAPS)' guideline advocates adherence to the modified Sydney protocol of two antral and two corpus biopsies without mandating a fifth biopsy from the incisura(197).

Though the modified Sydney score remains the best-known and most widely applied system for the uniform reporting of gastric preneoplastic lesions, it lacks the ability to offer prediction of the risk for gastric cancer development(199). To address this shortcoming, a validated scoring system was developed based upon the degree of gastric atrophy evident in biopsy specimens taken from the gastric antrum (including an angularis incisura sample) and that seen in specimens taken from the corpus(Table 1-1). This framework was produced by an international group of expert gastroenterologists and pathologists (the Operative Link for Gastritis Assessment (OLGA))(226).

Table 1-1 Operative link for gastritis assessment (OLGA) scoring matrix(226).

Atrophy score: 0 – no atrophy in biopsy specimens 1 – atrophy in 1-30% of specimens 2 – atrophy in 31-60% of specimens 3 – atrophy in >60%% of specimens		Corpus biopsy specimens - atrophy score *2 samples from corpus*			
		0	1	2	3
Antrum biopsy specimens – atrophy score *2 samples from antrum* *1 sample from incisura*	0	Stage 0	Stage I	Stage II	Stage II
	1	Stage I	Stage I	Stage II	Stage III
	2	Stage II	Stage II	Stage III	Stage IV
	3	Stage III	Stage III	Stage IV	Stage IV

Using this system, individuals with gastric atrophy can be categorised as '*low-risk*' (Stages I and II) or '*high-risk*' (Stages III and IV). The presence or absence of *H. pylori* organisms is included in the standardised report as is the presence of inflammatory lesions suggestive of undetectable *H. pylori* infection. The authors propose the surveillance of patients in the 'high-risk' category and this has been adopted in the previously mentioned MAPS guidelines(197) and validated as being an accurate predictor of prognosis of gastric preneoplasia in a 12-year follow-up study(227).

The reporting of gastric atrophy generally is prone to interobserver variation(207,228). To ameliorate this effect, Capelle et al. proposed a modified

system (operative link for gastric intestinal metaplasia (OLGIM)) based upon the presence of IM(229) for which interobserver reporting agreement is significantly greater(225,228). The authors proposed that an additional advantage of the OLGIM approach was that the number of patients categorised as 'high-risk' appeared to be lower, potentially reducing the number of individuals for whom surveillance would be indicated. In a subsequent prospective study however, although the OLGIM system (shown in Table 1-2) did exhibit the highest degree of interobserver agreement, a large proportion of individuals with high-risk gastric preneoplasia were excluded from the high-risk group suggesting insufficient sensitivity. In the same prospective study of 835 patients undergoing upper GI endoscopy the authors recommended a combination of OLGA and OLGIM scoring for staging chronic gastritis(228).

Table 1-2 Operative link for gastric intestinal metaplasia scoring matrix(229).

Intestinal metaplasia score: 0 – no IM in biopsy specimens 1 – mild IM in specimens 2 – moderate IM in specimens 3 – severe IM in specimens		Corpus biopsy specimens - IM score *2 samples from corpus*			
		0	1	2	3
Antrum biopsy specimens – IM score *2 samples from antrum* *1 sample from incisura*	0	Stage 0	Stage I	Stage II	Stage II
	1	Stage I	Stage I	Stage II	Stage III
	2	Stage II	Stage II	Stage III	Stage IV
	3	Stage III	Stage III	Stage IV	Stage IV

1.4.4.3 Noninvasive diagnosis and staging of gastric preneoplasia

Whilst endoscopy, adequate mucosal biopsy and rigorous histological assessment represent the best validated means of diagnosing and staging gastric preneoplasia, this approach does not meet the requirements of a screening test given its cost and inconvenience. For high-risk populations, noninvasive screening methods for the detection of gastric cancer are well established and include radiology and serological

tests(209,230). Whilst radiological modalities might be useful for detecting gastric mucosal abnormalities suggestive of cancer, they are not applicable to the diagnosis of gastric preneoplasia. Serological testing methods include serum pepsinogen concentrations and electrophoresis; the blood quininium resin test; *H. pylori* serology and serotyping and serum gastrin concentration.

The best-studied noninvasive markers of gastric mucosal preneoplasia are serum pepsinogens (PGs). Their usefulness in detecting CAG depends on the difference in topographical sites of secretion between PG1 and PG2 (see section 1.1.8). PG1 is secreted from oxyntic mucosa and PG2 by pyloric and duodenal mucosa. Gastritis leads an increase in serum concentrations of both pepsinogens but more so for PG2 hence PG1/2 ratio decreases. As CAG develops, loss of the normal oxyntic glands leads to a decrease in the concentration of PG1 and a more precipitous decrease in PG1/2 ratio is observed. A low serum PG1 concentration, PG1/2 ratio or both combined is therefore suggestive of CAG(231,232). The performance of PG1 concentration and PG1/2 ratio for detecting preneoplasia has been extensively investigated and widely varying results have been reported (233). A large, recent study in a high-risk population (South Korea) of 2558 individuals attending for population-based screening (for gastric cancer) by upper GI endoscopy also had serum pepsinogen 1 and 2 concentrations measured(234). The sensitivity and specificity of PG1/2 ratio for histologically proven CAG was 63.7% and 60.9% respectively (using a cutoff <3.0). Several groups have also examined the effect of adding *H. pylori* serological testing and/or serum gastrin (G17) concentration on the ability to detect preneoplasia. 'Compound' tests of this sort have been shown to improve test sensitivity and specificity in some series(235–237).

H. pylori serology is a useful contributor to the individual assessment of risk of the development of gastric cancer. It gives an indication not only of current *H. pylori* infection but also of previous infection and is independent of the gastric population of *H. pylori* bacteria. In patients with CAG, *H. pylori* infection may have been immunologically or medically cleared; or the bacterial population diminished as the microbial niche is lost during mucosal remodelling. In these higher-risk scenarios,

stool antigen or urea breath test methods may give negative results. *H. pylori* testing alone is recommended in the form of a 'test-and-treat' strategy in the Maastricht Consensus guidelines(238) for the management of younger patients with dyspepsia. This approach has been adopted by various national specialty organisations but rejected by some authors who point out the risk of early-onset gastric cancer in high-risk populations presenting with dyspepsia(239,240).

Serum G17 concentrations are relevant for two reasons: firstly the serum gastrin concentration offers an additional indicator for corpus atrophy and hypochlorhydria, where the absence of acid releases the negative-feedback 'brake' on gastrin secretion resulting in hypergastrinaemia. Secondly, low serum gastrin concentrations can offer some indication of antral atrophy – itself a preneoplastic 'condition'.

Several recent studies examining the usefulness of these combined tests – sometimes referred to as the 'serological biopsy' – for gastric preneoplasia have utilised a commercially available diagnostic kit. GastroPanel (Biohit Oyj, Helsinki, Finland) comprises serum ELISA kits for the determination of *H. pylori* IgG antibody concentration, serum G17 concentration and PG 1 and 2 concentrations; and their subsequent interpretation using a software-based analytical tool. The usefulness of this panel of noninvasive biomarkers is yet to be convincingly validated. A series in a European population (Spain) comprising 91 patients including 15 with CAG concluded that the diagnostic performance of the panel was inadequate for clinical use with sensitivity and specificity values of 50% and 80% respectively(241). These findings contrast with a larger, earlier series in Italy where the sensitivity and specificity of the GastroPanel for CAG in dyspeptic patients was 80% and 96% respectively.

1.4.4.4 Surveillance of gastric preneoplasia

Data supporting a specific follow-up regimen for gastric preneoplasia and its cost-effectiveness are scarce and demonstrative of considerable variation(197). The

aforementioned MAPS guideline provides a useful summary of current opinion on this subject.

High-grade dysplastic lesions are associated with a high-risk of metasynchronous invasive cancer or its early subsequent development(242). As such, they are staged and managed in much the same way as invasive cancer.

Low-grade dysplasia carries a lower risk of cancer development (in the region of 7% overall)(195,197) and so surveillance is recommended. The MAPS guideline suggests surveillance intervals of 12 months or less.

CAG and IM are relatively common and carry a relatively low risk of cancer development and so universal surveillance is not recommended. In the subgroup of patients with higher-risk preneoplasia (extensive CAG or IM) and/or with a family history of gastric cancer, surveillance is advised. Extent of CAG/IM should be determined histologically using the OLGA/OLGIM systems and/or serological phenotyping as discussed above. The guidelines suggest endoscopic assessment every 3 years(197).

1.5 Autoimmune atrophic gastritis

In section 1.4.3.1, I described the classical definition of gastric mucosal atrophy as representing a histological state in which there is a 'loss of appropriate glands'(199). In that section, I went on to describe the pathological entity of CAG, which results from chronic gastric mucosal inflammation (usually due to infection with *H. pylori*) and consists of loss of native glandular structures, which are replaced either with fibrosis or metaplastic glands.

A second, and distinct clinical entity in which chronic inflammation gives rise to chronic gastric atrophy is autoimmune atrophic gastritis (AIG). In this condition, autoantibody mediated T-cell destruction of parietal cells gradually leads to corpus-

specific atrophy with glandular metaplasia of varying types(243). The antrum is spared except in the relatively uncommon scenario in which chronic *H. pylori* infection has led to concomitant multifocal CAG.

1.5.1 Pernicious anaemia

Pernicious anaemia (PA) is the term given to the clinical syndrome to which AIG belongs. It is defined as the presence of anaemia, macrocytosis, low serum concentration of vitamin B_{12}, AIG and the presence of autoantibodies to intrinsic factor (IF) and/or gastric parietal cells (GPCs). The syndrome arises as a direct result of the gastric mucosal insult and the loss of intrinsic-factor secreting parietal cells culminates in failure of dietary B_{12} absorption. The associated symptoms are usually attributable to chronic vitamin B_{12} deficiency. Gastric hypochlorhydria often leads to failure of adequate dietary iron absorption and so associated iron deficiency is a common finding(244,245). Anti-GPC antibodies are found in 80-90% of cases with decreasing frequency as the disease progresses and GPC antigens are lost(246,247). These antibodies are commonly found in older individuals and in the context of other autoimmune diseases and so are not regarded as specific for PA. Anti-IF antibodies on the other hand are regarded as specific for the diagnosis(248). These are found in 40-80% of cases with the rate increasing with disease duration(249).

1.5.2 Epidemiology

Traditionally considered a disease of older, northern European women, AIG and PA is now recognised as having a broadly similar prevalence in all ethnicities and geographical locations though there is a female preponderance(243). The complexity of the diagnosis and frequency with which the syndrome is thought to go undiagnosed would suggest that prevalence figures are underestimated. A recent study reported an overall prevalence of 2% increasing to 4-5% in elderly females(250).

1.5.2.1 Risk of adenocarcinoma

As the metaplastic transformation seen in AIG comprises a similar spectrum of histopathological lesions as in CAG (IM and SPEM etc.), it seems reasonable to regard AIG as a preneoplastic condition. The risk of gastric adenocarcinoma however remains unclear with many small studies reporting conflicting data. A systematic review published in 2012 analysed six studies conducted in Europe and concluded that the annual risk of adenocarcinoma development was 0.27% with an overall relative risk of 6.8(251). There are no reliable data on which to base a strategy for endoscopic or non-invasive surveillance.

1.5.3 Development of type 1 gastric neuroendocrine tumours in autoimmune atrophic gastritis

Type 1 gastric neuroendocrine tumours are discussed in detail in section 1.6.3.

The risk of developing Type 1 gastric neuroendocrine tumours (NETs, carcinoids) as a result of AIG is unclear. Four European series have been published describing the incidence of type 1 gastric NETs in cohorts of individuals with established PA or AIG. The incidence rates in these cohorts varied from 3.6% to 7% at the first upper GI endoscopy.

1.6 Gastric neuroendocrine tumours

Gastric neuroendocrine tumours (NETs, carcinoids) are a heterogeneous group of neoplasms exhibiting dramatically varied natural histories and prognoses. They are increasing in incidence, presumably as upper GI endoscopy is becoming more commonplace and the equipment used ever more sensitive.

1.6.1 Classification

Three subtypes of gastric NET are widely recognised (Table 1-3)(252) though they are also classified according to the 2010 WHO classification of gastrointestinal neuroendocrine neoplasms (Table 1-4) which describes tumours by histological grade and mitotic and proliferative indices(253). The former classification takes into account clinical, biochemical and endoscopic variables and is more helpful in terms of prognostication. Although several gastric epithelial neuroendocrine cell types are recognised, gastric NETs are usually composed of ECL-cells(254) with G-cell and enterochromaffin cell tumours occurring vary rarely(253).

Table 1-3 Characteristics of the three types of gastric NET. AIG=autoimmune atrophic gastritis; PA=pernicious anaemia; ZES=Zollinger Ellison syndrome; MEN-1=multiple endocrine neoplasia type 1. Adapted from Burkitt et al.(255).

	Type 1	Type 2	Type 3	Type 4
Associated pathology	AIG and PA	ZES and MEN-1	Sporadic	Sporadic
Proportion of gastric NETs (from La Rosa et al.(256))	46%	6%	15%	<1%
Site	Corpus/Fundus	Corpus/Fundus	Any	Any
Typical number	Multiple	Multiple	Single	Single
Typical size of tumours	<10mm	<10mm	20-50mm	>20mm
Serum gastrin concentration	Increased	Increased	Normal	Normal
Gastric acid production	Decreased	Increased	Normal	Normal
Prognosis	Very good	Good	Poor	Very poor
Typical WHO grade	Grade 1	Grade 1	Grade 2>Grade1>NEC	Grade 2

Table 1-4 2010 WHO classification of gastric neuroendocrine neoplasms. NEC=neuroendocrine carcinoma; MANEC=mixed adenoneuroendocrine carcinoma. Adapted from La Rosa et al.(254).

WHO grade	Mitoses (per x10 HPF)	Ki67 index
Grade 1	<2	≤2%
Grade 2	2-20	3-20%
NEC	>20	>20%
MANEC	Variable	Variable

1.6.2 Epidemiology

The incidence of gastric NETs is increasing and they now represent approximately 4% of all NETs(257). A recent literature and cancer database review suggested that the annual incidence estimates from 10 European countries and the USA vary between 0.05 to 0.92 per 10,000 population(258). The same review estimated population prevalence in Japan of 0.05 per 10,000 people and in the USA of 0.3 per 10,000 people.

1.6.3 Type 1 gastric neuroendocrine tumours

1.6.3.1 Description and pathophysiology

Type 1 gastric NETs are usually found incidentally and arise on a background of AIG. As might be expected given the proclivity of AIG and PA for older females, type 1 gastric NETs are more commonly seen in older women(259). AIG results in parietal cell loss and hypochlorhydria. The negative feedback mechanism responsible for regulating gastrin secretion (described in section 1.1.7) - and which is dependent on gastric acid secretion - is lost. This results in G-cell hypertrophy and hypergastrinaemia, which in-turn leads to ECL-cell hyperstimulation, hypertrophy and proliferation. In the majority of AIG patients, micronodular ECL-cell hyperplasia

is observed(260). Interestingly, only a small minority of patients with AIG and hypergastrinaemia progress to the development of ECL-cell dysplasia and neoplasia. It is proposed therefore that hypergastrinaemia alone is insufficient for dysplastic transformation of ECL cells and several co-factors have been proposed.

The best characterised of these is the loss of heterozygosity (LOH) in the *MEN-1* gene. This gene encodes menin - a tumour suppressor. The best evidence for its contribution to NET formation comes from MEN-1 associated Zollinger-Ellison syndrome (ZES) in which an autosomal dominant defect in *MEN-1* leads to enhanced tumourigenesis. In this condition, gastric NET development is commoner than in the sporadic variant. LOH at 11q13-14 in *MEN-1* has been reported not only in MEN-1 associated ZES but in type 1 and type 3 gastric NETs(261). Growth factors contributing to dysplastic transformation include: α-human chorionic gonadotrophin which is found expressed in ECL-cell dysplasia but not in the ECL-cells of normogastrinaemic subjects(262); basic fibroblast growth factor (bFGF) and transforming growth factor-α (TGFα)(263,264).

1.6.3.2 Prognosis

Type 1 gastric NETs rarely metastasise and patients managed by surveillance and appropriate lesion resection have been shown to have 100% disease-survival(265). Without treatment of hypergastrinaemia however, this is a recurring tumour. Median recurrence-free survival is estimated at 24 months and an overall rate of neuroendocrine carcinoma development of 3% has been reported(265,266).

1.6.3.3 Treatment

There are limited data to guide management but guidelines published in 2012 offer a useful framework(265). A general approach of conservative management is recommended with regular endoscopic follow-up and lesion resection when indicated. Though some authors advise endoscopic resection of visible tumours,

there is no evidence that small (≤10mm) tumours exert unfavourable outcomes if simply monitored. For larger (>10mm) tumours, endoscopic ultrasound to exclude local invasion or nodal involvement is recommended prior to endoscopic or surgical resection. In the presence of local invasion or distal spread, it is advised that management should proceed along the same lines as for gastric cancer.

For larger tumours, surgical antrectomy (with or without tumour resection) to remove the source of hypergastrinaemia has been shown to effectively reduce both ECL-cell hyperplasia and gastric carcinoid tumour size and number in a proportion of patients(267,268). One drawback to this approach is the difficulty of predicting in advance whether or not the tumours in question are still 'gastrin-dependent' or if they have 'escaped' gastrin control and are proliferating independently. A strategy to stratify cases accordingly was proposed based upon the use of the octreotide suppression test. Our group reported a series of five cases in which the octreotide suppression test predicted response to antrectomy. In that series, one of the cases did not exhibit tumour regression despite a decrease in the serum gastrin concentration after surgery(269). It may be that response to novel gastrin/CCK2R antagonists might aid in the prediction of which patients might benefit from antrectomy.

Medical treatments for type 1 gastric NETs are still the subject of some debate. Jianu et al. reported the use of long-acting release octreotide for the treatment of type 1 gastric NETs for one year in five patients. Tumour load and ECL density was decreased on treatment and the effect was maintained after one year of follow-up. After five years of follow-up however, all five patients exhibited disease progression and one had developed an invasive cancer. The authors recommended that in patients treated with somatostatin analogues (SSAs), treatment should be continued indefinitely.

1.6.4 Type 2 gastric neuroendocrine tumours

Type 2 gastric NETs often complicate the natural history of ZES. In sporadic cases, they occur in fewer than 1% of cases whereas in ZES associated with MEN-1, there is a 20-30% risk of gastric NET development(270,271). These tumours share clinical and histological characteristics with the type 1 variant and are similarly dependent on hypergastrinaemia. They are distributed in the proximal stomach and are typically small (10-20mm) and multiple. Most cases behave (like type 1 NETs) indolently. One review suggested a lymph node involvement rate of 30%(271) and there is a risk of malignant transformation with rates of metastatic disease of up to 20% of cases; these are usually associated with the pro-tumourigenic MEN-1 variant(272,273).

Treatment of disease confined to the stomach is similar to that described for type 1 gastric NETs. Larger tumours can be resected endoscopically or surgically and multiple tumours have been effectively treated with somatostatin analogues(252,274). In suitable patients, localisation and resection of the culprit gastrinoma(s) is undertaken. In these cases, dependent gastric NETs are reported to have resolved following surgery(275).

1.6.5 Type 3 gastric neuroendocrine tumours

Sporadic type 3 gastric NETs are usually solitary and large (>20mm). They arise in non-atrophic gastric mucosa and are not dependent on hypergastrinaemia for development. They occur more commonly in males over the age of 50 years. Liver and lymphatic metastases are common and their presence correlates to tumour size in most cases. Metastatic disease is reported to occur in 50-70% of well-differentiated tumours and in up to 100% of poorly-differentiated tumours(252). In localised disease, the mainstay of treatment is surgical resection after appropriate staging in a similar fashion to the approach taken with gastric adenocarcinoma(265).

1.7 Netazepide (YF476)

1.7.1 Description and history

As outlined in section 1.5.4, both inhibition of gastrin synthesis (by targeting the somatostatin receptor with SSAs) and removal of gastrin producing tissue (by antrectomy) have been shown to be effective in treating type I gastric NETs in the short-term. The difficulty in administration (by regular, intramuscular injections) of SSAs weighed against the relative indolence of the condition diminishes its appeal as a long-term management strategy. Similarly, for small and (by implication) benign tumours, the risk of surgery would seem to outweigh the benefits for most patients. Alternative pharmacological strategies have been investigated including antagonists of the gastrin receptor.

Gastrin receptor antagonists (GRA) have been developed and trialled with limited success to date. Most have been limited by poor affinity with the CCK2 receptor, limited bioavailability, and inactivity when administered enterally. A recently developed compound - JNJ-26070109 – has been shown in animal models to have good receptor selectiveness and affinity, and high oral bioavailability(276). It inhibits gastric acid secretion and prevents PPI-induced rebound hyperacidity in rat models(277). This compound has yet to be tested in humans.

YF476 ((R)-1-2,3-dihydro-2-oxo-1-pivaloylmethyl-5-(2'-pyridyl)-1H-1, 4-benzodiazepin-3-yl-3-(3-methylamino-phenyl)urea) was developed in the UK by Ferring Pharmaceuticals from a series of benzodiazepine compounds(278) and later named ‘netazepide’. It is a highly potent, highly selective competitive antagonist of gastrin/CCK2R(279–281).

The drug was shown to inhibit pentagastrin stimulated gastric acid secretion in beagles(280). A study in healthy human volunteers taking netazepide twice daily for 7 or 14 days showed that the drug increased gastric pH in the first 24 hours of

administration in keeping with inhibition of gastric acid secretion. For reasons that remain unclear, when gastric pH was measured again after 7 days of dosing, the effect on pH had been lost despite ongoing elevation of plasma gastrin concentration(282).

Evidence for its potential effectiveness as a treatment for type I gastric NETs comes from animal models. The *Mastomys* rodent (*Praomys natalensis*) exhibits an accelerated response of the ECL cell to hypergastrinaemia. In response to the administration of H2-receptor antagonists (H2RAs) or PPIs, these animals develop ECL-cell tumours after approximately 2 months. In one study, four groups of animals were administered YF476 and Loxitidine (an H2RA, to induce hypergastrinaemia) alongside controls. The groups were designed to assess whether a) YF476 could prevent ECL-cell changes in response to hypergastrinaemia (YF476 & Loxitidine coadministered for 16 weeks); b) YF476 could prevent progression from hyperplasia to dysplasia or neoplasia both with (Loxitidine alone for 8 weeks followed by Loxitidine/YF476 coadministered for 8 weeks) and without (Loxitidine alone for 8 weeks followed by YF476 alone for 8 weeks) on-going hypergastrinaemia; c) YF476 could induce regression of ECL-cell neoplasia after a prolonged period of hypergastrinaemia (Loxitidine alone for 16 weeks followed by YF476 alone for 8 weeks). Gastrin and histamine were measured in peripheral plasma whilst gastric tissue was subjected to histological assessment and qPCR for ECL cell products (as a marker of ECL-cell activity). The outcomes were encouraging: there was a 60% reduction in tumour development and decreased ECL-cell activity in the group coadministered YF476 and Loxitidine. In animals treated with YF476 after a period of hypergastrinaemia, there was a reduction in tumour development compared with controls and decreased ECL-cell activity(283).

1.8 Study aims and objectives

1.8.1 Novel biomarkers of gastric preneoplasia following infection with *Helicobacter pylori*

Infection with *H. pylori* induces chronic gastric mucosal inflammation which can progress to gastric carcinogenesis. We designed an observational, cross-sectional study to investigate the role of gastric mucosal genes and proteins in the development of gastric preneoplasia and in so doing, identify novel biomarkers of these conditions.

Having identified one such potential biomarker (MMP-7) we then set out to determine the effect on its gastric mucosal expression of varying genotypes of genetic polymorphisms in the *MMP-7* gene.

1.8.2 A pilot study of the novel gastrin antagonist netazepide (YF476) for the treatment of type 1 gastric neuroendocrine tumours

Type 1 gastric NETs arise on a background of AIG and gastric hypochlorhydria as a result of hypergastrinaemia. Most behave indolently but some undergo malignant transformation, grow rapidly and give rise to metastases. Current treatment is conservative in the case of small tumours and surgical antrectomy to remove gastrin-secreting G-cells for the treatment of larger tumours. Medical treatment has been unsatisfactory to date. Netazepide (YF476) is a novel, potent, bioavailable ad orally-active gastrin/CCK2R antagonist.

We designed a phase 2, open-label pilot study to investigate the effectiveness in 8 patients of netazepide treatment for type 1 gastric NETs by means of endoscopic features, gastric mucosal biomarkers and circulating biomarkers of ECL-cell activity.

2 Materials and Methods

2.1 Investigating biomarkers for gastric mucosal preneoplasia development following chronic Helicobacter pylori infection

The study described here and in chapters 3, 4 and 5 was funded by the National Institute for Health Research (NIHR) via the Liverpool Biomedical Research Centre (BRC) and sponsored by the Royal Liverpool and Broadgreen University Hospitals NHS Trust (R&D No. 3592) and the University of Liverpool. Prior to initiation, local ethics committee (Liverpool (Adult) Research Ethics Committee REC:08/H1005/37) approval was obtained. Patients were recruited and samples processed by Dr. S. Murugesan (SM, Department of Gastroenterology, University of Liverpool, Liverpool, UK) (patient code numbers BRC1 – BRC1017) and Dr. A. R. Moore (ARM, author) (patient code numbers BRC1018 – BRC1400). SM performed serum gastrin radioimmunoassay (RIA) for all 1017 patients enrolled by him as well as the serum CagA enzyme-linked immunosorbent assay (ELISA) for those patients from the same cohort found to be *H. pylori* positive. ARM performed the same assays for the remaining patients as well as MMP-7, pepsinogen 1 and pepsinogen 2 ELISA for selected patients from the entire study cohort. Ms. L. Rainbow (LR) and Dr. I. A. Steele (IAS) (Department of Cellular and Molecular Physiology, University of Liverpool, Liverpool, UK) performed gastric tissue processing, mRNA extraction and reverse transcription and subsequent qPCR assay for candidate biomarkers.

Case report forms (CRFs) (appendix C) were used to record patient details and were securely stored at the Royal Liverpool University Hospital (RLUH) and will be destroyed 15 years after study completion. A materials transfer agreement was implemented to enable the transfer of biopsy and blood specimens to the Department of Cellular and Molecular Physiology, University of Liverpool to be used for study-related experiments and/or secure storage.

Study recruitment tool place between May 2008 and July 2011. Participants were recruited at the Department of Gastroenterology, RLUH before undergoing elective outpatient upper GI endoscopy. A patient information leaflet (PIL) explaining the study rationale and procedure was provided either by post in advance of the hospital attendance or in person upon arrival at the endoscopy unit (appendix B). All participants were given sufficient time to read the PIL, discuss the study and ask questions before agreeing to proceed. All included participants were aged 18 years or older, capable of giving informed written consent and had a clinical indication for upper GI endoscopy. The principle exclusion criteria were: haemodynamic instability or active bleeding at endoscopy; moribund or established terminal malignancy; hepatic cirrhosis; bleeding diathesis or current anticoagulation; pregnancy; HIV, hepatitis B or hepatitis C infection or other contraindication to endoscopy.

Patients recruited were referred either by their primary-care general practitioner (GP) or by another clinical service at the same hospital. In the case of GP referrals, these were subdivided into two groups: “2-week rule” referrals were reserved for those cases in which the referring clinician suspected an upper GI malignancy. The remainder were “routine” referrals of patients with upper GI symptoms. For each patient, a researcher completed a case record form (CRF). The patients identifying details (name, date of birth and hospital number) were recorded on both the CRF and in a subject enrolments log. All other records were anonymised. The CRFs and subject enrolments log were kept securely on hospital premises and accessed only by members of the research team. For each patient, a CRF was completed prior to endoscopy. This included demographic data, height and weight measurement and past medical & social history data {appendix C}. CRF data was subsequently transferred to an encrypted Microsoft Excel spreadsheet with only the study patient code number attached to each subject record. The results of the various analyses and assays described in this section were later added to the same spreadsheet along with the storage location of biological samples.

2.2 Collection of biological samples

Patients were fasted for at least 12 hours prior to endoscopy. Enrolled subjects underwent routine upper GI gastroscopy using Olympus Evis Lucera H240/H260 endoscopes (Olympus, Southend-on-Sea, UK) including pinch biopsy (Single-Use Radial Jaw 4 - Boston Scientific, Hemel Hempstead, UK) of gastric mucosa. Biopsies were obtained from the antrum and corpus (4 per site) for histopathological assessment and from the corpus (8 biopsies) for subsequent determination of real-time polymerase-chain reaction (PCR) abundance of putative biomarkers. Prior to the procedure, we drew 20mL of peripheral venous blood from each subject by simple venepuncture or via the intravenous cannula inserted for the purpose of sedation-drug administration. In the case of patients unable to tolerate endoscopy sufficiently to enable the collection of mucosal biopsies, blood samples were retained and analysed as described below though the resulting data was excluded from the final analyses. When recruited patients subsequently withdrew consent for participation in the study, any biological samples were discarded.

2.2.1 Histopathology specimens

Specimens for histology were first fixed and stored in 10% neutral-buffered formalin and then embedded in paraffin for slide preparation and staining with haematoxylin and eosin. Slides were prepared and reported on by the hospital histopathology service in the first instance. We subsequently cut additional slides from the paraffin blocks for submission to the study pathologist. A single, expert gastrointestinal (GI) pathologist (Dr. Laszlo Tiszlavicz, Department of Pathology, University of Szeged, Szeged, Hungary) examined all study specimens and prepared standardised reports (appendix A) incorporating descriptive diagnoses and scores for the modified Sydney classification(199), Padova classification(284) and modified Vienna classification(285).

2.2.2 *Helicobacter pylori* rapid urease testing

One mucosal biopsy each from the antrum and corpus was used to detect the presence of *H. pylori* by means of a rapid urease test (RUT) (*Pronto Dry*, MIC, Brignais, France). The RUT slides were read an hour after sample collection and the results recorded in the CRF.

2.2.3 Mucosal biomarker analysis specimens

In addition, eight biopsies were taken for RNA analysis from the gastric corpus and stored in RNAlater (Ambion, Austin, TX, USA). These were stored at -20°C prior to RNA extraction in batches (described in section 2.5.1).

2.2.4 Venous blood samples

20mL of peripheral venous blood was drawn as described above (section 2.2). 4.5mL was immediately decanted into EDTA tubes (BD Vacutainer – BD, Oxford, UK), transported on ice and stored at -20°C for subsequent genomic DNA extraction (described in section 2.3). 1mL was 'spotted' onto protein saver cards (Whatman 903 protein saver card – Sigma-Aldrich, Gillingham, UK) and stored at room temperature. 4.5mL was sent in serum gel tubes (*S-Monovette* – Sarstedt Ltd., Leicester, UK) to the microbiology laboratory at the host hospital for anti-*H. pylori* IgG ELISA (Biokit, Barcelona, Spain). The remaining 10mL was transported on ice in serum gel tubes and separated by centrifugation (7 minutes, 4°C, 800G). Three aliquots of the serum were stored at -20°C.

2.3 Genomic DNA extraction

Genomic DNA was separated and extracted using a magnetic bead separation technique (*chemagic* Magnetic Separation Module I (MSM-I) with *chemagic* DNA

Blood Kit (CMG-703-1), PerkinElmer *chemagen* Technologie GmbH, Baesweiler Germany). This technique uses proprietary polyvinyl-alcohol magnetic beads whose surface is prepared to bind nucleic acids. The MSM-I machine uses electromagnetism and mechanical agitation to bind DNA from whole blood and separate it via a series of buffers and solvents. Our 4.5mL samples of whole blood were processed in batches of 12 and the resulting 500µL aliquots of genomic DNA in elution buffer stored at 4°C. DNA concentration was determined using spectrophotometry. Prior to SNP genotyping, samples of standardised concentration (20ng/µL) were prepared. These were also stored at -20°C.

2.4 Serum sample analyses

2.4.1 Serum gastrin radioimmunoassay

Previously stored serum was used in radioimmunoassay (RIA) to determine the fasting serum gastrin concentration for each patient. RIA depends on competition for binding of radiolabelled gastrin with endogenous gastrin in the sample with a known concentration of gastrin antibody. Samples were assayed in duplicate and in batches. For study subjects with the study identification numbers BRC1017 to BRC1400, the samples were analysed in nine separate assays. These were performed by ARM and each included a serum sample taken from a stock provided by a non-patient volunteer and which acted as an interassay control.

Sodium barbitone buffer was prepared afresh prior to each assay from sodium azide (0.5g) and sodium barbitone (4.12g) dissolved in dH_2O and the pH adjusted to 8.4 before the addition of bovine serum albumin (1mL, Sigma-Aldrich, Gillingham, UK). Standards were prepared in concentrations of 1pM, 0.1pM and 0.01pM of synthetic human gastrin (synthetic human unsulphated heptadecapeptide gastrin (G17), Bachem, Bubendorf, Switzerland) and by serial dilution in sodium barbitone buffer. Labelled G17 ([125I]G17, PerkinElmer, Massachusetts, USA) was diluted to a standard radiation activity level (20000 com/mL). Charcoal suspension was prepared

by adding 10g of activated charcoal to 100mL dH_2O and later adding dextran (0.5g, D-4751, Sigma-Aldrich, Gillingham, UK) and skimmed fat-free milk powder (0.5g, Marvel International Food Logistics Ltd., London, UK) in solution with dH_2O. Standards and samples were added to the assay tubes with sodium azide buffer, label and L2 antigastrin antibody to a volume of 1mL; mixed and incubated at 4°C for 2 days. Samples were added to each assay tube at a volume of 50µL with 100µL each of label and L2 antigastrin antibody and 750µL of buffer up to a total volume of 1mL. The three standards were added in different volumes (to generate standards of 10 different concentrations) and the volume of buffer added varied to ensure that the total volume amounted to 1mL.

The assay was then 'separated' – 100µL charcoal was added to each tube before they were centrifuged (3000rpm, 10 minutes, 4°C). The resulting supernatant (containing the antibody-bound label) was poured into a separate tube, leaving the charcoal pellet (containing the unbound label). The radioactivity in each was measured to give the bound/free label ratio. The sample ratios were corrected for non-specific binding (calculated from the non-sample control tubes) and a standard curve plotted from the standards. Sample bound/free ratios were plotted against the standard curve (line of best fit, manually) to give the serum gastrin concentration. An example standard curve is shown in Figure 2-1. 'High' results, lying outside the linear portion of the standard curve were diluted and reanalysed in subsequent assays. Samples whose duplicate values differed by more than 10% were also reassayed.

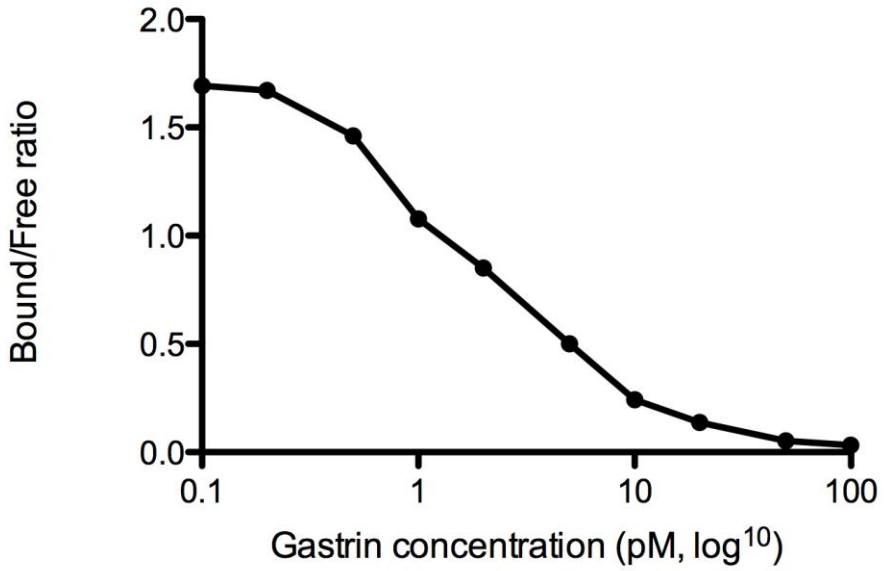

Figure 2-1 Standard curve from radioimmunoassay for subjects BRC1262-1346 inclusive

The mean serum gastrin concentration in the interassay control was 74.7pM (SEM 2.7, SD 8.2) with a coefficient of variation of 11.0%.

2.4.2 Serum CagA IgG enzyme-linked immunosorbent assay (ELISA)

CagA status of *H. pylori* was determined for all subjects with a 'positive' test result (histology, serology or gastric biopsy urease testing). We used a commercially available solid-phase sandwich-type ELISA kit (CagA IgG ELISA kit (GD33) Genesis Diagnostics Ltd, Littleport, UK) and conducted the assay according to the manufacturer's instructions. The kit comprises 96-well plates each well of which is coated with fixed, recombinant CagA protein. Standards of known concentrations of recombinant anti-CagA IgG were provided for inclusion in each assay and for the purposes of producing a standard curve.

Patient samples were diluted in the provided diluent to 1:200, and 100µL of diluted samples, standards or controls added to assay wells in duplicate. These were incubated at room temperature for 30 minutes before thorough washing with the

provided wash solution. 100µL of leporine anti-human CagA IgG antibodies conjugated to horseradish peroxidase (HRP) – the conjugate – was then added to each well and incubated for a further 30 minutes before repeated washing. In the final step, 100µL of tetramethylbenzidine (TMB) substrate was added and the plate incubated for 10 minutes. The reaction was terminated using provided sulphuric acid and the resulting colour change quantified by optical densitometry using a microplate reader (Tecan GENios Plus with XFLUOR4 software v4.51 – Tecan UK Ltd., Reading, UK) at an absorption wavelength of 450nm. Results were corrected against the blank absorbance value and plotted against the standard curve (line of best fit using GraphPad Prism v5.0a statistical software). Each sample was then categorised as being CagA positive (>7 U/mL), negative (<5.5 U/mL) or indeterminate. An example standard curve is shown in Figure 2-2. Samples yielding indeterminate results were reassayed. If found to be indeterminate a second time, they were grouped with negative samples for the purposes of data analysis.

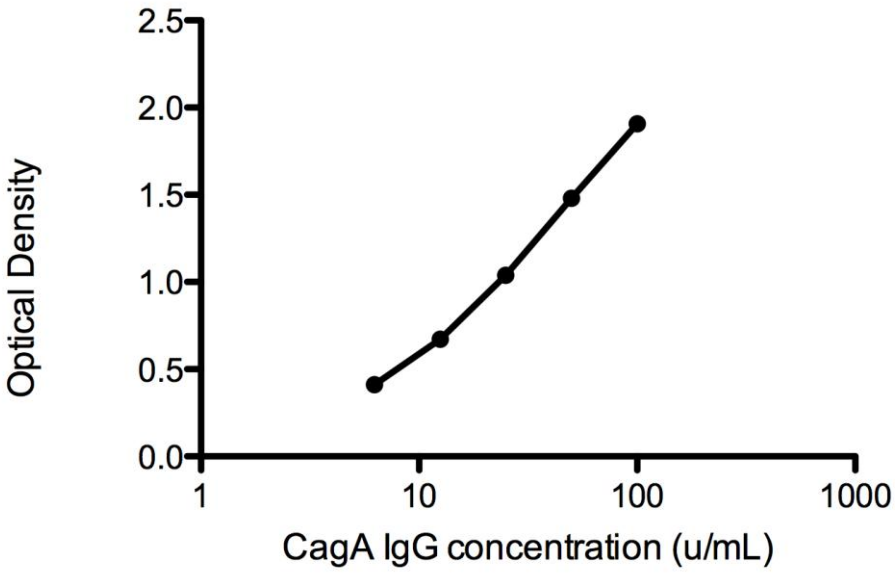

Figure 2-2 Standard curve from CagA ELISA for *H. pylori* positive subjects BRC1303-1387 inclusive

Each assay included positive and negative control samples and the former was used as an interassay control. There were a total of 13 assays performed including 584

samples from study subjects. The mean CagA IgG concentration was 33.3u/mL (SEM 0.95u/mL, SD 3.41u/mL). The coefficient of variation was 10.3%.

2.4.3 Serum MMP-7 enzyme-linked immunosorbent assay (ELISA)

We used another commercially available solid-phase sandwich-type ELISA kit (Human Total MMP-7 Quantikine ELISA Kit (DMP700) R&D systems, Abingdon, United Kingdom) and conducted the assay according to the manufacturer's instructions.

In this kit, recombinant MMP-7 was provided for reconstitution in dH_2O to give a solution of known concentration (100ng/mL). This was serially diluted to generate the standards required for production of a standard curve. These were added to the plate in the same way as the study serum samples.

Serum samples were diluted in the provided 'calibrator diluent' (100µL sample, 100µL diluent) and 50µL of the resulting solution added to the plate in duplicate along with 100µL of the provided 'assay diluent'. The assay plate was then incubated at room temperature for 2 hours on an orbital shaker (500rpm) before thorough washing with the provided wash buffer. 200µL of MMP-7 conjugate was added to each well and incubated for another 2 hours as before. After repeated washing, 200µL of the 'substrate solution' was added to each well and the plate incubated in the dark at room temperature for 30 minutes after which the reaction was terminated by the addition of the provided 'stop solution'. The resulting colour change was quantified by optical densitometry using a microplate reader (Tecan GENios Plus with XFLUOR4 software v4.51 – Tecan UK Ltd., Reading, UK) at an absorption wavelength of 450nm and using a reference wavelength of 620nm. Results were corrected against the blank absorbance value and plotted against the standard curve (line of best fit). An example standard curve is shown in Figure 2-3.

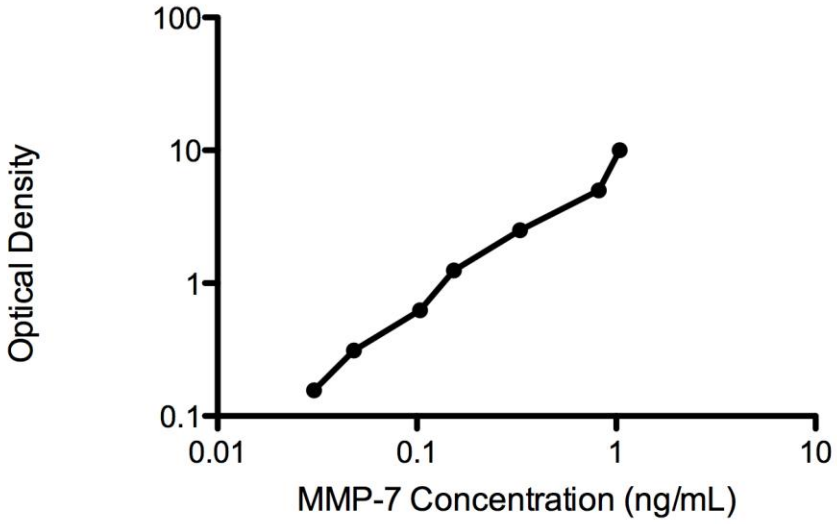

Figure 2-3 Standard curve from serum MMP-7 ELISA for selected subjects (assay number 1)

We performed 25 assays and included samples from 606 study subjects as well as samples from the subjects of other trials (including the netazepide trial whose results are reported in chapter 6). A serum sample taken from a stock provided by a non-patient volunteer was included in each assay and acted as an interassay control. The mean concentration in these controls was 1.90ng/mL (SEM 0.04ng/mL, SD 0.21). The coefficient of variation was 11.1%.

2.4.4 Serum Pepsinogen 1 enzyme-linked immunosorbent assay (ELISA)

We used another commercially available sandwich-type, solid-state ELISA kits (Pepsinogen I ELISA Kit (601010) - Biohit Healthcare Ltd. Ellesmere Port, United Kingdom) and conducted the assay according to the manufacturer's instructions.

Solutions of known concentrations of pepsinogen 2 (PG2) were provided to enable the production of a standard curve. Study subject serum samples were diluted 1 to 5 in the provided diluent buffer (100µL serum and 400µL buffer). 100 µL of the sample solutions and standards was added in duplicate to relevant wells, covered and the plate incubated for 1 hour at room temperature. After this, the wells were washed

thoroughly with the provided 'washing buffer' and 100 µL of the 'conjugate solution' added to each for incubation at room temperature for another hour. After repeated washing, 100 µL of the 'substrate solution' was added to each well and the plate incubated for a further 30 minutes in the dark at room temperature. The reaction was then terminated by the addition to each well of the provided 'stop solution'. The resulting colour change was quantified by optical densitometry using a microplate reader (Tecan GENios Plus with XFLUOR4 software v4.51 – Tecan UK Ltd., Reading, UK) at an absorption wavelength of 450nm. Results corrected for blank absorbance were plotted against the standard curve (line of best fit using GraphPad Prism v5.0a statistical software). An example standard curve is shown in Figure 2-4.

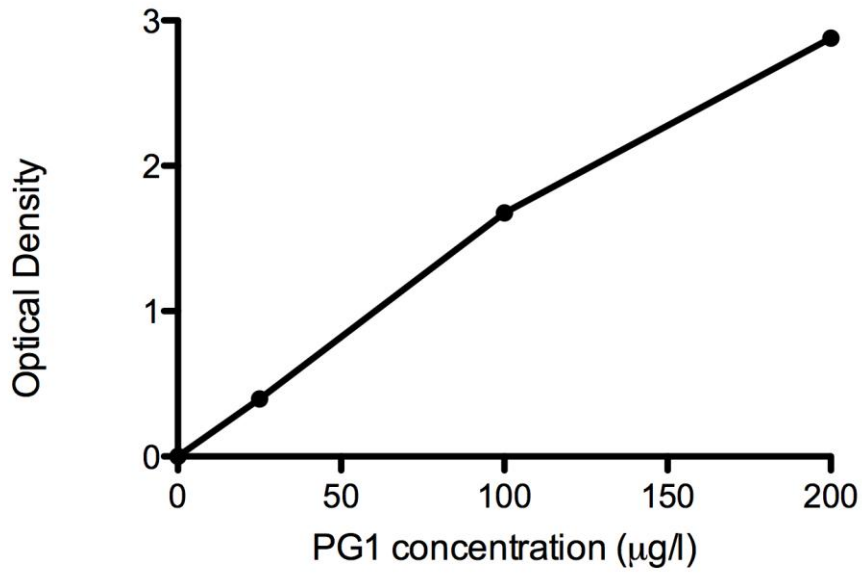

Figure 2-4 Standard curve from serum pepsinogen 1 ELISA for selected subjects (assay number 1)

We completed 10 assays and included samples from 397 study subjects. Each kit included a control sample of known concentration and which acted as an interassay control. The mean concentration of PG1 was 35.3µg/l (SEM = 0.47µg/l, SD = 1.48micg/l). The coefficient of variation was 4.2%.

2.4.5 Serum Pepsinogen 2 enzyme-linked immunosorbent assay (ELISA)

We used a commercially available sandwich-type, solid-state ELISA kits (Pepsinogen 2 ELISA Kit (601020) - Biohit Healthcare Ltd. Ellesmere Port, United Kingdom) and conducted the assay according to the manufacturer's instructions.

Solutions of known concentrations of pepsinogen 2 (PG1) were provided to enable the production of a standard curve. Study subject serum samples were diluted 1 to 10 in the provided diluent buffer (50µL serum and 450µL buffer). 100 µL of the sample solutions and standards was added in duplicate to relevant wells, covered and the plate incubated for 1 hour at 37°C. After this, the wells were washed thoroughly with the provided 'washing buffer' and 100 µL of the 'conjugate solution' added to each for incubation at 37°C for 30 minutes. After repeated washing, 100 µL of the 'substrate solution' was added to each well and the plate incubated for a further 30 minutes in the dark at room temperature. The reaction was then terminated by the addition to each well of the provided 'stop solution'. The resulting colour change was quantified by optical densitometry using a microplate reader (Tecan GENios Plus with XFLUOR4 software v4.51 – Tecan UK Ltd., Reading, UK) at an absorption wavelength of 450nm. Results corrected for blank absorbance were plotted against the standard curve (line of best fit using GraphPad Prism v5.0a statistical software). An example standard curve is shown in Figure 2-5.

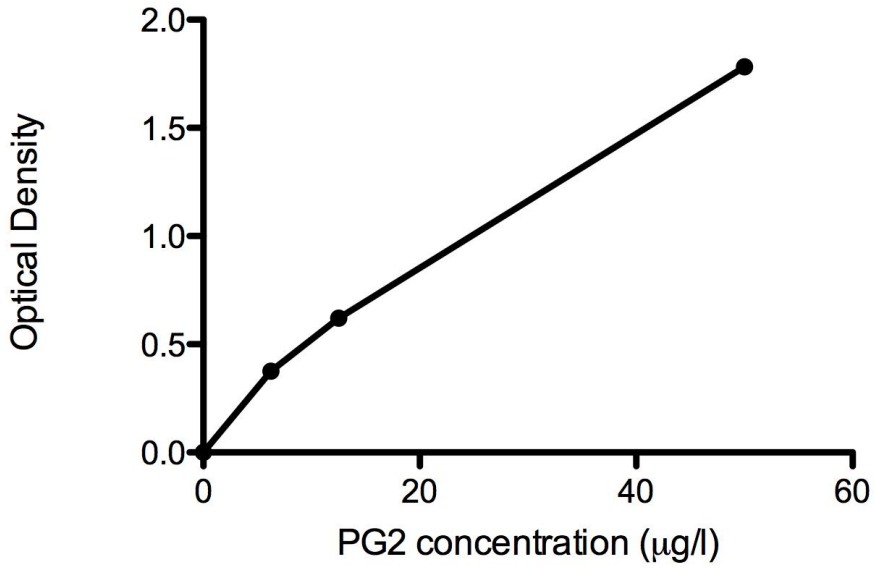

Figure 2-5 Standard curve from serum pepsinogen 2 ELISA for selected subjects (assay number 1)

We completed 10 assays and included samples from 397 study subjects. Each kit included a control sample of known concentration and which acted as an interassay control. The mean concentration of PG1 was 33.5μg/l (SEM = 0.12μg/l, SD = 0.37micg/l). The coefficient of variation was 1.1%.

2.5 Gastric mucosal biopsy sample RNA extraction and reverse transcription

2.5.1 RNA extraction

Gastric mucosal biopsy samples taken at endoscopy were stored in RNAlater as described in section 2.2.3. Prior to RNA extraction, samples were thawed and separated from RNAlater by blotting. We used an adaptation of the TRI Reagent Protocol(286). Samples were homogenised in Tri reagent (Sigma-Aldrich, Gillingham, UK) until no visible tissue particles remained. The resulting suspension was allowed to settle at room temperature for 5 minutes before 0.2mL chloroform was added and the samples shaken. The sample was allowed to settle for a further 5 minutes before centrifugation (5 minutes, 12000G, 4°C). The clear, aqueous phase of the

resulting separated suspension was transferred to a plastic tube and 0.5mL of isopropanol added. After standing for 5 minutes at room temperature, this solution was centrifuged (10 minutes, 12000G, 4°C) and the supernatant discarded to leave a 'pellet' of precipitated RNA. This was 'washed': first with 1mL 75% ethanol (-20°C) and centrifuged (5 minutes, 12000G, 4°C); then with 0.5mL 100% ethanol (-20°C). The ethanol was then poured off and the pellet allowed to air dry for 15 minutes. The RNA pellet was then dissolved in 50µL diethylpyrocarbonate (DEPC)-treated (DEPC) water (Sigma-Aldrich, Gillingham, UK). RNA concentration in the resulting solution was determined by spectrophotometry. Sufficient solution was immediately drawn for reverse transcription (section 2.5.2) and the remainder stored under ethanol in 2 aliquots.

2.5.2 Reverse transcription of extracted RNA

For reverse transcription, sufficient RNA solution to provide 2µg of RNA was added to a plastic tube. We added Oligo(dT) primer (Oligo(dT) primer 15 – Promega, Southampton, UK) and DEPC-treated water to a total volume of 39µL. The solution was briefly centrifuged to collect all liquid at the bottom of the tube and then incubated in a heating block for 5 minutes at 70°C after which the sample was allowed to cool in the block to 40°C to achieve RNA and primer annealing.

The cooled tube was then removed from the block and we added reaction buffer (12µL, 5X AMV buffer - Promega); deoxynucleotide solution set (5µL, 100mM DNTP set - Sigma-Aldrich, Gillingham, UK); ribonuclease inhibitor solution (1µL, RNAsin recombinant ribonuclease inhibitor - Promega); and reverse transcriptase solution (3µL, AMV reverse transcriptase – Promega). The solution was briefly centrifuged to collect all liquid at the bottom of the tube and then incubated in a heating block for 1 hour at 42°C. The reaction was terminated by briefly heating the reaction tube to 80°C in the heating block before cooling on ice. The resulting cDNA solution was then divided into 13 aliquots of 4.3µL and stored at -20°C. For this study, LR and IAS performed all RNA extraction and reverse transcription.

2.6 Quantitative (real-time) polymerase chain reaction for gastric mucosal gene products

We used quantitative (real-time) polymerase chain reactions (qPCR) to determine the abundance of putative biomarkers of gastric mucosal neoplasia. For this study, LR and IAS performed all qPCR assays using the Applied Biosystems 7500 real time PCR system (with 7500 System SDS software v1.4) using TaqMan chemistry double dye (5'-FAM, 3'TAMRA) probes.

2.6.1 Assay design

Primers and probes were selected using DNASTAR PrimerSelect software (DNASTAR Inc., Wisconsin, USA) and are shown in Table 2-1. Standards were produced by first amplifying the gene of interest by PCR before ligating this into pGEM-T Easy vector plasmid (Promega). Following restriction enzyme digestion and clone selection, *Escherichia coli* (*E. coli* DH5α, Invitrogen, Paisley, UK) were transformed to incorporate the plasmid. Plasmid-inclusive colonies were selected (by pGEM-T Easy blue/white selection), cultured and harvested. Resulting plasmid products were sequenced to confirm the presence of the genes of interest and were diluted to create standards of 6 known concentrations (100pg/µL, 10pg/µL, 1pg/µL, 0.1pg/µL, 0.01pg/µL and 0.001pg/µL). qPCR was used to optimise the concentrations of probes and of forward and reverse primers. Assay design, standard production and PCR optimisation was performed by IAS.

Table 2-1 Primer and probe sets employed in qPCR for determination of relative abundance of mucosal gene products.

Gene	Probe	Forward primer	Reverse primer
GAPDH	CGTCGCCAGCCGAGCCACA	GCTCCTCCTGTTCGACAGTCA	ACCTTCCCCATGGTGTCTGA
IGF-1	ACATGCCCAAGACCCAGAAGGAAGTACA	TGTATTGCGCACCCCTCAA	ACTCCCTCTACTTGCGTTCTTCA
IGF-2	CCCAGATACCCCGTGGGCAAGTTC	CCGTGCTTCCGGACAACTT	GGACTGCTTCCAGGTGTCATATT
MMP-1	TTGCAGCTCATGAACTCGGCCATTC	CCAACAATTTCAGAGAGTACAACTTACAT	TGAAGGTGTAGCTAGGGTACATCAAA
MMP-3	TTGCTGCTCATGAAATTGGCCACTCC	ACAAAGGATACAACAGGGACCAA	TAGAGTGGGTACATCAAAGCTTCAGT
MMP-7	CCTGTATGCTGCAACTCATGAACTTGGC	GGATGGTAGCAGTCTAGG GATTAACT	GGAATGTCCCATACCCAAAGAA
PAI-1	AGTTCAACTATACTGAGTTCACCACGCCCG	TGCCCATGATGGCTCAGA	GCAGTTCCAGGATGTCGTAGTAATG
PAI-2	CCAATGCAGTTACCCCCATGACTCCA	GGCCAAGGTGCTTCAGTTTAAT	TGAACCCACAGCTGGTAAAGTTC
TIMP-1	CCAGAACCGCAGTGAAGAGTT	GACGGCCTTCT CAATTCC	GGTATAAGGTGGTCTGGTTGACTTC
TIMP-3	CCGACATCGTGATCCGGGCC	CCAGGACGCCTTCTGCAA	CCCCTCCTTTACCAGCTTCTTC

2.6.2 Real-time qPCR conditions

All reactions were performed using the Applied Biosystems 7500 and TaqMan chemistry double dye probes as described above. We used Precision 2x qPCR MasterMix (Primerdesign Ltd., Southampton, UK) and total reaction volumes per well of 25µL. Reactions were conducted in 96-well plates with adhesive covers (MicroAmp Optical 96-well reaction plate and MicroAmp Optical Adhesive Film – Applied Biosystems). Each plate included non-template controls (NTCs), standards (for both the selected housekeeper gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and for the gene of interest) and study subject cDNA samples (for quantification of both housekeeper gene and gene of interest abundance) in triplicate. The thermal cycling conditions used are shown in Table 2-2.

Table 2-2 Thermal cycling conditions for qPCR assays.

Stage	Cycles	Temperature (°C)	Time (mins:secs)
1	1	50	02:00
2	1	95	10:00
3 (i)	40	95	00:15
3 (ii)		60	01:00

2.6.3 qPCR assay analysis

We used the 'standard curve' data analysis protocol from the Applied Biosystems SDS v1.4 software to control, monitor and harvest results from the qPCR assays. The software generates a standard curve from the wells designated as containing a standard of known concentration plotted as threshold cycle (CT) against concentration. An example standard curve is shown in Figure 2-6. The standard curve was used to determine the DNA concentration both of the housekeeper gene and the gene of interest in the reaction samples. From these values, a ratio of the two was calculated – the relative abundance of the gene of interest.

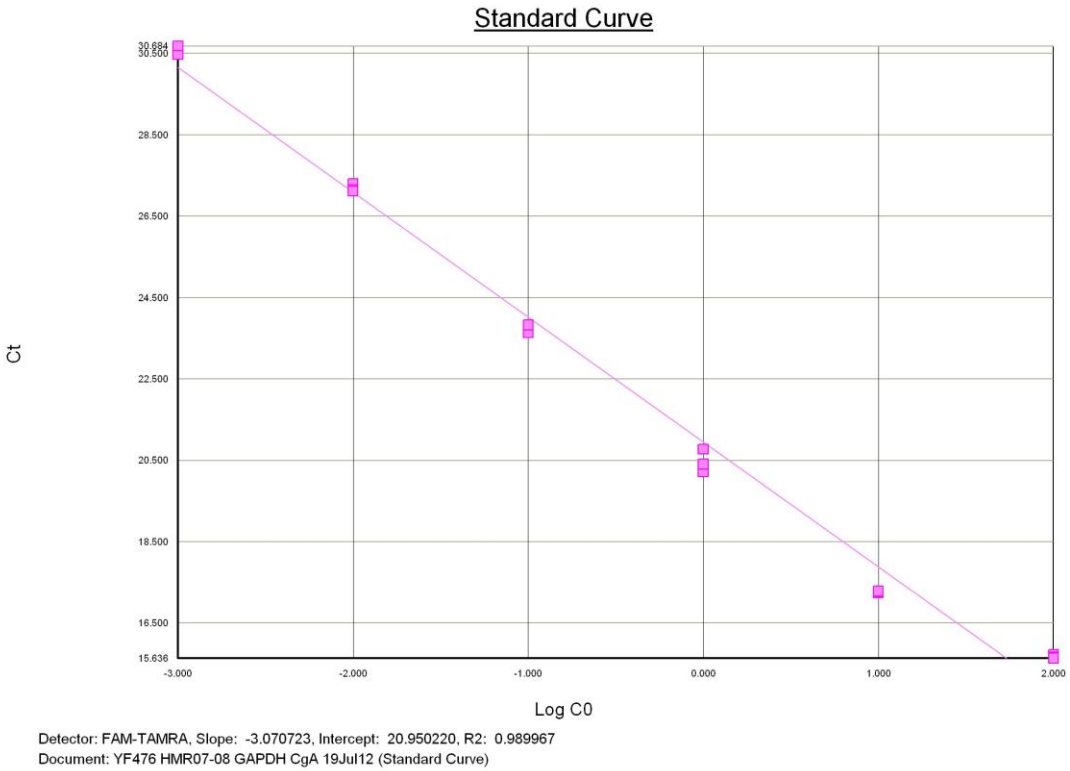

Figure 2-6 Standard curve from qPCR for GAPDH.

2.7 Determining the effect of MMP-7 gene SNP polymorphisms on the development of gastric mucosal preneoplasia

2.7.1 SNP selection

The present study was designed and funded to include genotyping for between five and ten individual SNPs. As our resources were therefore limited, we attempted to identify SNPs that were a) previously described in the literature as being associated with epithelial disease in humans or b) predicted to be 'functional' and c) useful as 'tagger' polymorphisms. SNP selection was performed by referring to several databases as follows:

2.7.1.1 Literature search

We searched the NCBI PubMed database (http://www.ncbi.nlm.nih.gov/pubmed, accessed November 2011) for reports of clinically relevant SNPs in the MMP-7 gene. Our principal search combined the following terms (with various suffixes shown in parentheses): “matrix metalloproteinase (7, -7)”; “matrix metallopeptidase (7, -7)”; “MMP (7, -7)” and “matrilysin” with: “polymorphism”; “variant’ and “SNP”. We also searched for reports of polymorphisms in the genes of other MMPs and TIMPs. We made note of the organ and disease associated with each reported polymorphism.

2.7.1.2 SNP database search

We interrogated the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/, accessed November 2011) for listed SNPs in the MMP-7 gene. Geneview was utilised to identify SNPs in the MMP-7 gene with both a) predicted ‘functionality’ i.e. those expected to alter the expression or structure of the MMP-7 protein and b) a minor allele frequency of greater than 0.05 in the reference European population. The results were saved for further analysis in Haploview.

2.7.1.3 SNP characteristics

Finally, we used Haploview haplotype analysis software(287) to further characterise our list of candidate SNPs. We examined the SNP linkage disequilibrium map and identified ‘tagging’. In identifying these SNPs, the presence of other polymorphisms in the haplotype can be predicted.

2.7.2 SNP genotype assays

Genomic DNA was extracted and diluted to an assay concentration of 20ng/µL as described in 2.3. We used generic 96-well plates in which to store a supply of the working solution of DNA for use in the series of SNP genotyping assays. These were laid out in such a way that each was identifiable by its pattern of blank wells. We included 10% random repeats to act as intra-assay controls. Prior to each assay, we prepared a generic 384-well PCR plate by pipetting 1µL of the working DNA solution from its 96-well storage plate. This method allowed us to combine samples and controls from four 96-well plates on one 384-well reaction plate. After pipetting the controls and samples, the solution was allowed to dry at room temperature for 12-24 hours, covered with lint-free tissue paper. Immediately before the assay was performed, we prepared an assay mix allowing for a reaction volume 5µL per well and comprising 2.5 µL TaqMan PCR master mix (Life Technologies Ltd.); 0.25µL 20x genotype assay mix (Life Technologies Ltd.) and 2.25µL micro-filtered dH_2O. The SNP genotype assay details are shown in Table 2-3. This was added to the wells and the plate sealed with optical adhesive covers (MicroAmp). The plate was read in an Applied Biosystems 7900 Fast real-time PCR system and with the supplied SDS software v2.2 before PCR. The thermal cycling conditions were identical to those described in Table 2-2. The plate was kept cool before repeat reading by the same software. The TaqMan allelic discrimination assays contain primer and probe sets to detect the SNP target of interest; and two distinct, allele-specific fluorescent dyes. The PCR system is able to detect the presence of one or both of these dyes in each well and so determine the SNP genotype of the sample in question. An example of these results is illustrated graphically in Figure 2.7.

Table 2-3 SNP genotyping assay context sequences.

SNP rs number	Celera ID	Context sequence
rs11568818	hCV27852953	ATTGGCAGGAAGCACACAATGTATT[**C/T**]GTCTTTCAAAGGATTTTTTTTTCTG
rs17352054	hCV34384229	GGTATCCTTAGTCAGAGTTTGACAT[**A/G**]TGATAAGGTGCACCATAAATATTTG
rs12285347	hCV32018626	AAAATGGCTTTTTATAGTCCTTTAA[**C/T**]GTGTGATGCAGAGCTGGTTATCTTC
rs11225297	hCV32018616	AAGTCTTATGGACTTCTATAAATAC[**A/T**]TAGTACCCTGGCTTTGGAATACAGA
rs11225307	hCV32018630	AAGTCTTATGGACTTCTATAAATAC[**A/T**]TAGTACCCTGGCTTTGGAATACAGA
rs17098318	hCV34384238	GGTATCCTTAGTCAGAGTTTGACAT[**A/G**]TGATAAGGTGCACCATAAATATTTG
rs10502001	hCV3210838	GGGCTTCTGCATTATTTCTATGACG[**C/T**]GGGAGTTTAACATTCCAGTTATAGG
rs10750646	hCV3210839	TAAATGTTTTGGGGAGAATTACACT[**A/G**]TACACTTTATAAAGCCTAGAAGTGT
rs11568819	hCV32018637	AACTAAAACGAGGAAGTATTACATC[**A/G**]TTATTGGCAGGAAGCACACAATGTA

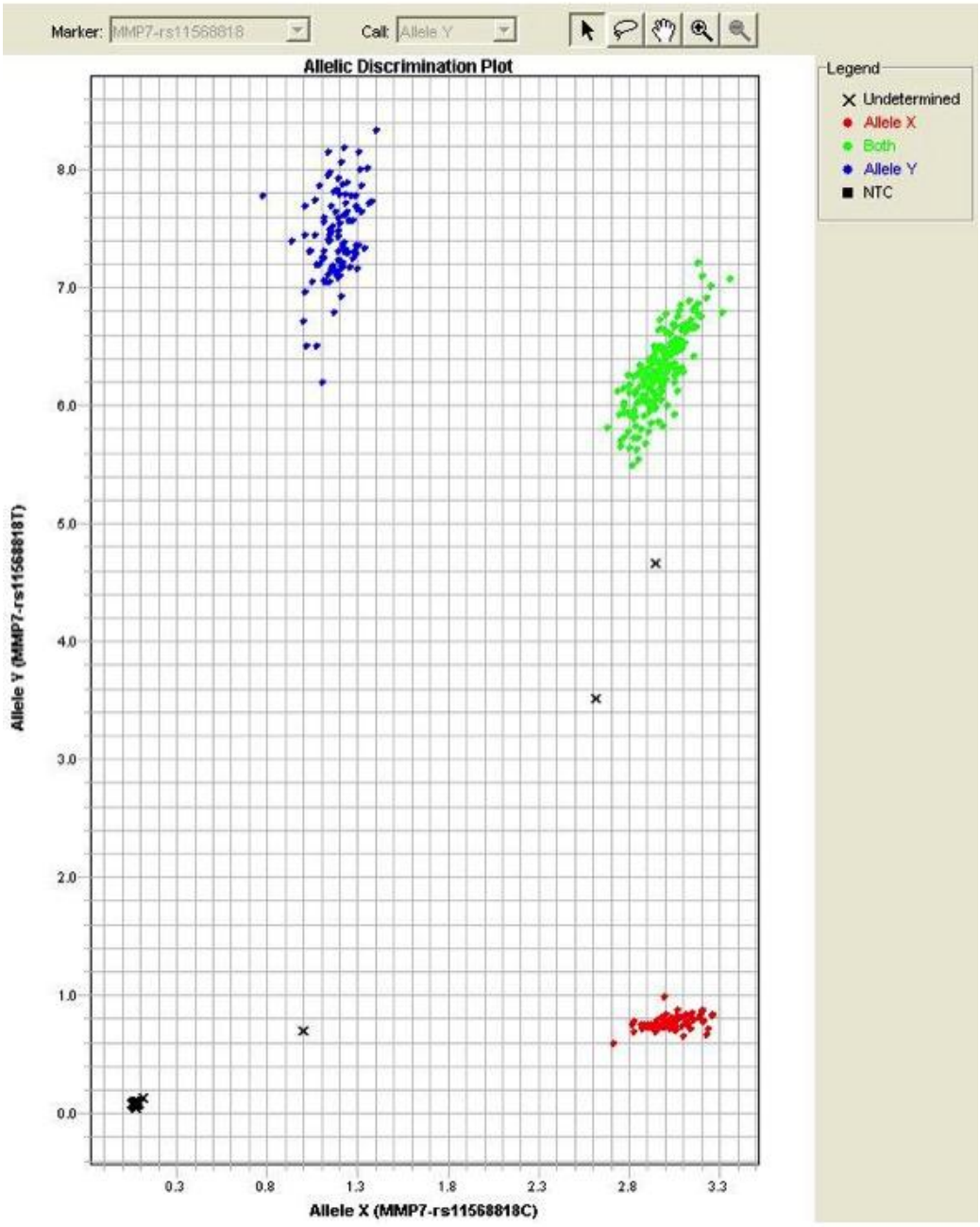

Figure 2-7 Graphical output from SDS software illustrating results of genotyping assay for rs-11568818.

2.8 Investigating the use of netazepide (YF476) for the treatment of type 1 gastric neuroendocrine tumours

The study described here and in chapter 6 was funded by Trio Medicines Ltd. (London, UK). ARM conducted patient recruitment, study visits and sample collection. The study was conducted in accordance with the ICH guidelines for Good Clinical Practice and the Helsinki Declaration of human studies. Approval was obtained from Cambridge East Research Ethics Committee and the Medicines and Healthcare Regulatory Authority (MHRA). All patients gave written informed consent prior to enrolment. The trial was registered at ClinicalTrials.gov, identifier NCT01339169, European Union Drug Regulating Authorities Clinical Trials (EudraCT) number 2007-002916-24.

2.8.1 Subject enrolment

Patients were recruited from a single centre (the professorial neuroendocrine gastroenterology clinic at the Royal Liverpool University Hospital, Liverpool, UK). They were considered eligible for inclusion if they had previously been diagnosed with histopathologically confirmed type I gastric NETs and chronic atrophic gastritis. Exclusion criteria included previous gastric surgery; treatment with somatostatin analogues; evidence of Zollinger-Ellison syndrome; prolonged QTc interval and pregnancy; and lactation or steroid contraceptive use in female subjects. The study was conducted in two stages as described in chapter 6.

Patients were eligible for enrolment into the second stage (extended dosing stage) if they had completed the first and were considered to have benefited from treatment with netazepide. We recruited 8 subjects for participation in the first stage and all of these completed both trial stages. The study (both stages) was conducted between November 2010 and February 2014.

2.8.2 Study design

We conducted an open-label, phase 2, pilot study. Trio Medicines Ltd. Supplied netazepide 25mg capsules. In both stages, patients were asked to take 50mg netazepide daily, orally and after breakfast. Patients were issued with diary cards in which they were asked to record the time of each dose along with the details of any deviation from the prescription, unexpected symptoms or new concomitant medicine use.

2.8.2.1 Stage 1

Stage 1 comprised 12 weeks of treatment with netazepide 50mg daily and a 12-week follow-up period (after discontinuing netazepide treatment). There were 6 study visits in this stage. The investigations performed at each visit are illustrated in Table 2-4.

Table 2-4 Investigations performed at stage 1 study visits.

Weeks	0	3	6	9	12	24
On treatment		•	•	•	•	
Blood tests	•	•	•	•	•	•
Endoscopy	•		•		•	•

Following enrolment and informed consent, patients attended for a baseline (week 0) assessment visit during which a detailed medical history was taken and a clinical examination was performed. This included the recording of vital signs, a 12-lead electrocardiograph (ECG) and urinalysis. As for all subsequent visits, the patients were fasted from food and fluids for at least six hours. A venous blood sample was taken for assessment of full blood count (FBC), urea & electrolytes (U&E), liver function tests (LFT), lipid profile, plasma glucose, plasma Chromogranin A (CgA) and serum gastrin concentrations. A baseline upper GI endoscopy was performed during which visible gastric NETs were counted, measured and photographed before pinch biopsies were taken from the mucosa of the gastric antrum, corpus and tumours.

Following this initial visit, patients were prescribed Netazepide 50mg once daily and issued with a diary card in which to record times of administration, side effects or symptoms and new concomitant medications.

Subsequent visits were conducted at three-week intervals (weeks 3, 6, 9 and 12). At each visit, a 12-lead ECG and urinalysis was performed and all blood tests were repeated. In addition, trough/peak plasma netazepide levels were measured. The diary card was reviewed and a capsule-count performed to evaluate drug compliance. A medical history and examination was carried out. After six and twelve weeks of treatment, upper GI endoscopy was performed as described below.

A final visit was carried out twelve-weeks after completion of the Netazepide course of treatment. At this visit, blood tests and endoscopy were repeated.

2.8.2.2 Stage 2

Stage 2 comprised 52 weeks of treatment with netazepide and 5 study visits. At 0, 12, 24, 36 and 52 weeks, we collected blood for gastrin, CgA, and netazepide assays along with 'safety' monitoring as before. We performed gastroscopy at baseline (0 weeks), 24 and 52 weeks as before. There was no formal follow-up study visit after this stage and patients were returned to routine clinic follow-up. The investigations performed at each study visit are summarised in Table 2-5. For one patient (patient 8), the interval between completing stage 1 and starting stage 2 was less than 6 months and for this individual, endoscopy was not repeated at week 0, stage 2.

Table 2-5 Investigations performed at stage 2 study visits.

Weeks	0	12	24	36	52
On treatment		•	•	•	•
Blood tests	•	•	•	•	•
Endoscopy	•		•		•

2.8.2.3 Informal follow-up

On completion of stage 2, patients were returned to routine follow-up in the neuroendocrine gastroenterology clinic. All 8 underwent routine endoscopy approximately one year after study completion and for completeness; we also report the findings from these procedures though they do not strictly fall within the remit of this study.

2.8.3 Collection and processing of biological samples

2.8.3.1 Endoscopy

Upper GI endoscopy was performed using Olympus Evis Lucera H240/H260 endoscopes (Olympus, Southend-on-Sea, UK). Procedures were carried out by the same endoscopist (Prof. D. M. Pritchard, Department of Gastroenterology, University of Liverpool, Liverpool, UK) and assistant (ARM) at each visit and for each patient. Visible tumours were photographed and their diameter estimated by comparison with an opened pair (9mm) of biopsy forceps (Single-Use Radial Jaw 4 - Boston Scientific, Hemel Hempstead, UK). Mucosal pinch biopsies were then taken from the gastric antrum, corpus and visible tumours for routine histopathology (four biopsies per site). Eight additional biopsies were taken from the gastric corpus and stored in RNAlater (Ambion, Austin, TX, USA) for subsequent assessment of biomarker mRNA abundance.

2.8.3.2 Histopathology and immunohistochemistry

Biopsy samples were fixed in 10% neutral buffered formalin and paraffin embedded prior to staining with haematoxylin and eosin. Samples were also processed for immunohistochemical detection of synaptophysin, Ki67 and chromogranin A using monoclonal mouse antihuman synaptophysin antibody at 1:80 (NCLSynap299, Leica

Microsystems Inc. IL, USA), monoclonal mouse antihuman Ki67 antibody at 1:200 (NCL-Ki67-MM1, Leica Microsystems Inc. IL, USA) and polyclonal rabbit antihuman CgA antibody at 1:8000 (A0430, Dako, Denmark) respectively. Specimens were reported by the same, expert gastrointestinal histopathologist (Prof. F. Campbell, Royal Liverpool University Hospital, Liverpool).

2.8.3.3 Plasma Chromogranin A Concentration

4ml venous blood was collected in EDTA tubes and centrifuged to separate plasma (4°C, 1500G for 10min) which was stored at -80 °C until assay. Plasma CgA concentration was measured using ELISA (Chromogranin A ELISA Kit K0025, DAKO, Denmark). Hammersmith Medicines Research, London, UK, performed CgA ELISA assays.

2.8.3.4 Serum Gastrin Concentration

2.5ml venous blood was collected in serum tubes and allowed to clot at room temperature for at least 20 minutes. Serum was then separated by centrifugation (4°C, 1500G for 10min) and stored at -20°C until assay. Serum samples were assayed for total amidated gastrin concentrations by RIA and ELISA. ELISA was performed using the Siemens Immulite2000 Gastrin chemiluminescent, enzyme-labelled immunometric assay (Siemens Healthcare Diagnostics Inc., NY, USA). These were performed by Hammersmith Medicines Research, London, UK. RIA was performed by ARM at the University of Liverpool and as described in section 2.4.1.

2.8.3.5 Gastric Mucosal Biomarkers

Gastric corpus mucosal biopsies were stored in RNAlater at -20°C before undergoing RNA extraction and reverse transcription as described in section 2.5. Real-time qPCR was performed using TaqMan chemistry and the 7500 real-time PCR system as

described in 2.6. Histidine decarboxylase (HDC), CgA and MMP-7 mRNA abundances were determined relative to GAPDH. The primer and probe sets employed are listed in Table 2-6. For stage 1, RNA extraction, reverse transcription and biomarker qPCR assays were performed by ARM who also designed and produced primers and probes for the histidine decarboxylase qPCR assay. For stage 2, Dr. B. N. Parsons (Department of Gastroenterology, University of Liverpool, Liverpool, UK) performed RNA extraction, reverse transcription and qPCR assays.

Table 2-6 Primer and probe sets employed in qPCR for determination of relative mRNA abundance of mucosal gene products.

Gene	Probe	Forward primer	Reverse primer
GAPDH	CGTCGCCAGCCGAGCCACA	GCTCCTCCTGTTCGACAGTCA	ACCTTCCCCATGGTGTCTGA
MMP-7	CCTGTATGCTGCAACTCATGAACTTGGC	GGATGGTAGCAGTCTAGG GATTAACT	GGAATGTCCCATACCCAAAGAA
CgA	CCAGCCCCATGCCTGTCAGCC	GATACCGAGGTGATGAAATGCA	TCCTTCAGTAAATTCTGATGTCTCAGA
HDC	CTCTGTTAAACTCTGGTTCGTGATTCGGTCC	CCCTGAGCCGACGGTTT	GTACCATGTCTGACATGTGCTTGA

2.8.3.6 Serum Netazepide Concentration

4ml venous blood was collected in lithium-heparin tubes and centrifuged to separate plasma (4°C, 1500G for 10min) within 30 minutes of sampling. Trough samples were collected along with other fasting blood samples. Peak samples were collected 1 hour after observed administration of 50mg Netazepide. Plasma was stored at -20 °C until assay. Plasma Netazepide concentrations were measured at Huntingdon Life Sciences, UK, by a validated liquid chromatographic-tandem mass spectrometric method as previously described(288).

3 Gastric mucosal preneoplasia following *Helicobacter pylori* infection

3.1 Introduction

The gram-negative bacterium *Helicobacter pylori* colonises the stomachs of approximately half of the world's population(289). It universally induces a state of chronic inflammation in the gastric mucosa of its host and whilst most infected individuals remain asymptomatic, a minority go on to develop additional mucosal diseases such as peptic ulceration and mucosa-associated lymphoid tissue (MALT) lymphoma. A further 1% of hosts exhibit mucosal remodelling which culminates in the development of gastric adenocarcinoma. The disease phenotype displayed following infection is dependent on the interaction between bacterial, host and environmental factors.

We designed a cross-sectional study of patients referred to the Royal Liverpool University Hospital, Liverpool, UK for upper GI endoscopy. Our aims were: 1) to establish the prevalence both of *H. pylori* infection and of gastric preneoplasia in this select population; 2) to collect data pertaining to relevant environmental contributors to gastric epithelial disease and 3) determine the transcript abundance of several genes and their proteins involved in *H. pylori* induced gastric preneoplasia with a view to developing a clinically applicable biomarker of the same.

3.2 Materials and Methods

The study methodology is described in detail in chapter 2 and is summarised here for convenience. Patients attending the Royal Liverpool University Hospital for outpatient upper GI endoscopy were recruited prior to undergoing the procedure. They answered a questionnaire (appendix C), which included demographic information, height and weight, past medical history, family history, concurrent medication use, tobacco and alcohol use and previous *H. pylori* testing or treatment.

Study participants provided fasting samples of blood and gastric mucosal biopsies in addition to those obtained as part of the routine endoscopy.

Gastric corpus and antral biopsies were fixed, cut and stained before examination by a single, expert gastrointestinal pathologist. This study pathologist produced standardised reports including categorization by modified Sydney score and Vienna classification.

Serum was stored in aliquots at -20°C. Anti-*H. pylori* IgG antibody serotype was determined by ELISA. Study subjects with one or more positive test results for *H. pylori* were also serotyped for CagA by ELISA. Fasting serum gastrin concentration was determined by radioimmunoassay (RIA). We selected smaller groups of study participants based on *H. pylori* and pathology results for serum pepsinogen 1 and 2 concentration analyses. These were also determined by ELISA.

3.3 Study cohort characteristics

3.3.1 Demographics

Over the time period described, 1400 patients were recruited. These were predominantly female (57.5%) and Caucasian (98.4%). The mean age was 58 years, the median 60 years and the interquartile range 48-70 years.

3.3.2 Mode of referral and symptoms

Most patients were referred by their GP (56%) rather than from another department at the hospital. Of these 764 outside referrals, 66% were referred using the so-called "2-week rule" – an administrative mechanism to ensure that patients with symptoms suggestive of possible malignancy are investigated quickly.

We recorded the symptoms reported by the patients and given as indications for upper GI endoscopy. Most patients (55.6%) reported a single, predominant symptom. The commonest symptom to be reported alone was dysphagia, closely followed by the rather less specific symptoms of dyspepsia, heartburn and abdominal pain. The relative frequency with which solitary symptoms were reported is shown in Figure 3-1. Overall, these three non-specific symptoms were the most commonly reported. The overall frequency with which all symptoms were reported is shown in Figure 3-2.

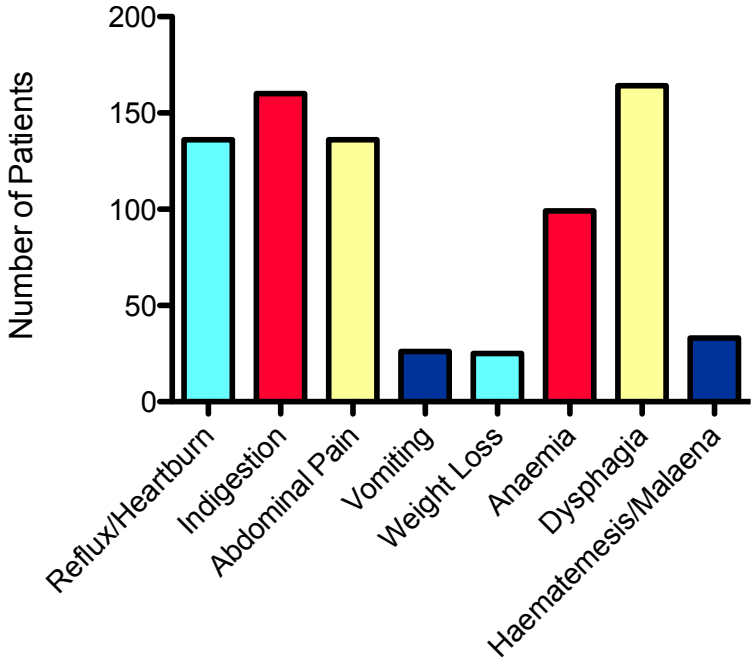

Figure 3-1 Frequency with which solitary symptoms were reported.

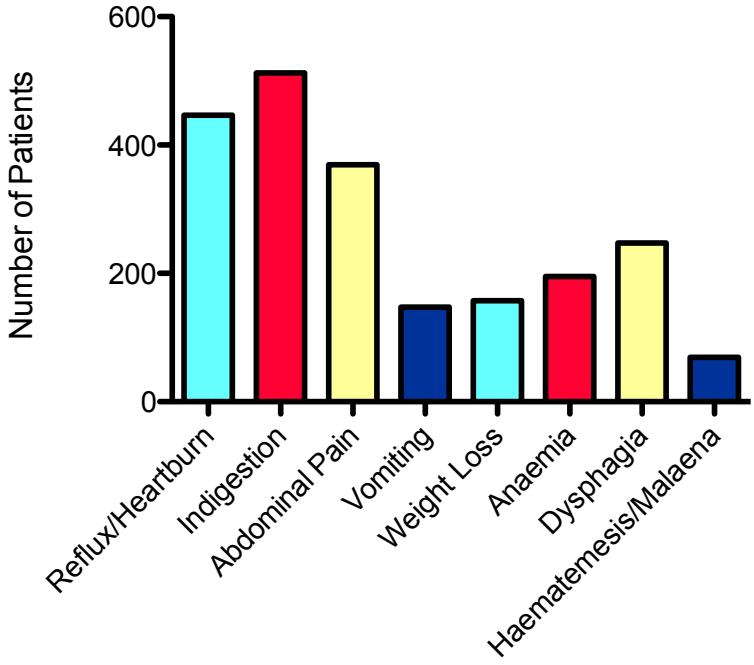

Figure 3-2 Overall frequency with which symptoms were reported

3.3.3 Previous medical history

We asked participants about their previous surgical and medical histories. In addition to recording major, general comorbidities, we specifically asked about previous upper gastrointestinal surgery; diabetes mellitus; ischaemic heart disease; cerebrovascular disease; inflammatory arthritis and pulmonary disease.

Previous upper GI surgery was reported by 67 patients (4.8%). The largest proportion of these (48%) comprised of procedures performed to address the complications of peptic ulcer disease (PUD). The relative frequencies of indications for surgery are shown in Figure 3-3. Of the upper GI malignancies (n = 17), most were gastric adenocarcinoma (7) with 2 cases of gastric stromal tumours and one of a gastric neuroendocrine tumour. There were 4 cases of oesophageal malignancy and 3 of pancreatic cancer treated by Whipple's procedure.

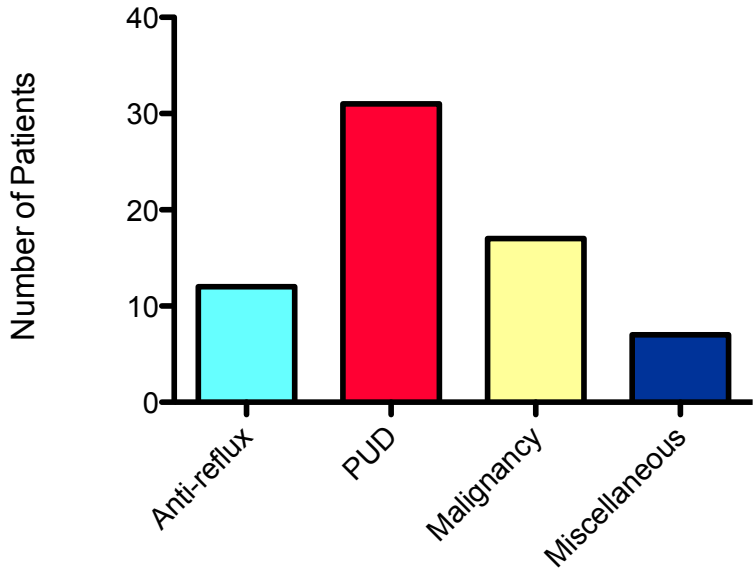

Figure 3-3 Indications for surgery.

Diabetes mellitus (DM) featured in the medical histories of 164 patients (11.7%). All but 9 of these reported Type-2 DM. Ischaemic heart disease was reported by 124 (8.9%) patients and cerebrovascular disease by 51 (3.6%). In these 'cardiovascular disease' groups combined, 24% were also diabetic. Chronic pulmonary disease was

reported by 215 (15.4%) of patients. Only 10 patients (0.7%) gave a history of inflammatory arthropathy.

Previous malignancy was reported by 65 (4.6%) patients. The tumour sites are listed in Table 3-1.There were also 32 (2.3%) patients with inflammatory bowel disease and 7 (0.5%) with coeliac disease. Prior to the endoscopy for which they were attending hospital, 12 (0.9%) patients were known to have Barrett's oesophagus. 6 (0.4%) patients gave a history of Familial Adenomatous Polyposis (FAP).

Table 3-1 Previous malignancies by site.

Primary Neoplasia Site	Number of patients - total	Number of patients - current
Breast	18	1
Colorectal	14	0
Prostate	7	7
Lung	2	1
Gastric	11	0
Oesophageal	6	0
Pancreas	3	0
Other	4	0

3.3.4 Family & Social history

We recorded family history of gastric pathology and asked about tobacco and alcohol use.

A family history of upper GI pathology was given by 302 (21.6%) subjects. Included in this group were the 69 (4.9%) patients who had a first degree relative with gastric malignancy and an additional 28 (2%) who reported gastric malignancy in a second degree relative. Also included were 162 (11.6%) patients who reported a first or second degree relative with a history of peptic ulceration, though of these only 18 (1.3%) recalled a close family member having been found to have *H. pylori* infection.

Regular use of alcohol was reported by 776 (55.4%) patients – 47.3% of women and 65.4% of men. Of the female participants, 53 (6.6%) admitted to drinking more than the UK recommended weekly limit (14 units/week for women, 21 units/week for men(290). The same figure for male patients was 77 (12.9%).

Tobacco smoking was reported for 730 (52.1%) including 346 (24.7%) who were current smokers. Smoking history is summarised in Table 3-2.

Table 3-2 Smoking history.

	Non- smoker	**Ex-smoker**	**Smoker**
Female	408 (50.7%)	191 (23.7%)	206 (25.6%)
Male	262 (44.0%)	193 (32.4%)	140 (23.6%)
Total	670 (47.9%)	384 (27.4%)	346 (24.7%)

3.3.5 Concomitant medications

We specifically asked about the use of proton-pump inhibitors; H2-receptor antagonists, aspirin, clopidogrel and non-steroidal anti-inflammatory analgesics.

If patients reported the use of one of these classes of drug, we also noted the dose and the time of last use. 50 (3.6%) patients reported recent use of non-steroidal anti-inflammatory analgesics (NSAIDs) and 211 (15.1%) patients reported recent aspirin use. Although these agents typically have half-lives of less than 24 hours, their effects on the gastric mucosa may persist for several weeks(291). For this reason, we considered patients to be NSAID users if they reported having taken a non-aspirin NSAID within 4 weeks of the study endoscopy. The drugs whose use was reported are listed in Table 3-3. 211 (15.1%) patients reported recent aspirin use and 9 patients stated that they had used aspirin and NSAIDs concurrently in the preceding month.

PPI use was reported by 732 (52.3%) subjects. Again, PPIs typically have short half-lives (<12 hours), but their effects on GI physiology and the resultant mucosal changes might be present for much longer. We included anyone reporting PPI use within 2 weeks. The drugs reported are listed in Table 3-4.

Table 3-3 Number of patients who reported NSAID use.

NSAID	Number of patients	% of total
Diclofenac	3	0.21
Diclofenac/Misoprostol	3	0.21
Celecoxib	15	1.07
Etoricoxib	3	0.21
Ibuprofen	16	1.14
Meloxicam	2	0.14
Naproxen	8	0.57

Table 3-4 Number of patients who reported PPI use.

PPI	Number of patients	% of total
Esomeprazole	57	4.07
Lansoprazole	368	26.29
Omeprazole	290	20.71
Pantoprazole	10	0.71
Rabeprazole	7	0.50

3.4 Endoscopic findings

Of the study cohort of 1400 patients, 12 provided blood samples but did not undergo a complete endoscopy examination, either because they withdrew consent early during the procedure or because a proximal lesion precluded access to the stomach. For the remaining 1388 patients, significant endoscopy findings as reported by the endoscopist were recorded in the database. For the purposes of our analyses, these were then reclassified into “primary findings” and “others”. A “primary finding” was a significant mucosal abnormality reported at endoscopy in the stomach or duodenum. We also included Barrett’s oesophagus and oesophageal malignancy in this category. Where two such abnormalities were observed, we counted only the major abnormality e.g. if gastric ulceration and duodenitis were both present, we counted that case as gastric ulceration(292). Non-mucosal findings (e.g. hiatus hernia) thought unlikely to have a bearing on our subsequent analyses were counted as “others”. The primary findings along with the frequency with which they were reported are listed in Table 3-5.

It was hypothesised at the outset that Barrett’s oesophagus might exhibit a similar relationship to the expression of our proposed biomarkers to that seen in gastric preneoplasia. As the table illustrates, we identified 67 suspected cases of Barrett’s oesophagus by endoscopy alone, though 44 of these accompanied another upper GI abnormality. For clarity, we have given both the total number of cases reported at endoscopy and those reported as the only significant abnormality seen.

Table 3-5 Primary endoscopic findings.

Primary endoscopic findings	Number of patients	
	n	%
Normal	443	31.9
Gastric malignancy	12	0.9
Oesophageal malignancy	9	0.6
Gastric atrophy and/or intestinal metaplasia	14	1.0
Barrett's oesophagus ONLY	23	1.7
Gastritis and/or duodenitis	684	49.3
Gastric and/or duodenal ulcer(s)	54	3.9
Gastric polyp(s)	64	4.6
Duodenal atrophy	6	0.4
Duodenal polyp(s)	1	0.1
Duodenal stenosis	1	0.1
Oesophagitis ONLY	61	4.4
Vascular lesion(s)	13	0.9
Gastrointestinal stromal tumour	3	0.2
Barrett's oesophagus ALL	67	4.8

3.5 Helicobacter pylori status

As described above, patients were tested for *H. pylori* using three methods: serological analysis, rapid urease testing (RUT) of a pinch-biopsy specimen of gastric mucosa and by histopathological staining and examination.

There were 17 patients entered into the database from whom we obtained samples of blood but no gastric biopsies because the patient withdrew consent before these were taken (n=15) or because a proximal lesion precluded access to the stomach (n=2). 16 patients underwent the gastroscopy procedure, but a rapid urease test result was not recorded. In 3 cases, the histology was not reported because of mishandled samples. 9 patients were entered into the database despite our being unable to obtain a blood sample. A further 39 patients did not have an *H. pylori* serology result reported because of mishandled or mislabeled samples.

Taking into account these "not done" *H. pylori* tests, we have a complete set of results for 1322 (94.4%) study patients. These results are shown in Table 3-6. The hospital laboratory reports the *H. pylori* antibody IgG serology result qualitatively. In cases where the antibody titre is neither sufficiently high to be considered "positive" nor sufficiently low to be considered "negative", then the result is reported as "indeterminate". 46 (3.3% of total cohort) such results were returned and in our analyses, we regarded these results as being "negative".

Table 3-6 *H. pylori* test results. n=number of patients.

n=1322	Urease		Histology		Serology	
	n	%	n	%	n	%
Positive	245	18.5	288	21.8	573	43.3
Negative	1077	81.5	1034	78.2	749	56.7

Comparisons between the various testing methodologies are shown in Figure 3-4.

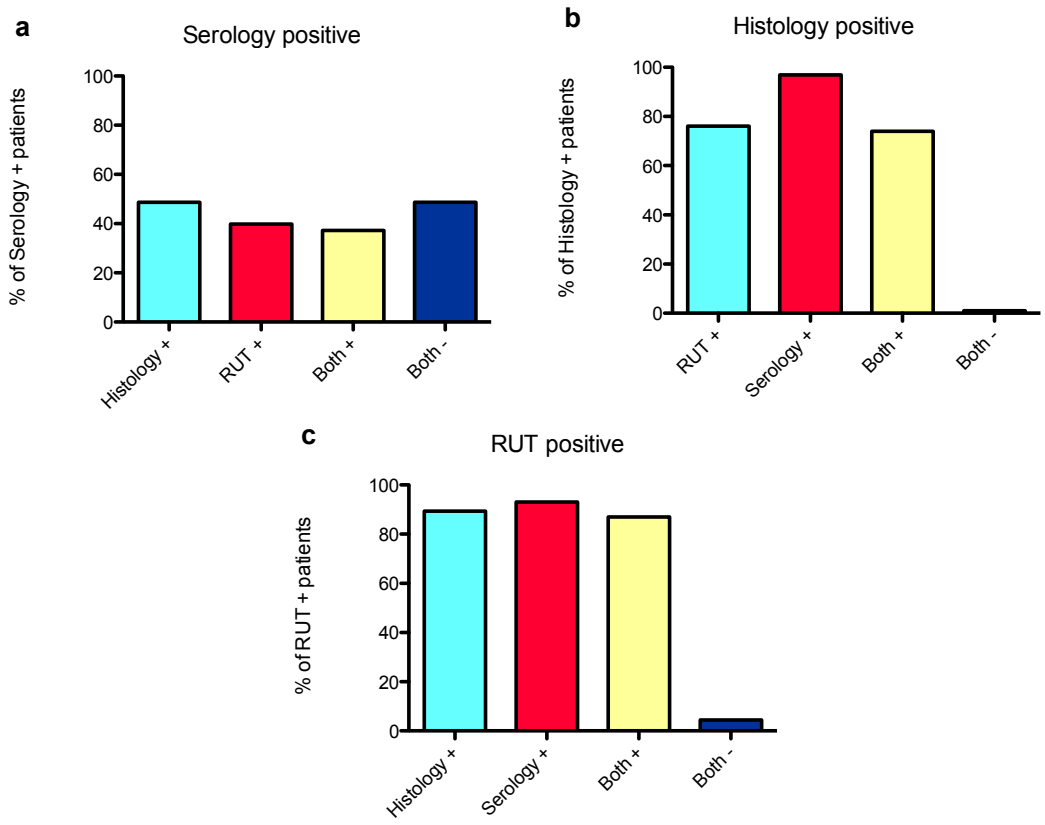

Figure 3-4 Comparison between three methods of *H. pylori* test. a) In seropositive, b) in histology positive and c) in RUT positive patients

As might be expected, approximately half (48.7%) of seropositive patients exhibited no evidence of active *H. pylori* infection by histology or RUT. Though some false-positive serology (and false-negative histology/RUT) results might be expected, this figure largely reflects the rate at which *H. pylori* has been cleared either by host-immunity or with antimicrobial eradication treatment. In our study, 158 subjects reported previous eradication therapy for *H. pylori* of whom only 100 (63.3%) were seropositive. Of these, 27 (27%) were found to be infected with *H. pylori* on histological examination.

Using histology as the "gold standard", the overall sensitivity and specificity of RUT in our study cohort were 76.0% and 97.5% respectively. In those patients taking a PPI, sensitivity of RUT was 60.4% compared with 84.5% in those not taking such agents. Similarly, in patients with positive CagA serology, RUT sensitivity was 83.9% compared with 71.0% in CagA negative *H. pylori* infected subjects. We observed no effect on RUT performance of the presence of preneoplastic mucosal changes.

3.5.1 Topographical distribution of *H. pylori* colonisation

As outlined in chapter 2, our sample collection protocol described separate antrum and corpus mucosal biopsies for histological assessment both by our study pathologist and by the local hospital pathologists. Based on this, we were able to estimate the topographical distribution of *H. pylori* organisms in infected subjects (Table 3-7)

Table 3-7 Topographical distribution of *H. pylori* colonisation on histological examination.

Site of infection	**Number of patients**	
	n	**%**
Pangastric	199	69.1
Corpus only	59	20.5
Antrum only	30	10.4

3.5.2 CagA status in *H. pylori* infected patients

CagA serology was performed on 584 patients whose *H. pylori* testing had yielded at least one positive result. 210 (36.0%) were seropositive for CagA. Of the 306 with evidence of current infection (RUT and/or histology positive), 129 (42.1%) were CagA positive. For those individuals with positive *H. pylori* serology, but no evidence of current infection (RUT and histology negative) i.e. "previous" infection, the number found to be CagA positive was 81 (29.1%). These data are shown in Figure 3-5.

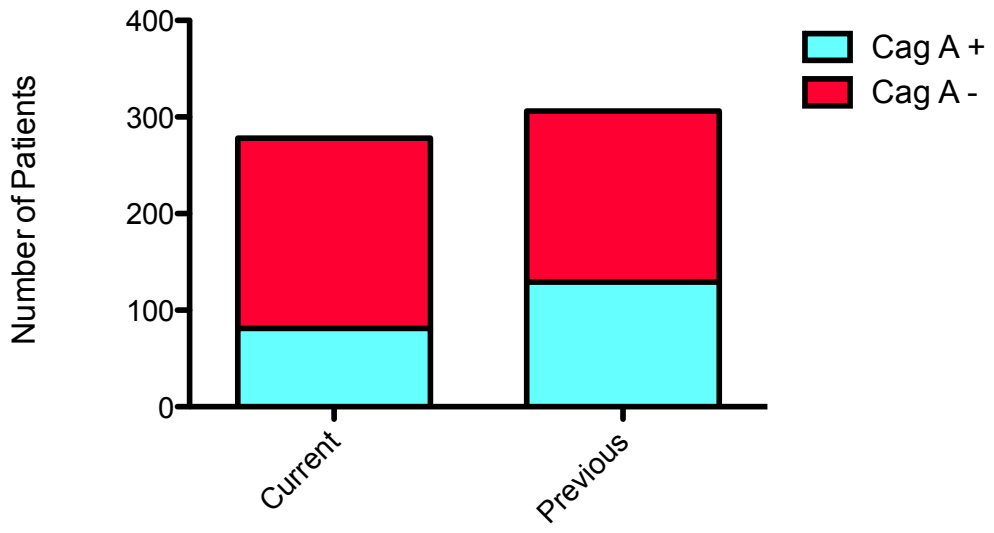

Figure 3-5 CagA status in patients with current and previous evidence of *H. pylori* infection.

3.6 Gastric mucosal inflammation and preneoplasia

Gastric mucosal biopsy specimens were obtained during endoscopy as described above (section 2.2). Of the 1400 patients in the study database, 19 had no histology reported by the study pathologist. An additional 11 had analyses reported from antral mucosal samples only and 18 from the corpus only. Of the antral-only set, one patient had antral intestinal metaplasia (IM) and one had gastric adenocarcinoma and IM. Of the corpus-only set, one patient had atrophy and one had atrophy and IM. Excluding the patients without histology data, there are 1381 patients included in the following analyses.

In total, there were 338 (24.5%) patients with gastric mucosal preneoplastic changes, dysplasia or cancer. These are listed in Table 3-8 in order of increasing severity. Patients with more "severe" mucosal changes have been counted only in the most severe category e.g. if a sample was reported to exhibit both "atrophy" and "low-grade dysplasia", it has been included only in the "low-grade dysplasia" category. Similarly, if pathology was reported in both antrum and corpus specimens, that case was included in the pangastric category only.

Table 3-8 Primary findings at histopathological assessment.

Primary histopathological findings	Number of patients	
	n	%
Atrophy - antrum	19	1.4
Atrophy - corpus	45	3.3
Atrophy - pangastric	5	0.4
Intestinal metaplasia - antrum	156	11.3
Intestinal metaplasia - corpus	42	3.0
Intestinal metaplasia - pangastric	45	3.3
Low-grade dysplasia - antrum	6	0.4
Low-grade dysplasia - corpus	5	0.4
Low-grade dysplasia - pangastric	0	0.0
High-grade dysplasia - antrum	0	0.0
High-grade dysplasia - corpus	0	0.0
High-grade dysplasia - pangastric	3	0.2
Gastric adenocarcinoma	12	0.9

3.6.1 Age and gastric preneoplasia

We examined the rates of preneoplasia in the different age groups of patients recruited to our study. We divided patients by age into the following groups: <20, 20-29, 30-39, 40-49, 50-59, 60-69, >69 years old. The proportion of patients with preneoplasia increased with age as shown in Figure 3-6a. The mean age of patients with preneoplasia was 64 years (median = 66 years) and was significantly higher (mean = 56, median = 57 years) than that of patients without preneoplasia ($p<0.0001$, two-tailed Mann-Whitney test) Figure 3-6b.

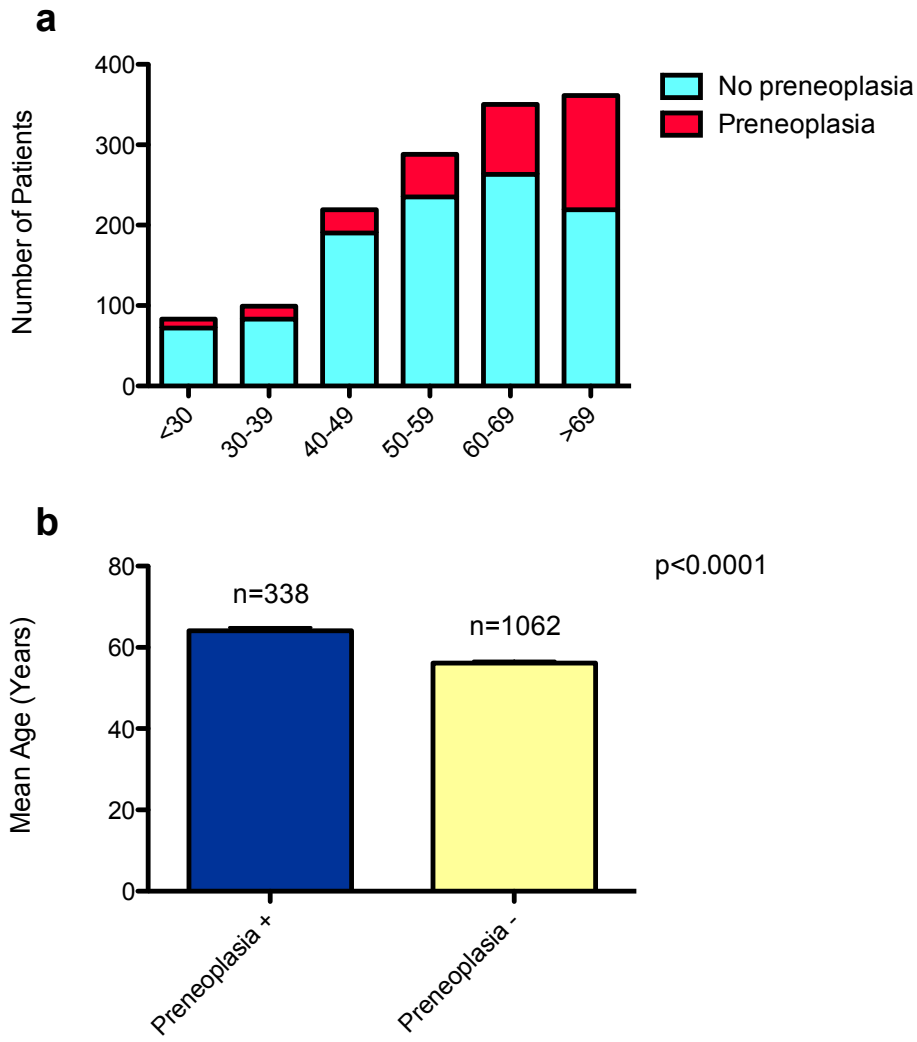

Figure 3-6 a) Proportion of patients with gastric preneoplasia by age group and b) mean age in patients with and without gastric preneoplasia (two-tailed Mann-Whitney test).

3.6.2 *H. pylori* and gastric preneoplasia

H. pylori infection was identified by at least one test in 231 (68.3%) of the 338 patients with histologically confirmed preneoplasia or cancer. 131 (38.8%) had evidence of current *H. pylori* infection and 100 (29.6%) were positive by *H. pylori* serology only, suggesting previous infection. 107 (31.7%) gave negative results for all three modes of *H. pylori* testing. In comparison, of 1062 patients without preneoplasia or cancer, 384 (36.6%) were *H. pylori* positive – 191 (18.0%) for current infection and 193 (18.2%) for previous infection. The difference in prevalence of *H.*

pylori infection between the two groups was significant (p<0.0001, OR 3.812, 95% CI 2.248-3.378, two-sided Fisher's exact test) and the data are illustrated in Figure 3-7.

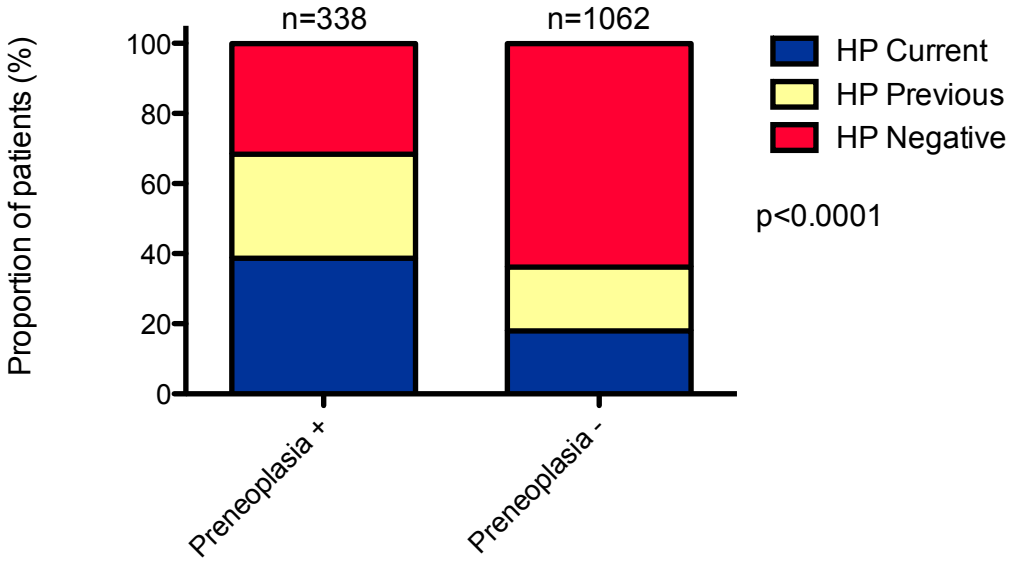

Figure 3-7 Proportions of patients with evidence of current, previous or no *H. pylori* infection in groups with and without preneoplasia (two-tailed Mann-Whitney test).

As has been previously reported, the tendency to develop *H. pylori* associated preneoplastic changes in gastric mucosa is enhanced by more virulent strains of the organism. In our cohort, of 338 patients with preneoplasia or cancer, 174 (51.5%) were cagA serology positive. Of the 532 patients without preneoplasia or cancer who were tested for cagA, only 168 (31.6%) were seropositive. The association between cagA positivity and preneoplasia was significant (p<0.0001, OR 2.125, 95% CI 1.495-3.020, two-sided Fisher's exact test). The data are shown in Figure 3-8.

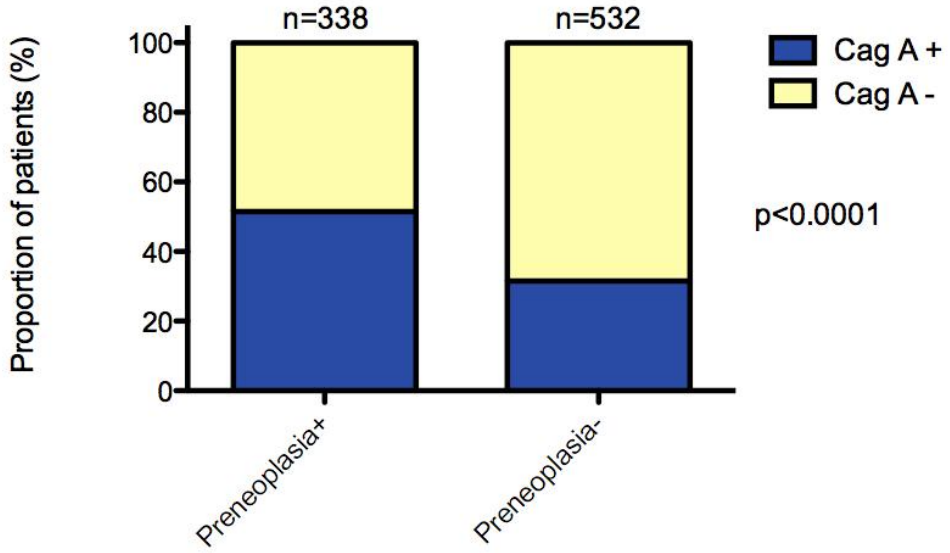

Figure 3-8 Proportion of patients with CagA seropositivity in groups with and without preneoplasia (two-tailed Mann-Whitney test).

3.6.3 Serum gastrin concentration and gastric preneoplasia

All but 13 of the 1400 patients recruited into the study had their serum analysed for gastrin concentration by radioimmunoassay as described in chapter 2. Patients were classified into three groups based on fasting serum gastrin concentration: normal (<40pM), moderately elevated (40-100pM) or high (>100pM). The data are summarised in Figure 3-9. The proportion of patients with fasting serum gastrin concentrations above "normal" i.e. >40pM was significantly higher in those with preneoplasia or cancer (56.8%) than in those without (40.8%) ($p<0.0001$, OR 1.764, 95% CI 1.375-2.261, two-sided Fisher's exact test). The association between high (>100pM) fasting serum gastrin concentrations and preneoplasia or cancer was also significant ($p<0.0001$, OR 2.355, 95% CI 1.722-3.220, two-sided Fisher's exact test). The mean fasting serum gastrin concentrations in the preneoplasia and control groups were 121.5pM (median = 46.0pM) and 62.1pM (median = 31.0) respectively ($p<0.0001$, two-tailed Mann-Whitney test).

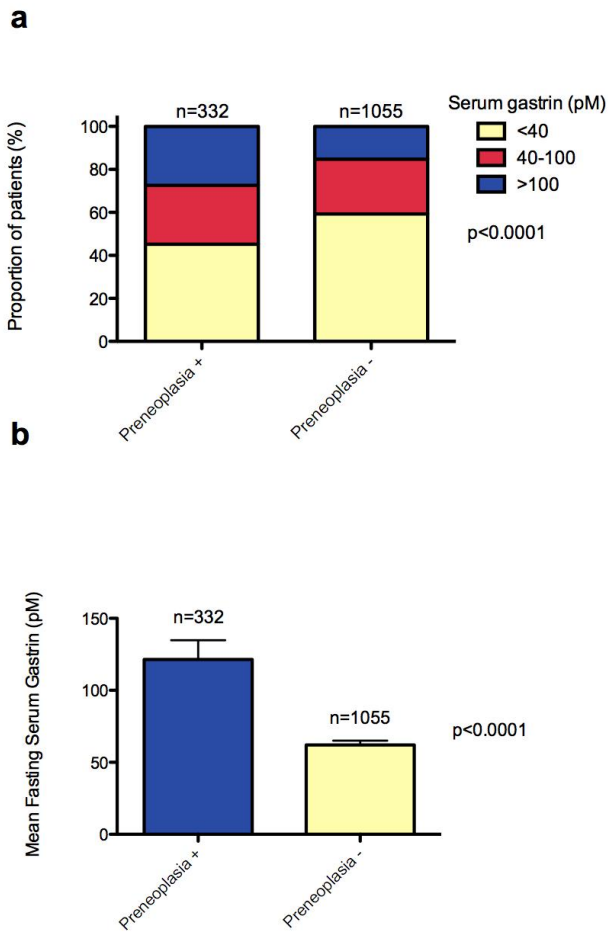

Figure 3-9 a) The proportion of patients with fasting serum gastrin concentration in normal, moderately elevated and high ranges in those with and without gastric preneoplasia and b) mean gastrin concentration in patients with and without gastric preneoplasia (two-tailed Mann-Whitney test).

Of the patients in our cohort with histologically proven preneoplasia, 317 had both complete histology data and a serum gastrin concentration recorded. 158 had preneoplastic pathology identified only in antral biopsy specimens compared with 84 only in corpus samples and 75 in both (pangastric). The mean concentrations of fasting serum gastrin in these groups were 65.7pM, 230.0pM and 107.6pM (median = 36.0pM, 90.0pm and 50.0pM) respectively. The difference between each of these three groups was significant (<0.0001, repeated two-tailed Mann-Whitney tests). There was no significant difference in mean serum gastrin concentration between the antrum-only preneoplasia group and the no preneoplasia group (Figure 3-10).

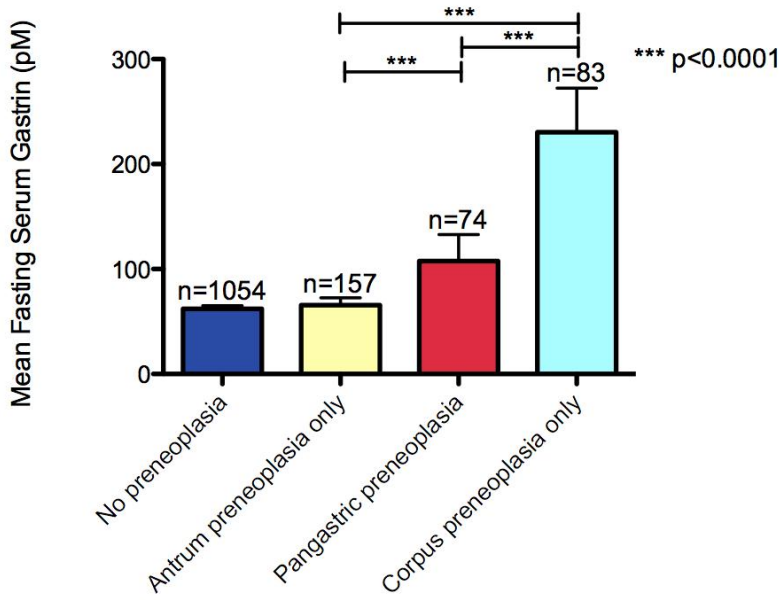

Figure 3-10 Comparison of mean fasting serum gastrin in preneoplasia groups by site (repeated two-tailed Mann-Whitney tests).

Other causes of hypochlorhydria have also been shown to induce hypergastrinaemia(293). Perhaps most relevant to our study are PPI use and *H. pylori* infection. We therefore evaluated the effects of these variables in our study population as follows:

We firstly examined the effect of PPI use in both subjects with normal gastric histology (Figure 3-11a) and those with gastric preneoplasia (Figure 3-11b).

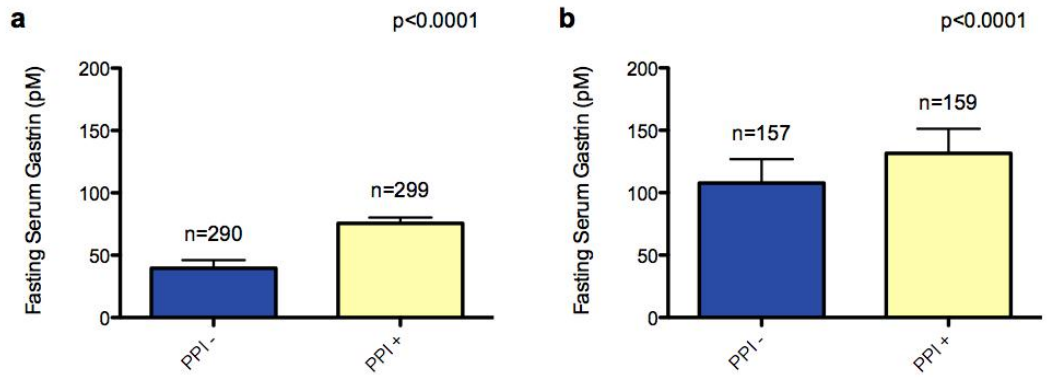

Figure 3-11 Mean fasting serum gastrin concentration with and without PPI use in a) subjects with normal gastric histology and b) subjects with gastric preneoplasia (two-tailed Mann-Whitney tests).

In the 'normal' histology group, the mean fasting serum gastrin concentration in PPI naïve subjects was 39.6pM (median 20.0pM, SEM 6.5), compared with 75.6pM (median 50.0pM, SEM 4.6) in PPI users and the difference was highly statistically significant ($p<0.0001$, two-tailed Mann-Whitney test). Similarly, in the 'preneoplasia' group, mean fasting serum gastrin concentration in PPI naïve subjects was 107.7pM (median 30.0pM, SEM 19.2) and 131.6pM (median 61.8pM, SEM 19.6) in PPI users. The difference here was also highly statistically significant ($p<0.0001$, two-tailed Mann-Whitney test).

To determine the effect of *H. pylori* infection on fasting serum gastrin concentration, we performed similar analyses. Subjects were regarded as currently infected if *H. pylori* organisms were reported on study histology. In such cases, the histopathological changes induced are commonly referred to as 'gastritis B'. We compared mean fasting serum gastrin concentration in the 'normal' histology group with that in the 'gastritis B' group (Figure 3-12a). In the preneoplasia group, we compared *H. pylori* positive and *H. pylori* negative subjects (Figure 3-12b).

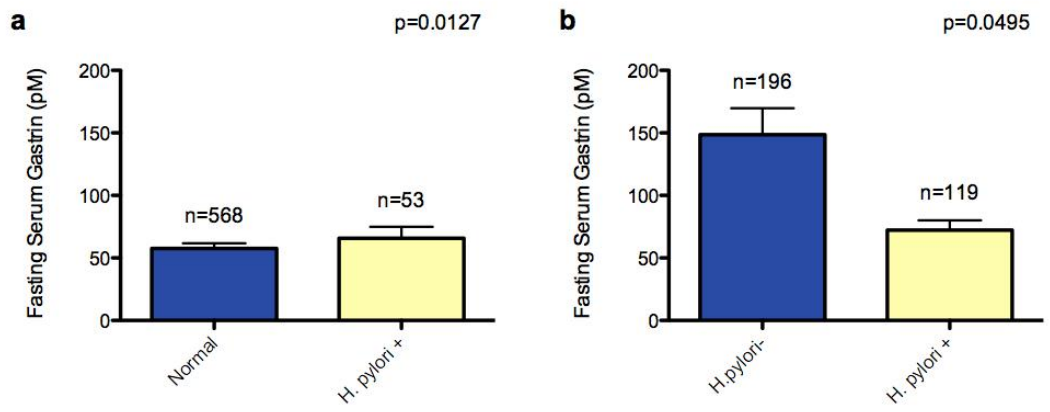

Figure 3-12 Mean fasting serum gastrin concentration for a) subjects with normal histology and those with *H. pylori* associated gastritis and b) *H. pylori* negative and *H. pylori* positive patients with coexisting preneoplasia (two-tailed Mann-Whitney tests).

In the group comprising *H. pylori* negative subjects with normal gastric histology, the mean fasting serum gastrin concentration was 57.6pM (median 29.5pM, SEM 4.1), compared with 65.7pM (median 36.0pM, SEM 9.2) in the 'gastritis B' group and the difference was statistically significant (p=0.0127, two-tailed Mann-Whitney test).

There was an unexpected pattern observed in patients with histologically proven gastric preneoplasia. Here, the mean fasting serum gastrin concentration in *H. pylori* negative subjects was 148.5pM (median 46.8pM, SEM 21.3) and was greater than that observed in *H. pylori* positive subjects where the equivalent value was 72.2pM (median 39.0pM, SEM 7.9). The difference was also statistically significant (p=0.0495, two-tailed Mann-Whitney test).

Revisiting our earlier comparison of 'normal' and 'preneoplasia' groups but excluding *H. pylori* positive subjects or PPI users amplifies the observed effect of preneoplasia on fasting serum gastrin concentration (Figure 3-13). In the 'normal' histology group, the mean serum fasting gastrin concentration was 39.7pM (median 20.0pM, SEM 6.8). The value for the preneoplasia group was significantly higher (mean 148.4 pM, median 32.0, SEM 32.9, $p<0.0001$, two-tailed Mann-Whitney test).

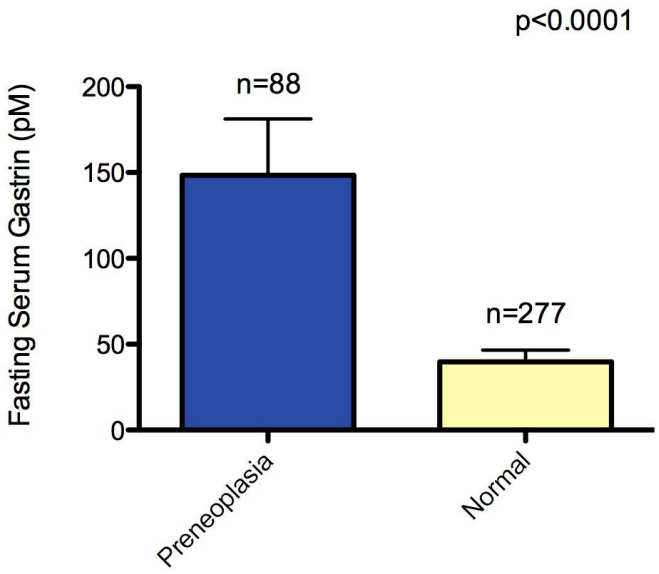

Figure 3-13 Mean fasting serum gastrin concentration in subjects with gastric preneoplasia compared with those with normal histology, excluding *H. pylori* positive subjects and PPI users (two-tailed Mann-Whitney test).

3.7 Serum pepsinogen 1 and 2 concentration and presence of gastric preneoplasia

As outlined above, estimation of the concentration of serum pepsinogens (PG) 1 and 2 is a well-established method of screening for gastric mucosal preneoplasia – the so-called "serological biopsy". We selected samples from our cohort to represent the major preneoplasia pathology groups as well as controls who had normal gastric histology. Of the study subjects with complete histology results, we performed PG 1 and 2 ELISA for 397. The pathology groups and number of patients included are shown in Table 3-9.

We excluded all patients with gastric mucosal pathology except gastritis (of any aetiology) and compared serum PG1 and PG2 concentrations from those with proven, current *H. pylori* infection and those without. There were 122 subjects included in this analysis: normal (n=85), gastritis C (n=5) and gastritis B (n=32). 39 of these had yielded a positive test for current *H. pylori* infection (i.e. rapid urease test or histology positive). The mean serum PG1 concentration, PG2 concentration and

PG1/2 ratio for the *H. pylori* negative group were 96.2µg/l, 8.6µg/l and 11.9 (median = 89.5 µg/l, 7.9 µg/l and 11.8) respectively compared with the same values for the *H. pylori* positive group which were 133.8 µg/l, 17.1 µg/l and 8.5 (median = 110.5 µg/l, 13.6 µg/l and 7.8) respectively. The differences between groups were statistically significant for serum PG1 concentration ($p=0.0011$), PG2 concentration ($p<0.0001$) and PG1/2 ratio ($p<0.0001$, two-tailed Mann-Whitney tests for each). The results are shown in Figure 3-14.

Table 3-9 Gastric pathology groups and numbers of included patients.

Gastric histopathology group	**Number of patients**	
	n	**%**
Normal	86	21.7
Gastritis B - antrum	10	2.5
Gastritis B - corpus	1	0.3
Gastritis C - antrum	2	0.5
Gastritis C - corpus	3	0.8
Gastritis B - pangastric	34	8.6
Foveolar hyperplasia	19	4.8
Atrophy - antrum	13	3.3
Atrophy - corpus	43	10.8
Atrophy - pangastric	5	1.3
Intestinal metaplasia - antrum	73	18.4
Intestinal metaplasia - corpus	40	10.1
Intestinal metaplasia - pangastric	45	11.3
Low-grade dysplasia - antrum	3	0.8
Low-grade dysplasia - corpus	5	1.3
High-grade dysplasia - corpus	1	0.3
High-grade dysplasia - pangastric	2	0.5
Gastric adenocarcinoma	12	3.0

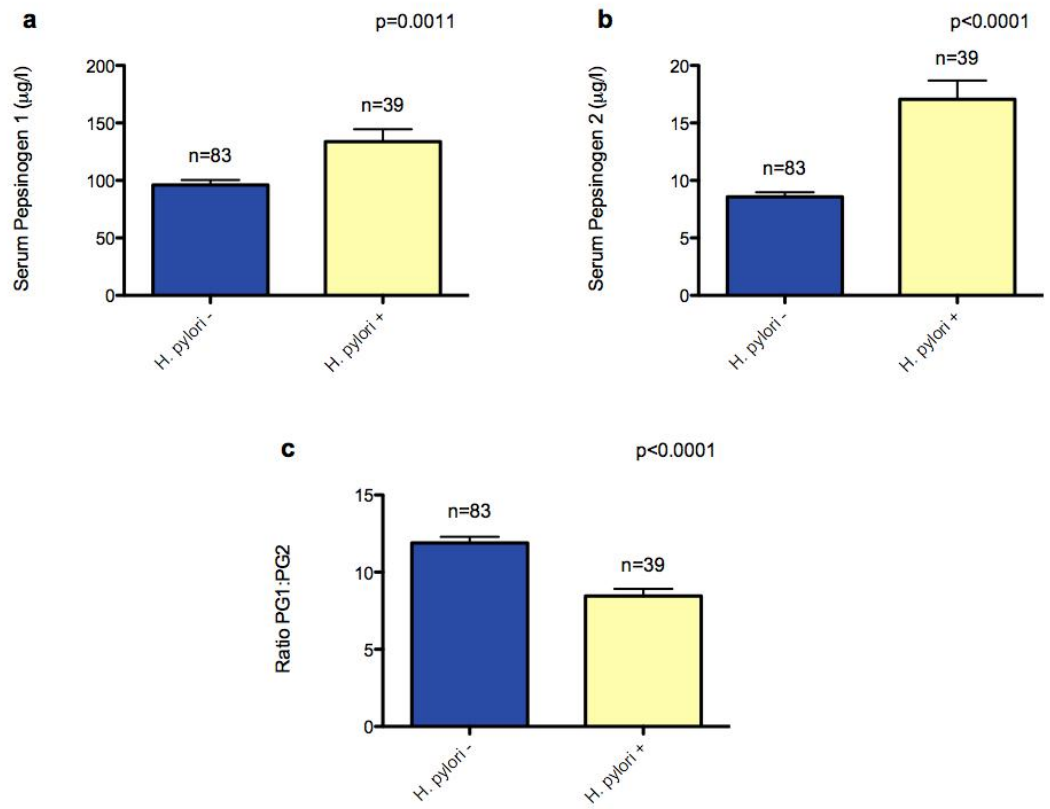

Figure 3-14 a) Serum pepsinogen 1 concentration, b) serum pepsinogen 2 concentration and c) pepsinogen 1/2 ratio in subjects with normal or *H. pylori* associated gastritis only (two-tailed Mann-Whitney tests).

As the premise for PG ratio use in the detection of preneoplasia is based upon the predilection of chronic atrophic gastritis (and the subsequent more "severe" lesions) for a distribution skewed towards the proximal stomach, we assessed the effect of lesion topography on serum PG concentration. For this analysis, we compared histologically normal (n=86) with antral preneoplasia (n=89); corpus preneoplasia (n=89); pangastric preneoplasia (n=52) and gastric adenocarcinoma (n=12). The results are summarised in Table 3-10 and illustrated in Figure 3-15.

Table 3-10 Comparison of serum pepsinogen 1 & 2 concentrations and pepsinogen 1/2 ratio for different disease distributions.

	PG 1 (µg/l)			PG 2 (µg/l)			Ratio PG1:PG2		
	Mean	Median	p-value	Mean	Median	p-value	Mean	Median	p-value
Normal	98.89	88.22	-	9.23	8.10	-	11.54	11.23	-
Antrum preneoplasia	137.7	112.5	0.0002	18.33	15.15	<0.0001	8.80	7.90	<0.0001
Corpus preneoplasia	98.2	82.16	0.1440	16.0	13.16	<0.0001	5.90	5.84	<0.0001
Pangastric preneoplasia	106.3	98.13	0.7836	16.66	13.38	<0.0001	6.77	6.57	<0.0001
Adenocarcinoma	108.8	109.8	0.7410	21.01	12.88	0.0014	6.84	7.83	0.0017

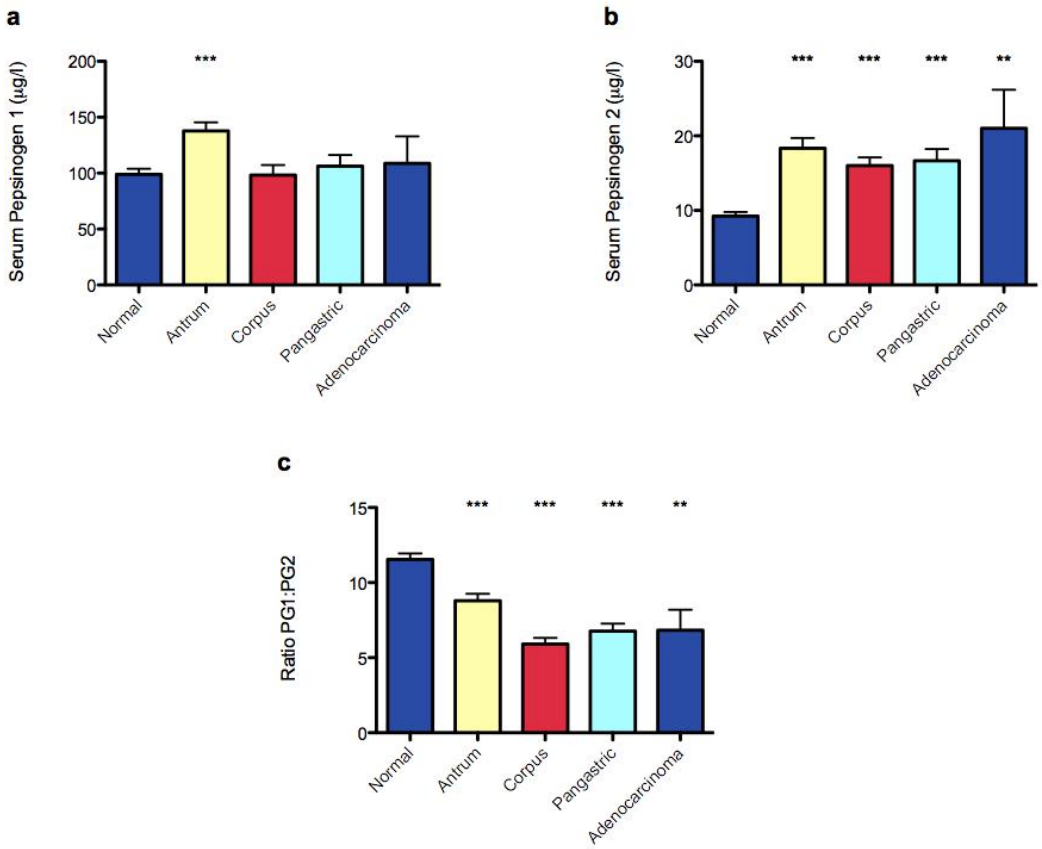

Figure 3-15 a) Serum pepsinogen 1 concentration, b) serum pepsinogen 2 concentration and c) pepsinogen 1/2 ratio comparison for different disease distributions (two-tailed Mann-Whitney tests).

As before, each pathology group was compared with “Normal” controls. There was no significant difference in serum PG 1 concentration between control and pathology groups. In contrast, both serum PG 2 concentrations and PG 1/2 ratios were significantly different between controls and pathology groups ($p<0.0001$, two-tailed Mann-Whitney test). For both ‘tests’, the magnitude of this effect was not diminished by the inclusion of less “severe” preneoplasia cases in the comparison group and there was no significant difference observed in either PG 2 concentration or PG 1/2 ratio between the pathology group comprising “severe” mucosal changes and that containing “all” preneoplasia. Our study was not designed to capture cases of gastric adenocarcinoma and so the sample size is small (we included only 12 cases in the entire cohort). Nevertheless, both PG 2 concentration and PG 1/2 ratio were significantly different in the gastric adenocarcinoma group as compared with normal controls ($p=0.0014$ and 0.0017 respectively, two-tailed Mann-Whitney test).

In our cohort therefore, it seems that serum PG 1 concentration has no discriminant value when comparing subjects with normal gastric histology to those with gastric preneoplasia. Both serum PG 2 concentration and PG 1/2 ratio seem to discriminate between these two groups and so we have examined the usefulness of these “tests” as diagnostic or screening tools. The receiver operating characteristic (ROC) curve for both measures for the presence of gastric preneoplasia are shown in Figure 3-16.

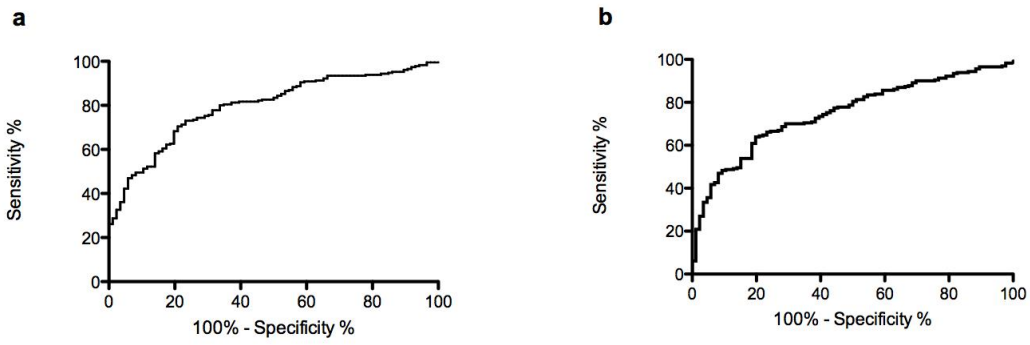

Figure 3-16 ROC curves for a) PG ratio and b) serum PG 2 concentration in gastric preneoplasia.

The PG 1/2 ratio performed better as a diagnostic test than did the PG 2 concentration, with areas under the curves (AUCs) of 0.80 ($p<0.0001$, 95% CI: 0.7455

to 0.8473 Clopper/Hanley method) and 0.75 (p<0.0001, 95% CI: 0.6947 to 0.8061 Clopper/Hanley method) respectively.

The ROC curves for PG ratio in groups including “all” neoplasia, “severe” neoplasia and gastric adenocarcinoma are shown in Figure 3-17. The AUCs were 0.80 (p<0.0001, 95% CI: 0.7455 to 0.8473), 0.77(p<0.0001, 95% CI: 0.7158 to 0.8301) and 0.78 (p=0.0017, 95% CI: 0.6291 to 0.9329) respectively.

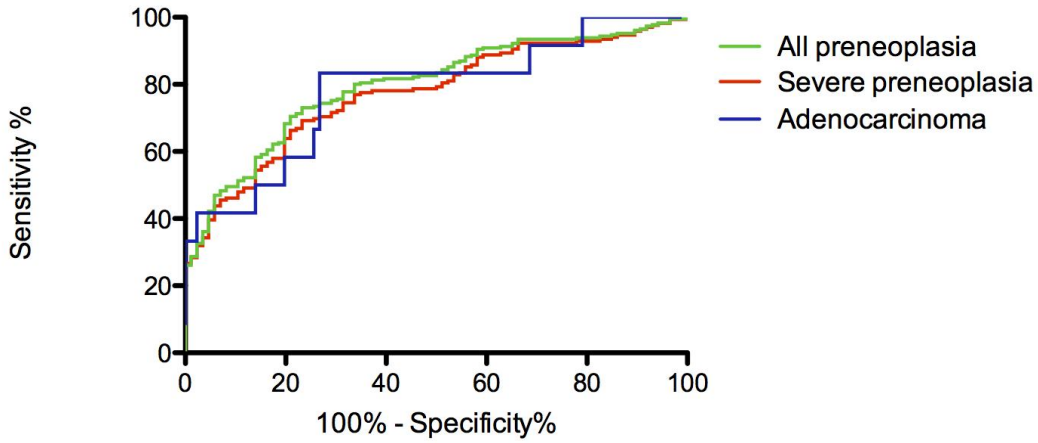

Figure 3-17 ROC curve for PG ratio in all preneoplasia, severe preneoplasia and gastric adenocarcinoma.

The manufacturer of the PG 1 & 2 ELISA assay kits used in this study suggests that a PG 1/2 ratio of less than 3.0 is suggestive of “advanced corpus atrophy” and the same level is accepted as an arbitrary cutoff in the majority of relevant studies(237,294). In our cohort, a cutoff of <3.0 yielded a sensitivity and specificity of 15.7% and 100% respectively. Finding the optimal cutoff in our study by drawing the tangent to the curve in parallel with its axis gives a PG 1/2 ratio cutoff of 8.8 with a sensitivity and specificity of 70.0% and 79.1% respectively (likelihood ratio 3.34).

We also evaluated the addition of elevated fasting serum gastrin concentration and *H. pylori* seropositivity as additional positive tests. Using the same cutoff for PG 1:2 ratio of <8.8 for the diagnosis of preneoplasia as defined above, we first determined the effect of adding fasting hypergastrinaemia as an additional positive test i.e.

patients with “normal” PG 1:2 ratio (>8.8) were considered as giving a positive test if they exhibited a high fasting serum gastrin concentration. We used cutoffs for serum gastrin of 40pM (moderately elevated) and 100pM (high) and evaluated their effects on the performance of a combined diagnostic test separately. The data are summarised in Table 3-11. Inclusion in the “positive test” group of patients with normal PG 1/2 ratio but fasting serum gastrin concentration of ≥40pM increased sensitivity (from 70% to 85%) but at the expense of specificity (from 79% to 70%). The addition of patients with “high” fasting serum gastrin concentration however improved the performance of the combined test. Sensitivity increased (from 70% to 74%) with unaltered specificity. The resulting effect on the predictive values was to increase both positive predictive value (PPV) (from 51% to 52%) and negative predictive value (NPV) (from 89% to 91%). These improvements are marginal, but suggest that future panels of biomarkers for gastric preneoplasia should include both pepsinogen 1/2 ratio and fasting serum gastrin concentration for the best performance.

Table 3-11 Diagnostic performance of pepsinogen 1/2 ratio alone and in conjunction with serum gastrin concentration. PPV = positive predictive value; NPV = negative predictive value.

	Pepsinogen 1/2 ratio only		Pepsinogen 1/2 ratio & serum gastrin ≥40pM		Pepsinogen 1/2 ratio & serum gastrin ≥100pM	
	Test +	Test -	Test +	Test -	Test +	Test -
Preneoplasia +	160	70	195	35	170	60
Preneoplasia -	18	68	26	60	18	68
Sensitivity %	69.6		84.8		73.9	
Specificity %	79.1		69.8		79.1	
PPV %	50.7		46.4		52.2	
NPV %	89.4		93.7		90.7	

We adopted the same approach to assess the effect of *H. pylori* testing in combination with the PG 1/2 ratio on the performance of the ‘diagnostic test’ for the detection of gastric preneoplasia. Commercially available kits marketed for this purpose include a test to determine *H. pylori* serology status as this would be in

keeping with the other noninvasive tests described (i.e. based on blood testing). For completeness, we also assessed the contribution of *H. pylori* histology status. By definition, this would not be considered a noninvasive test, as it would conventionally require an upper GI endoscopy examination to obtain gastric mucosal biopsies. We have included it however as there are alternative noninvasive means of testing for 'current' *H. pylori* infection (e.g. C^{13} urea breath testing or *H. pylori* faecal antigen testing). These modes of testing lack the sensitivity of histological examination for the diagnosis *H. pylori* infection, but we have included the latter as a surrogate for the former. We also assessed CagA seropositivity in place of *H. pylori* seropositivity. The data are summarised in Table 3-12. Both *H. pylori* histology positivity and CagA seropositivity adversely affected the performance of the combined diagnostic test by their inclusion. In both cases, sensitivity and specificity were diminished. In contrast, combining *H. pylori* serology positivity and PG 1/2 ratio proved to be more useful for the diagnosis of gastric preneoplasia than PG 1/2 ratio alone. Sensitivity was increased (from 70% to 88%) for a small decrease in specificity (79% to 74%). Positive and negative predictive values were both improved (50% to 52%; 89% to 95% respectively).

Table 3-12 Diagnostic performance of pepsinogen 1/2 ratio in combination with *H. pylori* serology, histology and CagA serology. PPV = positive predictive value; NPV = negative predictive value.

	Pepsinogen 1/2 ratio & *H. pylori* serology +		Pepsinogen 1/2 ratio & *H. pylori* histology +		Pepsinogen 1/2 ratio & *H. pylori* CagA +	
	Test +	Test -	Test +	Test -	Test +	Test -
Preneoplasia +	203	27	180	50	173	57
Preneoplasia -	22	64	21	65	19	67
Sensitivity %	88.3		78.3		75.2	
Specificity %	74.4		75.6		77.9	
PPV %	51.6		49.8		51.3	
NPV %	95.4		91.8		91.1	

To conclude our assessment of the utility of conventional noninvasive biomarkers of gastric preneoplasia in our study cohort, we combined the best performing tests: PG

1/2 ratio, fasting serum gastrin concentration and *H. pylori* serology. As before, a subject was considered to have yielded a positive test if the serum PG 1/2 ratio was <8.8 and/or the fasting serum gastrin ≥100pM and/or the *H. pylori* serology was positive. The data are summarised in Table 3-13. The combined test outperformed any of the individual tests or other combinations.

Table 3-13 Diagnostic performance of pepsinogen 1/2 ratio in combination with *H. pylori* serology and fasting serum gastrin concentration. PPV = positive predictive value; NPV = negative predictive value.

	Combined test	
	Test +	Test -
Preneoplasia +	207	23
Preneoplasia -	22	64
Sensitivity %	90.0	
Specificity %	74.4	
PPV %	52.1	
NPV %	96.0	

3.8 Discussion

In the present study, we report the effects of various host, environmental and microbial factors on the development of gastric preneoplasia. Our study included a large number of participants. We hypothesised that a large number of subjects would be required in order to include a sufficient population with *H. pylori* and gastric preneoplasia for meaningful analysis. In the event, 1400 study volunteers yielded large numbers with gastric preneoplasia (388, 24.5%).

As discussed in chapter 1, in the UK population, demands on healthcare providers are inexorably increasing and outpatient endoscopy services are no exception. Noninvasive tools, which might dispense with the requirement to proceed to upper GI endoscopy, would reduce the burden on these resources and avoid the inconvenience, discomfort and risk associated with these procedures. An obvious limitation of the present study is that it is based on a relatively homogeneous group

of patients recruited at a single centre. The study does however benefit from the inclusion of only symptomatic individuals who have been referred for upper GI endoscopy, which is arguably the most important group for identification of individuals at risk of cancer development.

Another major advantage of our study design is the means by which we obtained and assessed gastric mucosal histology specimens. The participation of a single, specialist GI pathologist and their use of standardised and validated scoring and reporting systems proved invaluable in mitigating the effects of subjectivity and eliminating inter-observer variation. There is considerable variation in terminology apparent in the literature. In this thesis, I have presented the histological results using the terminology agreed upon by our research group with our expert GI pathologist in advance of the study commencing. In this context, the terms 'gastritis B' and '*H. pylori* positive gastritis' are used interchangeably. The limitations of histology as a means of testing for *H. pylori* are discussed on page 138. We recognise that the presence of gastritis in the absence of *H. pylori* organisms does not exclude *H. pylori* infection as the aetiology in every case but in the interests of clarity, we have used the term 'gastritis C' to describe this pathological cohort.

Safety and ethical considerations dictated the number of gastric mucosal biopsies that we were able to obtain from each subject. Gastric mucosal preneoplastic changes are frequently distributed unevenly. As a means of diagnosing and determining disease topography therefore, mucosal pinch biopsies are susceptible to sampling errors. The recommended sampling protocol for clinical diagnosis would include 5 pinch biopsies including one from the angulus incisura (197,199) whereas we were limited to 4 specimens.

We tested the majority of our subjects for evidence of *H. pylori* infection by three methods. We were able to evaluate the performance of a commonly used diagnostic technique (RUT) in the process. We observed (and previously reported) RUT accuracy in the overall study cohort and that sensitivity is adversely affected by concurrent PPI use and CagA negative status. Rates of current and previous *H. pylori*

infection and CagA positivity were broadly in line with similar population based studies in similar populations(295–297) though it should be noted that our mode of testing (ELISA) has been shown to lack sensitivity when compared with western blot assay(298). We selected ELISA for this application in the present study due to resource constraints.

In section 3.5, I have described how the results of the anti-*H. pylori* IgG ELISA are reported (in accordance with the manufacturer's instructions) as being 'positive', 'negative' or 'indeterminate', and how we then grouped the small number (46 cases) of 'indeterminate' results with the 'negative' results for the purposes of our analyses. The rationale for doing so was pragmatic – we considered that the effect on subsequent analyses of including 'false positive' indeterminate cases would be more deleterious to our results than that of excluding 'false negative' cases. We recognise that this distinction might adversely influence the sensitivity of the diagnostic biomarker panel described in this chapter but consider that any effect would be small given the low number of cases involved.

In section 3.5, we also reported that 48.7% of subjects found to be seropositive for *H. pylori* exhibited no histological evidence of current infection. We categorised these individuals as having been 'previously infected' and proposed that this group comprises those individuals in whom *H. pylori* infection has been cleared either by means of antimicrobial treatment or by host immunity. We recognise however that some of these individuals will have been misclassified in this way as a result of the vagaries of the two testing modalities in question. As described, a small number of false-positive serology might be expected but the impact of false-negative histology/RUT is likely to be greater. In particular, *H. pylori* detection by histology or RUT is known to be less sensitive in the presence of hypochlorhydria and the commonest cause of this is PPI use.

We found histological evidence of gastric mucosal preneoplasia in 24.5% of patients analysed. Our observations corroborated our understanding and expectations of the factors associated with preneoplasia development, namely: patients with gastric

preneoplasia were more likely to be infected with *H. pylori* or be seropositive for this organism (suggesting previous infection); the effect of *H. pylori* on preneoplasia incidence was potentiated by CagA positivity; and preneoplasia was detected more frequently in older subjects.

The effect of gastric preneoplasia on fasting serum gastrin concentration was also in line with previous observations. Gastric preneoplasia was associated with elevated fasting serum gastrin concentrations and the effect was greater for corpus predominant disease than for pangastric disease, which was – in turn – greater in effect than antral predominant disease.

Other causes of gastric hypochlorhydria were also associated with an increase in fasting serum gastrin concentration. Recent PPI use conferred an increase in fasting serum gastrin concentration both in subjects with normal gastric histology and those with gastric preneoplasia.

Similarly, the well-reported(36) effect of *H. pylori* infection upon fasting serum gastrin concentrations was replicated in our study. Current *H. pylori* infection (as evidenced by RUT or histology positive testing) was associated with a significant increase in fasting serum gastrin concentration in subjects with normal gastric mucosal histology. Somewhat unexpectedly, this effect was reversed in those patients who had gastric preneoplasia. We speculated that this phenomenon might result from the loss of *H. pylori* infection that is often seen in gastric atrophy following chronic gastritis.

Serum pepsinogen 1 and 2 concentrations (and the pepsinogen 1/2 ratio) have been well validated(197) in some (particularly far-Eastern) populations as a noninvasive means of diagnosing early gastric adenocarcinoma and preneoplastic lesions. The so-called serologic biopsy has been less well studied in Western populations for the identification of preneoplasia. Sensitivity, specificity and negative predictive value of serum pepsinogen 1/2 ratio for the detection of gastric preneoplasia were similar to previously published data from both Asian and European populations.

Recent studies have also examined the use of a "GastroPanel" for the detection of gastric preneoplasia(241). However, the authors concluded that this compound test was insufficiently accurate for clinical use. Our compound test performed better in this study and the populations were similar. We speculate that this may be due to the improved accuracy of gastrin radioimmunoassay over the ELISA test that was used in GastroPanel, as the former detects all amidated gastrins whereas the latter is said to be specific for only one peptide, namely gastrin-17.

In the present study, we evaluated the performance of a combined diagnostic test for gastric preneoplasia comprising assays for serum PG1 and PG2 concentration, serum gastrin concentration and *H. pylori* serology. The analyses described in section 3.7 were conducted using a defined 'normal' histology group as controls. We recognise that excluding confounding factors such as concurrent PPI use and *H. pylori* seropositivity from this control group might potentiate the observed performance of the diagnostic panel. Further biomarker panel development would include testing in 'real-world' groups to determine clinical usefulness in target populations.

4 Novel Biomarkers of Gastric Mucosal Preneoplasia

4.1 Introduction

Following infection with *H. pylori*, a small minority of individuals goes on to develop intestinal type gastric adenocarcinoma. Gastric cancer is very often diagnosed at an advanced stage and is associated with very high mortality. The disease develops via a series of well-defined preneoplastic steps (known as Correa's oncogenic cascade) and a means to reliably detect the presence of these preneoplastic conditions might offer opportunities for surveillance and early treatment. Upper GI endoscopy is invasive, costly and probably unreliable for the detection of gastric preneoplasia if not performed by properly trained endoscopists and adequate gastric mucosal biopsies taken from appropriate sites. Current noninvasive screening tests are insufficiently sensitive and/or specific to be used in routine clinical practice.

There is therefore a need to identify novel biomarkers of gastric preneoplasia which might be used both in high-risk populations for population screening; and in low-risk populations for screening of symptomatic individuals. Such a biomarker might be useful in determining the risk of gastric cancer posed to a given individual and assist in determining the required surveillance or treatment strategy.

Our group amongst others has previously described the role played by several genes and proteins of the gastric epithelium in carcinogenesis. We aimed to assess the changes in expression of these genes and proteins in the presence of gastric preneoplasia in order to determine their potential utility as biomarkers.

4.2 Materials and Methods

The study methodology is described in detail in chapter 2 and is summarised here for convenience.

Gastric corpus mucosal pinch biopsies were taken during each study endoscopy and stored immediately in RNAlater. These were stored at -20°C prior to RNA extraction and reverse transcription. The resulting cDNA was assayed by real-time qPCR. Each PCR plate incorporated standards of known concentrations, both for the gene of interest and for the housekeeper gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The abundance of both gene transcripts was determined from the standard curves. Relative gene transcript abundance was calculated from the ratio of candidate gene to housekeeper gene mRNA abundance.

We began the study with a list of candidate genes which are listed in Table 4-1.

Table 4-1 Candidate biomarker genes.

Candidate Gene	Candidate Gene (abbreviation)	Origin
Insulin-like growth factor-1	IGF-1	Mesenchymal
Insulin-like growth factor-2	IGF-2	Mesenchymal
Matrix metalloproteinase-1	MMP-1	Both
Matrix metalloproteinase-3	MMP-3	Both
Matrix metalloproteinase-7	MMP-7	Epithelial
Plasminogen activator inhibitor-1	PAI-1	Both
Plasminogen activator inhibitor-2	PAI-2	Both
Tissue inhibitor of metalloproteinases-1	TIMP-1	Both
Tissue inhibitor of metalloproteinases-3	TIMP-3	Both
Tissue inhibitor of metalloproteinases-4	TIMP-4	Both
Urokinase plasminogen activator	uPA	Both
Vimentin	-	Mesenchymal

4.3 Selection of biomarker gene

The above panel of candidate genes was tested following an initial phase of recruitment, which yielded a 'test group' – a subset of the final study cohort. In this phase, 354 samples were selected from the first 425 patients recruited to represent the relevant pathology groups. For these and subsequent biomarker analyses, pathology groups were separated along the lines described in chapter 3, with the important distinction that cases with oesophageal mucosal pathology (oesophageal malignancy and Barrett's oesophagus) were assigned to their own groups for

separate analyses. This was done on the basis that the expression of genes of interest could be altered in oesophageal mucosal disease as well as in that of the gastric mucosa. One difficulty encountered in implementing this distinction was that not all patients whose endoscopy report included mention of Barrett's oesophagus (BO) had biopsies of the distal oesophagus taken to substantiate the diagnosis. These cases were therefore excluded from both the gastric 'control' group, and from the 'Barrett's oesophagus' pathology group as the endoscopic diagnosis of BO is notoriously unreliable. When there was an unsubstantiated case of BO in the presence of gastric preneoplasia, then that case was counted in the relevant gastric pathology group.

As described previously, there were 19 cases in whom a complete set of serum and biopsy samples were not obtained, hence the subsequent analyses were performed on a total cohort of 1381 cases. As outlined previously, there were 29 additional cases whose gastric histology dataset was incomplete (i.e. only antral or only corpus samples were taken/analysed). Once cases of gastric cancer, oesophageal cancer and histologically proven BO were counted, there were 18 cases of endoscopically reported BO without histological support for that diagnosis. Of these, there was histological evidence of gastric preneoplasia in 6 individuals and these cases were included in the relevant gastric pathology group. The remaining 12 cases were removed from further analyses. The 'pathology' groups in this analysis are shown in Table 4-2. One of the groups listed is 'normal gastric histology but *H. pylori* histology positive'. This group comprises those individuals in whom the study histopathologist reported normal mucosa, but in whom the local hospital pathologist had identified *H. pylori* organisms. We amalgamated small groups into larger categorical groups for some analyses. For example, reactive gastritis and foveolar hyperplasia were combined into 'benign disease' and intestinal metaplasia, atrophy and dysplasia (of any grade) were combined into 'preneoplasia'.

Patient samples were assayed by qPCR for each putative biomarker gene's expression and for that of the reference gene (GAPDH). The relative mRNA abundance values for each gene of interest were calculated by normalising the

mRNA abundance measured by PCR to that of the reference gene in order to control for discrepancies in the quantity of cDNA included in each assay.

Table 4-2 Gastric pathology groups and numbers of included patients. FH=foveolar hyperplasia; IM=intestinal metaplasia; LGD=low-grade dysplasia; HGD=high-grade dysplasia

	'Test' group n=354		**Total cohort** n=1381	
Gastric histopathology group	**Number of patients**		**Number of patients**	
	n	**%**	**n**	**%**
Normal gastric histology	99	28.0	444	32.2
Normal histology but *H. pylori* serology+	33	9.3	140	10.1
Normal histology but *H. pylori* histology+	0	0.0	21	1.5
Gastritis B - Antrum	5	1.4	23	1.7
Gastritis B - Corpus	7	2.0	30	2.2
Gastritis B - Pangastric	24	6.8	79	5.7
Gastritis C - Antrum	21	5.9	39	2.8
Gastritis C - Corpus	4	1.1	5	0.4
Gastritis C - Pangastric	7	2.0	7	0.5
FH - Antrum	37	10.5	165	11.9
FH - Corpus	2	0.6	7	0.5
FH - Pangastric	4	1.1	7	0.5
Atrophy - Antrum	7	2.0	19	1.4
Atrophy - Corpus	12	3.4	55	4.0
Atrophy - Pangastric	2	0.6	5	0.4
IM - Antrum	34	9.6	111	8.0
IM - Corpus	4	1.1	16	1.2
IM - Pangastric	4	1.1	19	1.4
IM and Atrophy - Antrum	4	1.1	24	1.7
IM and Atrophy - Corpus	16	4.5	44	3.2
IM and Atrophy - Pangastric	3	0.8	5	0.4
LGD - Antrum	3	0.8	6	0.4
LGD - Corpus	1	0.3	5	0.4
LGD - Pangastric	0	0.0	0	0.0
HGD - Antrum	0	0.0	0	0.0
HGD - Corpus	0	0.0	1	0.1
HGD - Pangastric	2	0.6	2	0.1
Barrett's oesophagus	8	2.3	54	3.9
Endoscopy report of Barrett's oesophagus	2	0.6	12	0.9
Oesophageal cancer	3	0.8	11	0.8
Gastric cancer	3	0.8	12	0.9
Gastric ulcer only	0	0.0	10	0.7
Antrum MALT only	0	0.0	3	0.2

Using the 'test group' of patients, we compared the relative mRNA abundances of each gene of interest in four groups – histology normal, benign gastric disease, gastritis B (*H. pylori* related gastritis) and preneoplasia. The results are shown in Figure 4-1.

In the 'test group', only 7 of the 12 proposed biomarker genes exhibited a significant difference ($p<0.0001$, Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test) in mRNA relative abundance when comparing subjects with normal gastric histology to those with gastric mucosal preneoplasia (IGF-1, IGF-2, MMP-1, MMP-7, TIMP-1, TIMP-4 and uPA). Of these, only 3 (TIMP-1, MMP-1 and MMP-7) also exhibited a significant increase when comparing benign disease to preneoplasia groups and in these three, only MMP-7 showed a highly statistically significant change ($p<0.0001$, Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test).

In the same cohort, mRNA abundances were significantly increased in 'benign disease' when compared with 'normal' samples for only 7 of the 12 genes of interest (IGF-1, IGF-2, MMP-1, MMP-7, TIMP-1, TIMP-4 and uPA). Of these, 6 also exhibited a significant difference when comparing 'normal' to 'gastritis B' subjects (IGF-1, IGF-2, MMP-1, MMP-7, TIMP-1, and uPA). Only the three previously highlighted genes of interest (TIMP-1, MMP-1 and MMP-7) yielded a significant difference in all categories described and MMP-7 showed the greatest difference across all four comparisons. In the 'test group', none of the proposed biomarkers were shown to discriminate between subjects with gastritis B and those with gastric preneoplasia.

On the basis of these findings, MMP-7 was selected as the most promising biomarker and was studied further in the entire study cohort.

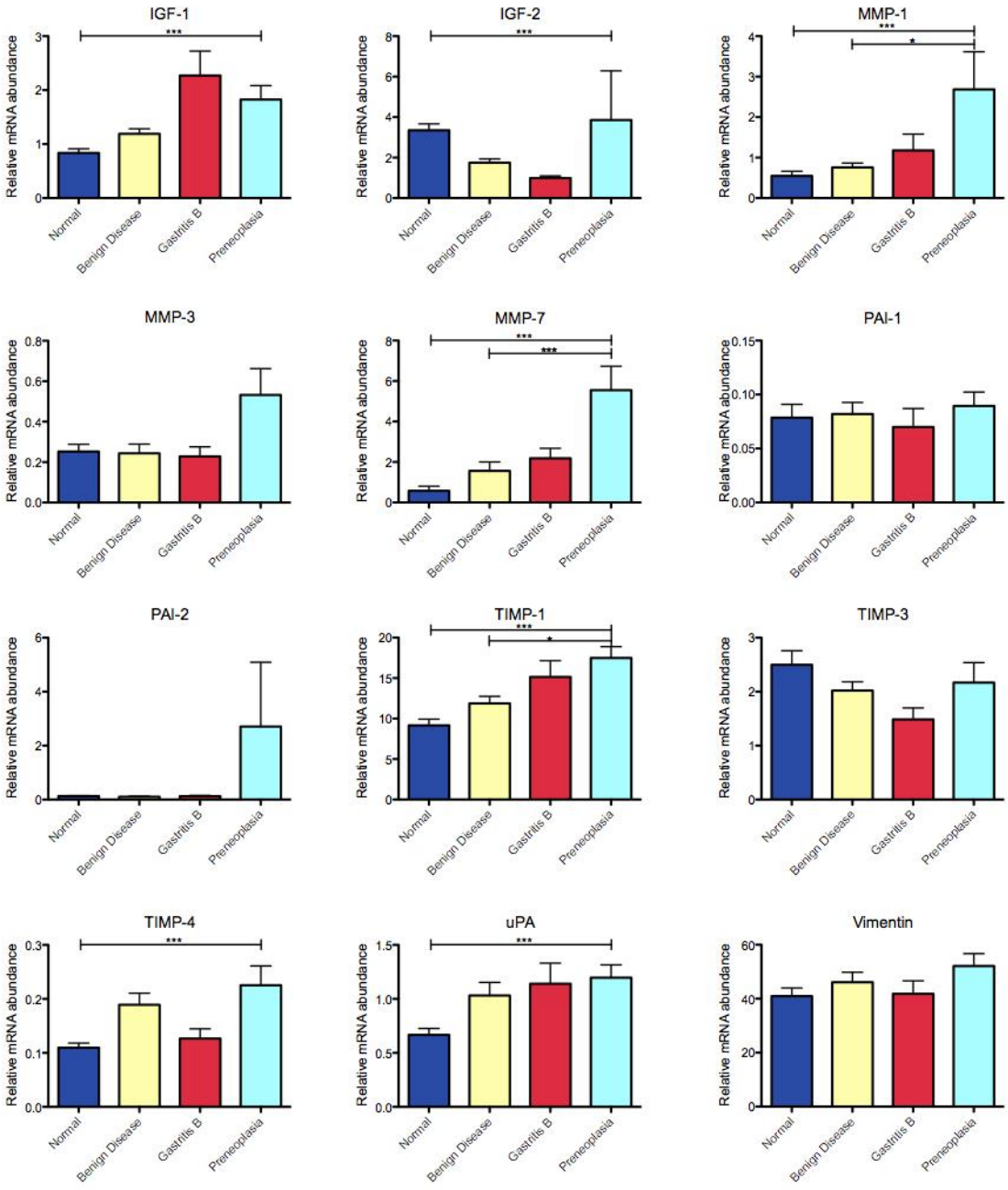

Figure 4-1 Mean relative abundance of mucosal biomarker mRNA in subjects with normal gastric histology, benign disease, *H. pylori* associated gastritis (gastritis B) and preneoplasia (Kruskal-Wallis 1-way ANOVA).

4.4 Mucosal expression of MMP-7

As described above, cDNA obtained from gastric corpus mucosal pinch biopsies was assayed for MMP-7 relative abundance by qPCR. Samples were selected for assay based on the histology group to which they belonged. In total, 917 of 1381 samples were assayed for MMP-7 relative abundance. The numbers assayed by group are summarised in table 4-3. The mean value across the entire cohort was 3.10 (SD 35.80, SEM 1.182, median 0.3972). We performed Grubbs' test to identify outliers with a two-sided significance level of 0.05. Only one outlier was identified. That subject had been assigned to the 'foveolar hyperplasia – antrum' pathology group and the relative abundance of MMP-7 was 1057.23 as compared to a mean in that group excluding the outlier of 1.51. The patient information for the outlier was examined (including local hospital pathology report, medication history and medical history), but no explanation for such a high mucosal expression of the gene was identified. The result was assumed to be spurious and to simplify further analysis this patient was excluded.

4.4.1 Control Group

As outlined in section 2.1, we collected data for a large number of variables for each patient, many of which were included based on the suspicion that they might influence the expression of putative biomarkers. In order that accurate assessments of the effect of these variables on mucosal MMP-7 expression could be made, a 'control' group was required. We generated this group from the subjects whose gastric histology was reported as "normal" by both the study and local hospital pathologists. We then excluded patients whose medical history included chronic inflammatory diseases, chronic obstructive pulmonary disease (COPD), ischaemic heart disease (IHD) or active cancer. We also removed patients who had reported recent use of NSAIDs, aspirin or PPIs and those who had elevated fasting serum gastrin concentrations (>40pM). There were 36 patients who had gastric MMP-7 mRNA relative abundance measured and who fulfilled the above criteria. They are

referred to in the subsequent analyses as the ‘control’ group. The mean values for relative abundance of MMP-7 mRNA was 0.549 (SEM ± 0.148) in the ‘normal histology’ group as compared to 0.230 (SEM ± 0.054) in this ‘control’ group. The difference between the means of the two groups was significant (p=0.044, two-tailed unpaired t-test/Welch’s correction). The data are illustrated in Figure 4-2.

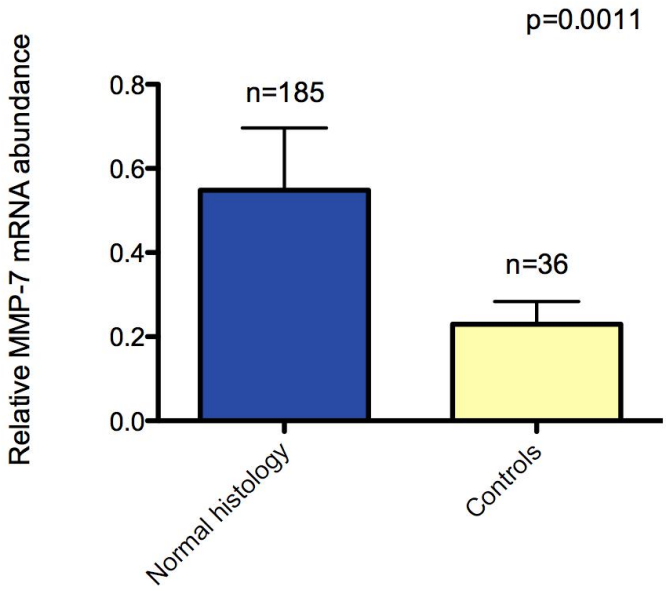

Figure 4-2 Relative mRNA abundance in subjects with normal gastric histology compared with the 'refined' control group (two-tailed unpaired t-test)

Table 4-3 Gastric pathology groups and numbers of patients included for MMP-7 assay.

Gastric histopathology group	Total in group	Number assayed	Proportion assayed (of group)	Proportion assayed (of total cohort)
	n	**n**	**%**	**%**
Normal gastric histology	444	185	41.7	13.4
Normal histology but *H. pylori* serology+	140	70	50.0	5.1
Normal histology but *H. pylori* histology+	21	7	33.3	0.5
Gastritis B - Antrum	23	15	65.2	1.1
Gastritis B - Corpus	30	30	100.0	2.2
Gastritis B - Pangastric	79	77	97.5	5.6
Gastritis C - Antrum	39	34	87.2	2.5
Gastritis C - Corpus	5	5	100.0	0.4
Gastritis C - Pangastric	7	7	100.0	0.5
FH - Antrum	165	120	72.7	8.7
FH - Corpus	7	7	100.0	0.5
FH - Pangastric	7	7	100.0	0.5
Atrophy - Antrum	19	17	89.5	1.2
Atrophy - Corpus	55	53	96.4	3.8
Atrophy - Pangastric	5	5	100.0	0.4
IM - Antrum	111	98	88.3	7.1
IM - Corpus	16	16	100.0	1.2
IM - Pangastric	19	18	94.7	1.3
IM AND Atrophy - Antrum	24	15	62.5	1.1
IM AND Atrophy - Corpus	44	44	100.0	3.2
IM AND Atrophy - Pangastric	5	5	100.0	0.4
LGD - Antrum	6	6	100.0	0.4
LGD - Corpus	5	5	100.0	0.4
LGD - Pangastric	0	0	-	-
HGD - Antrum	0	0	-	-
HGD - Corpus	1	1	100.0	0.1
HGD - Pangastric	2	2	100.0	0.1
Barrett's oesophagus	54	29	53.7	2.1
Endoscopy report of Barrett's oesophagus	12	7	58.3	0.5
Oesophageal Cancer	11	9	81.8	0.7
Gastric Cancer	12	10	83.3	0.7
Gastric Ulcer only	10	10	100.0	0.7
Antrum MALT only	3	3	100.0	0.2

4.5 Mucosal expression of MMP-7 and gastric mucosal pathology

We compared the mean relative mRNA abundance of mucosal MMP-7 in our various gastric pathology groups with that in the control group.

Our aims were to examine the effect of the presence of gastric preneoplasia on the mucosal expression of MMP-7; to determine how gastric corpus mucosal MMP-7 expression varied with topographical distribution of gastric mucosal preneoplasia; to determine how mucosal MMP-7 expression varied with "severity" of gastric mucosal preneoplasia and to establish whether the relative mRNA abundance of MMP-7 could reliably distinguish between patients with gastric mucosal preneoplasia and those with either histologically normal biopsy specimens; "benign" gastric mucosal changes (including reactive gastritis (gastritis C) and foveolar hyperplasia) or *H. pylori* associated gastritis.

The comparisons of relative mRNA abundance of MMP-7 are shown for the major groups in Figure 4-3.

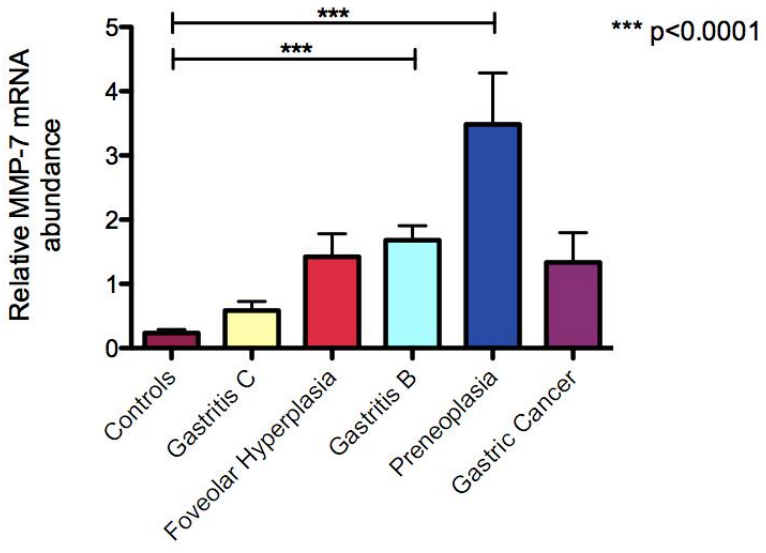

Figure 4-3 Mean relative mRNA abundance of MMP-7 in controls (n=36) and major gastric pathology groups: gastritis C (n=46); foveolar hyperplasia (n=133); gastritis B (n=122); preneoplasia (n=285) and gastric cancer (n=10) (Kruskal-Wallis 1-way ANOVA).

As described before, mean relative mRNA abundance of MMP-7 in the control group was 0.2298 (median 0.0905, SEM 0.0537). There were small observed increases in the abundances seen in gastritis C (mean 0.5839, median 0.1221, SEM 0.1452), foveolar hyperplasia (mean 1.423, median 0.2014, SEM 0.3569) and gastric cancer (mean 1.336, median 0.8375, SEM 0.4612) groups though none were statistically significant (Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test).

There was however a highly statistically significant difference ($p<0.0001$, Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test) between the relative mRNA abundance of MMP-7 in controls and pathology groups for both *H. pylori* associated gastritis B (mean 1.423, median 0.2014, SEM 0.3569) and for the combined preneoplasia group (mean 3.485, median 0.7322, SEM 0.7996).

For completeness, we also compared mucosal mRNA abundance of MMP7 in these pathology groups with the 'normal' histology group described in section 4.4.1,in place of the 'control' group. The results are shown in Figure 4-4. As before, there was a highly statistically significant difference ($p<0.0001$, Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test) between the mean relative mRNA abundance of MMP-7 in the 'normal histology' group and that in both *H. pylori* associated gastritis B and combined preneoplasia groups.

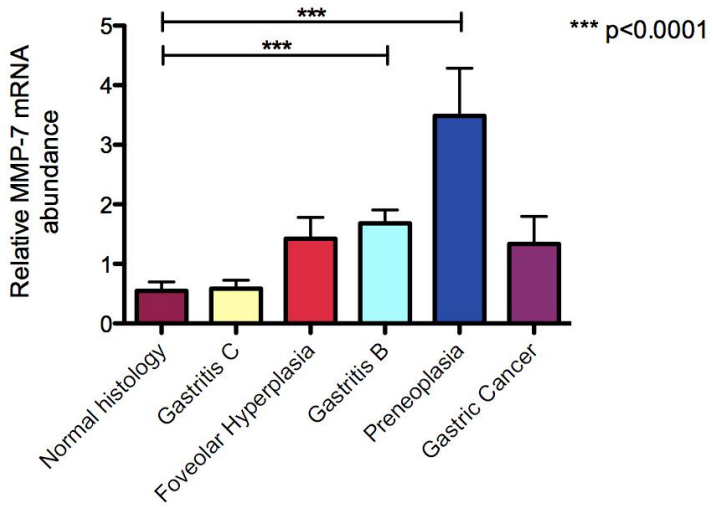

Figure 4-4 Mean relative mRNA abundance of MMP-7 in normal histology group (n=185) and major gastric pathology groups: gastritis C (n=46); foveolar hyperplasia (n=133); gastritis B (n=122); preneoplasia (n=285) and gastric cancer (n=10) (Kruskal-Wallis 1-way ANOVA).

4.5.1 Variation in mucosal MMP-7 expression with distribution of disease

As described in chapter 3, we were able to separate study subjects with gastric mucosal disease into groups depending on the topographical distribution of their disease as determined by histological examination of antral and corpus biopsies separately. Relative abundance of mucosal MMP-7 mRNA was measured in gastric tissue obtained from the corpus only and so the effect on MMP-7 relative mRNA abundance of topographical variation is important to determine. If “antral only” gastric mucosal preneoplasia is seen to result in an increase in corpus mucosal MMP-7 expression, then our data from this patient group will contribute to the validation or otherwise of the gene as a potential biomarker for preneoplasia. If however, MMP-7 mRNA expression in corpus mucosa is influenced only by mucosal remodelling at the same site then we ought only to interpret corpus-inclusive disease groups. We therefore compared antral-only, corpus-only and pangastric preneoplasia groups with controls. The data are illustrated in Figure 4-5.

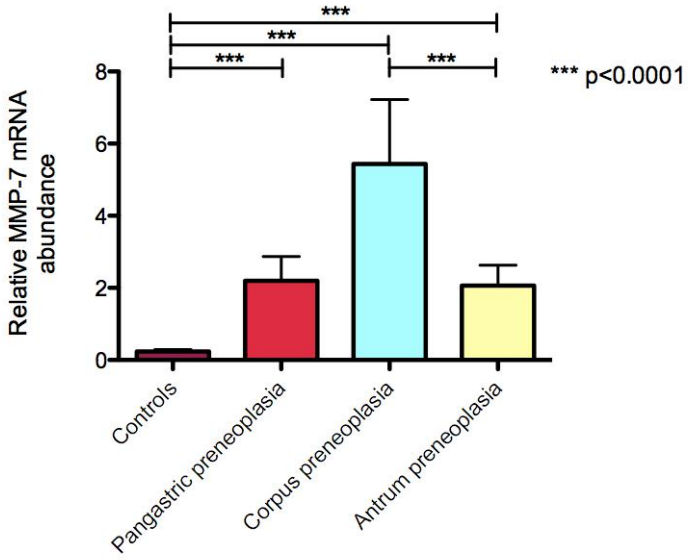

Figure 4-5 Mean MMP-7 relative mRNA abundance for controls (n=36) and different preneoplasia disease distributions: pangastric (n=30); corpus predominant (n=119); antrum predominant (n=136) (Kruskal-Wallis 1-way ANOVA).

There was a significant difference observed in the relative mRNA abundance of MMP-7 in all groups when compared with controls ($p<0.0001$, Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test). However the effect was substantially greater in the corpus-only pathology groups than in either the antrum-only (where the difference itself was also statistically significant) or pangastric groups. The data are summarised in Table 4-4.

For completeness, we again repeated these analyses using the 'normal histology' group as a comparator in place of the 'control' group. The data are illustrated in Figure 4-6. As before, there was a significant difference observed in the relative mRNA abundance of MMP-7 in all groups when compared with the normal histology group ($p<0.0001$, Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test). Similarly, the effect was substantially greater in the corpus-only pathology groups than in either the antrum-only or pangastric groups.

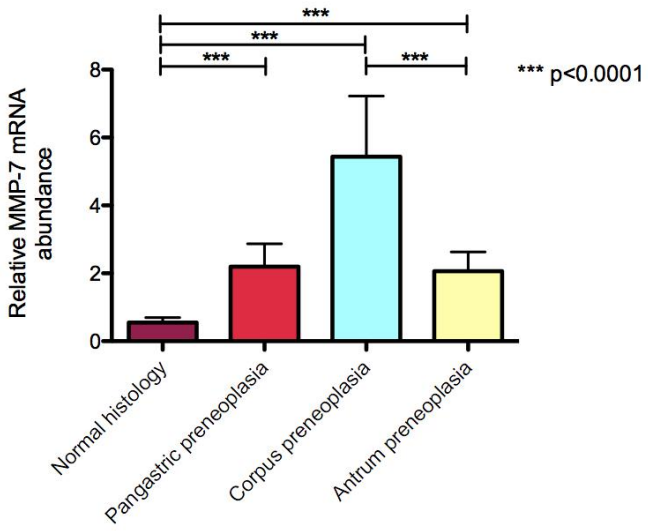

Figure 4-6 Mean MMP-7 relative mRNA abundance for normal histology group (n=185) and different preneoplasia disease distributions: pangastric (n=30); corpus predominant (n=119); antrum predominant (n=136) (Kruskal-Wallis 1-way ANOVA).

Table 4-4 Relative abundance of MMP-7 in different disease distributions.

	Controls	Antrum	Corpus	Pangastric
Number of patients	36	136	119	30
Minimum	0.01243	0.00812	0.023	0.02393
25% Percentile	0.04154	0.1317	0.518	0.2204
Median	0.09052	0.397	1.424	0.7194
75% Percentile	0.2913	1.062	4.269	2.078
Maximum	1.539	60.98	200.2	16.27
Mean	0.2298	2.061	5.436	2.198
Std. Deviation	0.3221	6.604	19.46	3.686
Std. Error	0.05368	0.5663	1.784	0.6729
Fold change	1.000	8.969	23.655	9.565
Lower 95% CI	0.1208	0.9411	1.904	0.8214
Upper 95% CI	0.3388	3.181	8.969	3.574

These results are interesting – we might have expected an attenuated increase in corpus expression of MMP-7 when the histology reflected antral predominant disease and the data confirm this. The effect seen in pangastric disease is however

somewhat unexpected. We anticipated a similar effect on corpus MMP-7 expression in corpus-inclusive disease whether the antrum was involved or not, but observed a smaller increase in the 'pangastric' group than in the 'corpus only' group. The number of subjects in the 'pangastric' group is small hence the difference between the relative mRNA abundance of MMP-7 in this and the 'corpus only' group was not statistically significant. Further study would be required to adequately characterise this response.

4.5.2 Mucosal expression of MMP-7 and severity of preneoplasia

As described earlier, we separated study subjects with gastric preneoplasia into pathology groups reflecting the severity of mucosal pathology. We compared these groups with controls and each other to determine the effect of preneoplastic disease severity on mucosal MMP-7 expression. The data are shown in Figure 4-7.

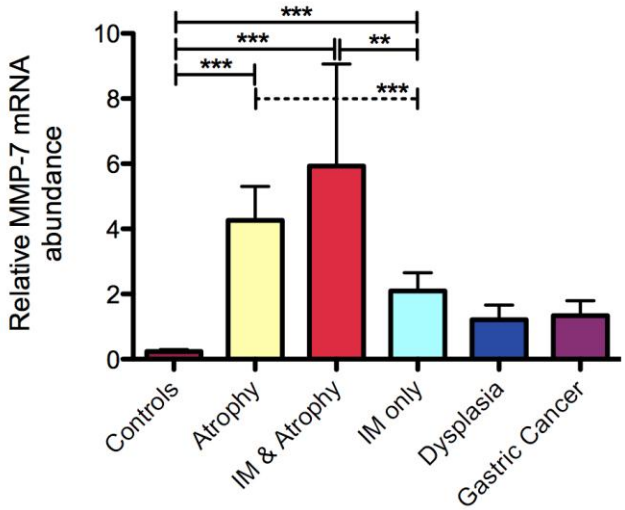

Figure 4-7 Mean relative mRNA abundance of MMP-7 for controls (n=36) and groups of different mucosal preneoplastic disease 'severity': atrophy (n=75); IM & atrophy (n=64); IM only (n=132); dysplasia (n=14) and gastric cancer (n=10) (Kruskal-Wallis 1-way ANOVA).

There was a significant increase in the relative mRNA abundance of MMP-7 in 'atrophy', 'intestinal metaplasia & atrophy' and 'intestinal metaplasia alone' groups ($p<0.0001$, Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test). We observed a smaller increase in MMP-7 mRNA abundance in the 'dysplasia' and

‘gastric cancer’ groups though these were not statistically significant. Interestingly, the magnitude of the change in expression was smaller in the ‘intestinal metaplasia only’ group than in the groups showing histological evidence of gastric atrophy and the differences between the former and latter groups were statistically significant. These data are summarised in

Table 4-5.

As before, we repeated this analysis using the 'normal histology' group as a comparator in place of the 'control' group. The data are illustrated in Figure 4-8. The increases observed in MMP-7 mRNA abundance remained highly statistically significant ($p<0.0001$, Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test).

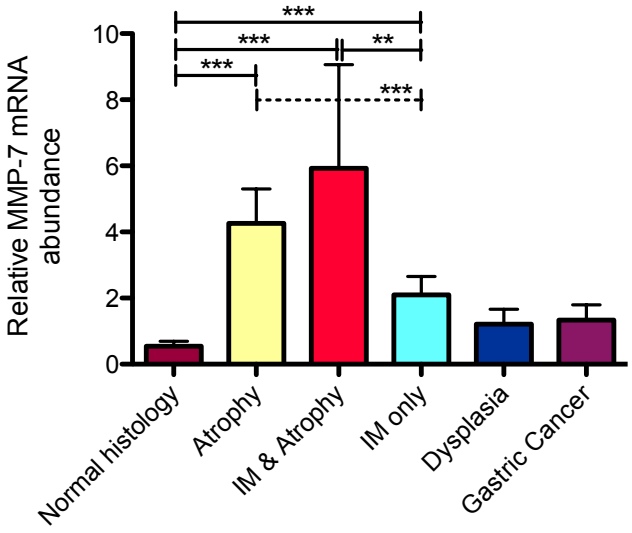

Figure 4-8 Mean relative mRNA abundance of MMP-7 for normal histology group (n=185) and groups of different mucosal preneoplastic disease 'severity': atrophy (n=75); IM & atrophy (n=64); IM only (n=132); dysplasia (n=14) and gastric cancer (n=10) (Kruskal-Wallis 1-way ANOVA).

Table 4-5 Comparison of MMP-7 relative mRNA abundance between groups of varying gastric preneoplastic severity.

	Controls	Atrophy	IM & Atrophy	IM only	Dysplasia	Gastric Cancer
Number of values	36	75	64	132	14	10
Minimum	0.01243	0.06839	0.04009	0.00812	0.03477	0.03443
25% Percentile	0.04154	0.4234	0.4388	0.1262	0.08343	0.09882
Median	0.09052	0.9963	1.188	0.4225	0.3697	0.8375
75% Percentile	0.2913	3.631	4.027	1.411	1.856	2.722
Maximum	1.539	50.33	200.2	60.98	5.622	4.099
Mean	0.2298	4.266	5.929	2.097	1.215	1.336
Std. Deviation	0.3221	9.009	25.06	6.418	1.663	1.459
Std. Error	0.05368	1.04	3.132	0.5586	0.4445	0.4612
Fold change	1.000	18.564	25.801	9.125	5.287	5.814
Lower 95% CI	0.1208	2.193	-0.3308	0.9915	0.2551	0.2924
Upper 95% CI	0.3388	6.339	12.19	3.202	2.176	2.379

To mitigate for any potential effects of the topographical variation in mucosal disease distribution, we performed the same analysis for patients with corpus-only disease. The data are shown in Figure 4-9.

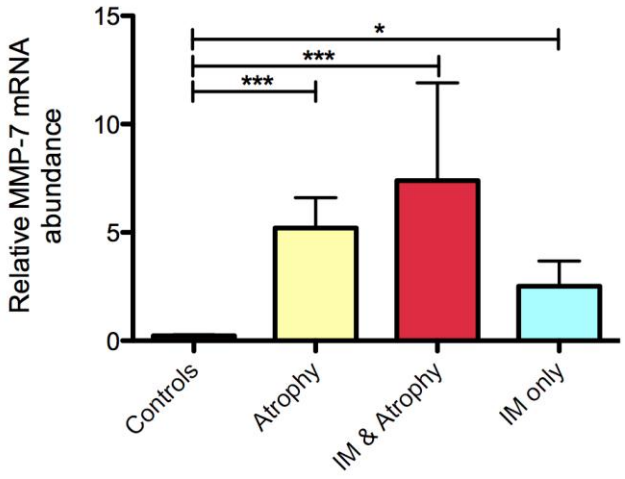

Figure 4-9 Mean relative mRNA abundance of MMP-7 in controls (n=36) and groups of varying corpus preneoplastic severity: atrophy (n=53); IM & atrophy (n=44); IM only (n=16) (Kruskal-Wallis 1-way ANOVA).

The same variation in MMP-7 expression was seen in corpus-only disease as in the overall preneoplasia analysis. The relative mRNA abundance of MMP-7 was significantly higher in corpus only preneoplasia groups than in controls ($p<0.0001$, Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test). Additionally, the relationship between pathology groups was preserved – 'atrophy and 'IM & atrophy' both exhibited a significantly higher relative mRNA abundance of MMP-7 than did the 'IM only' group. The data are summarised in Table 4-6. In the overall analysis outlined above, the 'atrophy', 'IM & atrophy' and 'IM only' groups exhibited a fold-change in mean MMP-7 relative mRNA abundance compared with controls of 18.6, 25.8 and 9.1 respectively. In the corpus-pathology only analysis, the corresponding values were similar at 22.7, 32.2 and 11.0 respectively.

Table 4-6 Comparison of MMP-7 relative mRNA abundance between groups of varying corpus preneoplastic severity.

	Controls	Atrophy	IM & Atrophy	IM only
Number of values	36	53	44	16
Minimum	0.01243	0.06839	0.04009	0.023
25% Percentile	0.04154	0.5616	0.8211	0.1894
Median	0.09052	1.911	1.432	0.5463
75% Percentile	0.2913	5.498	4.403	3.282
Maximum	1.539	50.33	200.2	18.67
Mean	0.2298	5.206	7.39	2.516
Std. Deviation	0.3221	10.23	29.92	4.664
Std. Error	0.05368	1.405	4.511	1.166
Fold change	1.000	22.654	32.158	10.949
Lower 95% CI	0.1208	2.387	-1.707	0.03102
Upper 95% CI	0.3388	8.024	16.49	5.002

4.5.3 Comparative analysis of MMP-7 expression in preneoplastic and benign gastric mucosal disease

The altered mucosal expression of MMP-7 in gastric preneoplasia suggests that the gene products might be useful biomarkers of the same. In the case of gastric preneoplasia however, the performance of any putative biomarker is influenced to some degree by not only the ability to discriminate between 'disease' and 'normal', but also to distinguish preneoplastic mucosal disease from pathological changes unrelated to neoplasia development. We therefore compared the relative mRNA abundances of MMP-7 in such so-called 'benign' groups with the previously defined preneoplasia groups. The results are shown in Figure 4-10.

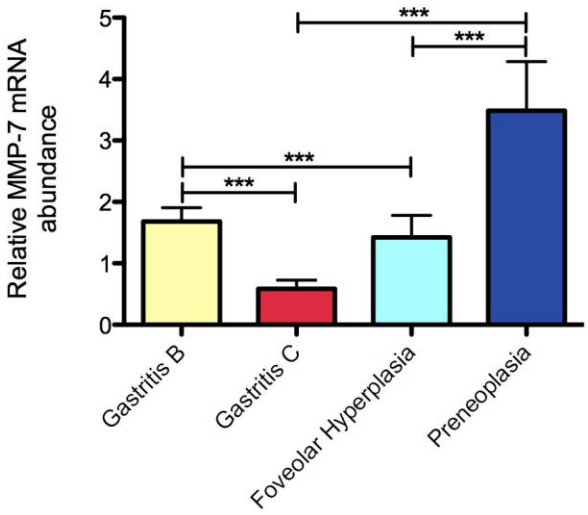

Figure 4-10 Mean relative mRNA abundance of MMP-7 in benign gastric disease groups: gastritis B (n=122); gastritis C (n=46) and foveolar hyperplasia (n=133), compared with preneoplastic disease (n=285) (Kruskal-Wallis 1-way ANOVA).

The relative mRNA abundance of MMP-7 was significantly higher in the combined preneoplasia group when compared to either of the 'benign' groups i.e. reactive gastritis (Gastritis C) or foveolar hyperplasia. The same was true when comparing *H. pylori* associated gastritis (Gastritis B) to benign groups. The observed difference between gastritis B and preneoplasia groups was not however statistically significant.

In view of the discrepancy between the MMP-7 expression in preneoplasia groups of different severity, we compared these groups to the benign groups individually. The results are illustrated in Figure 4-11.

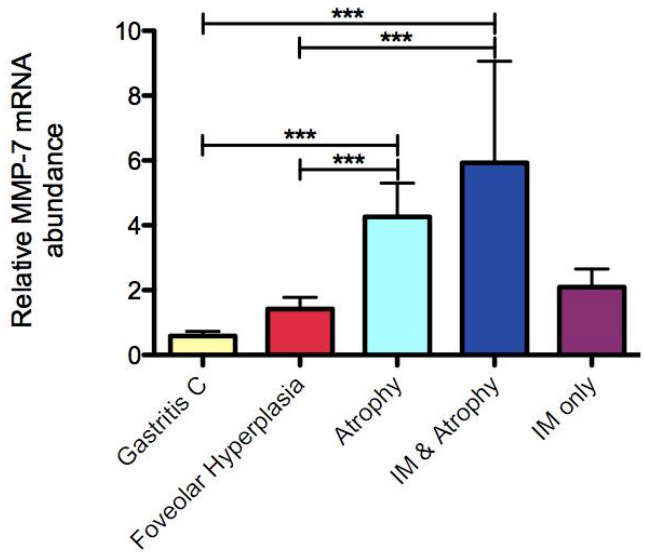

Figure 4-11 Mean relative mRNA abundance of MMP-7 in benign gastric disease and pathology groups of varying preneoplastic severity: gastritis C (n=46); foveolar hyperplasia (n=133); atrophy (n=75); IM & atrophy (n=64) and IM only (n=132) (Kruskal-Wallis 1-way ANOVA).

Unsurprisingly (given the results described in section 4.3.2), the 'atrophy' and 'IM & atrophy' groups exhibited the largest differences in MMP-7 expression when compared to the 'benign' groups and these differences were highly statistically significant. There was a small and statistically insignificant difference in the relative mRNA abundance of MMP-7 in the 'IM only" preneoplasia group when compared with either of the 'benign' groups. This pattern was unaltered when we repeated the analyses with corpus-only disease groups. The implication of this finding is that for patients with gastric mucosal intestinal metaplasia, a 'diagnostic test' based on the gene products of MMP-7 might lack specificity and thus positive predictive value.

4.5.4 Mucosal expression of MMP-7 and Barrett's oesophagus

As described earlier, we recorded both endoscopic and histological diagnoses of Barrett's oesophagus on the basis that oesophageal mucosal disease might exert an effect on gastric MMP-7 expression. Thus, cases of oesophageal disease including neoplasia and Barrett's oesophagus were excluded from analysis of gastric mucosal MMP-7 expression. For completeness, we examined the effect on expression of MMP-7 in the gastric corpus of the presence of Barrett's oesophagus. The data are illustrated in Figure 4-12.

We found that corpus expression of MMP-7 was significantly altered in the presence of Barrett's oesophagus. As described before, mean relative abundance of MMP-7 in the control group was 0.2298 (median 0.0905, SEM 0.0537). In the Barrett's oesophagus group, the mean relative abundance of MMP-7 was 1.752 (median 0.4176, SEM 0.952) and was significantly greater (p=0.0014, Mann-Whitney test) than controls. The differences between the preneoplasia (mean 3.485, median 0.7322, SEM 0.7996) and gastritis B (mean 1.681, median 0.7018, SEM 0.2249) groups were also statistically significant (p=0365 and 0.0269 respectively).

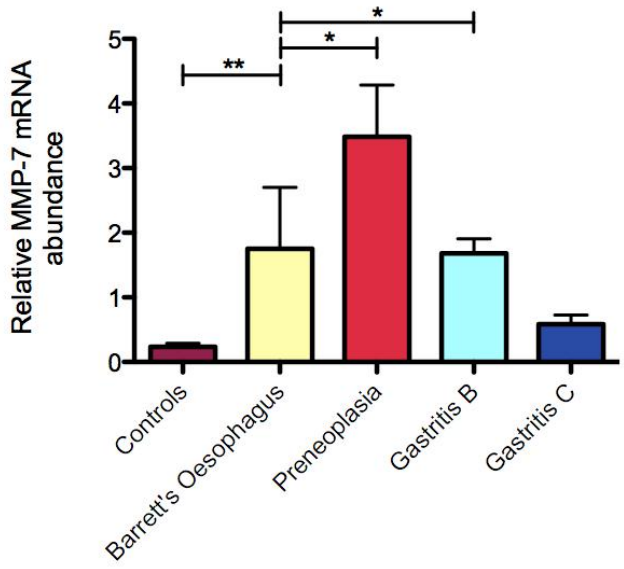

Figure 4-12 Mean relative mRNA abundance of gastric MMP-7 in Barrett's oesophagus (n=29) compared with preneoplastic (n=285) and benign disease groups gastritis B (n=122); gastritis C (n=46), and controls (n=36) (two-tailed Mann-Whitney test).

To mitigate the effects of hypergastrinaemia on gastric mucosal MMP-7 expression on this analysis (described in section 4.5.6), we removed subjects whose fasting serum gastrin concentration was greater than 40pM. There were 12 individuals in this group with 'normal' fasting serum gastrin concentration. The repeated analysis is illustrated in Figure 4-13. In the normogastrinaemic Barrett's oesophagus group, the mean relative mRNA abundance of MMP-7 was 1.261 (median 0.4714, SEM 0.746) and was significantly greater (p=0.0128, Mann-Whitney test) than controls. There was no statistically significant difference between this normogastrinaemic Barrett's oesophagus group and the other described pathology groups.

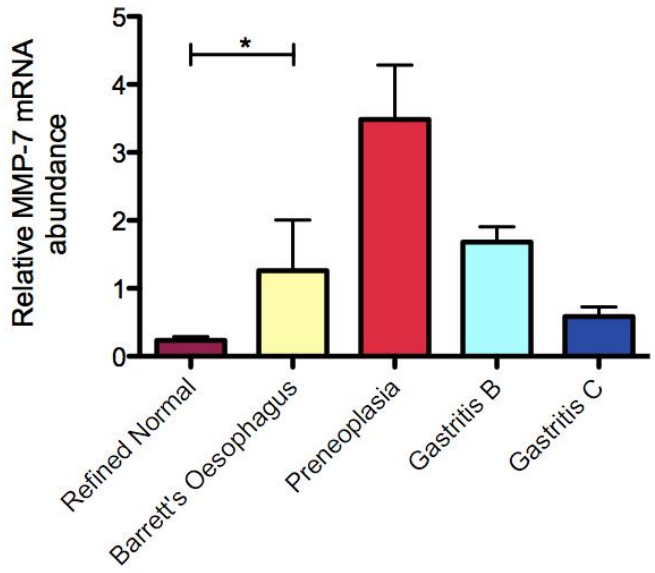

Figure 4-13 Mean relative mRNA abundance of MMP-7 in normogastrinaemic Barrett's oesophagus (n=12) compared with preneoplastic (n=285) and benign disease groups gastritis B (n=122); gastritis C (n=46), and controls (n=36) (two-tailed Mann-Whitney test).

4.5.5 Mucosal expression of MMP-7 and *H. pylori* CagA serovar

Several authors have previously described the relationship between *H. pylori* induced gastric inflammation and MMP-7 expression. The data above demonstrate a significant increase in mucosal expression of MMP-7 in our study cohort when *H. pylori* associated inflammation (Gastritis B) was present. As outlined in chapter 3, we also serotyped all *H. pylori* positive patients by CagA status. We therefore compared

the effect of CagA positive *H. pylori* infection on MMP-7 expression to CagA negative *H. pylori* infection and controls. The results are illustrated in Figure 4-14. We examined the difference in relative abundance of gastric MMP-7 for CagA negative and positive subjects in a) all *H. pylori* positive (by histology or RUT) subjects, b) all subjects reported to have *H. pylori* associated gastritis (gastritis B) by the study pathologist and c) all subjects reported to have corpus-inclusive *H. pylori* associated gastritis (gastritis B) by the study pathologist. In each case, both CagA positive and CagA negative subjects exhibited significantly higher MMP-7 expression than controls ($p<0.0001$, two-tailed Mann-Whitney tests). The expected and observed higher relative abundance in CagA positive subjects was not however statistically significant in any group (two-tailed Mann-Whitney tests).

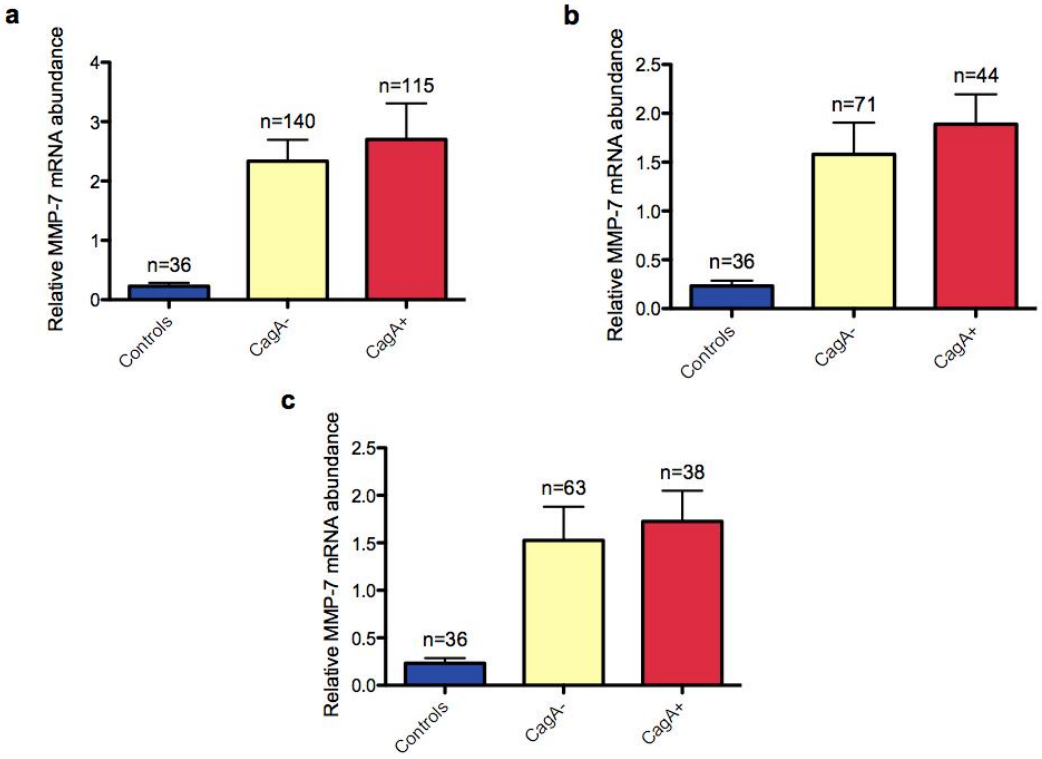

Figure 4-14 Mean relative mRNA abundance of MMP-7 in CagA positive and negative patients compared with controls for a) all *H. pylori* positive subjects, b) all subjects with *H. pylori* associated gastritis on histology and c) all patients with *H. pylori* associated gastritis involving the corpus on histology (two-tailed Mann-Whitney tests).

4.5.6 Mucosal expression of MMP-7 and fasting serum gastrin concentration

Our own group has previously reported an association between hypergastrinaemia and increased MMP-7 expression in human and murine stomachs(126). We therefore examined our study cohort for evidence of the same effect. For all subjects for whom both fasting serum gastrin concentration and mucosal MMP-7 relative mRNA abundance were measured (n=916), we observed a significant increase in the latter in those with 'moderate' (fold change in mean=1.38, p=0.0006) and 'severe' (fold change in mean=3.79, p<0.0001, two-tailed Mann-Whitney tests) hypergastrinaemia. When combining 'moderate' and 'severe' hypergastrinaemic groups, the fold change in mean relative mRNA abundance of MMP-7 was 2.47 (p<0.0001, two-tailed Mann-Whitney test). The results are illustrated in Figure 4-15.

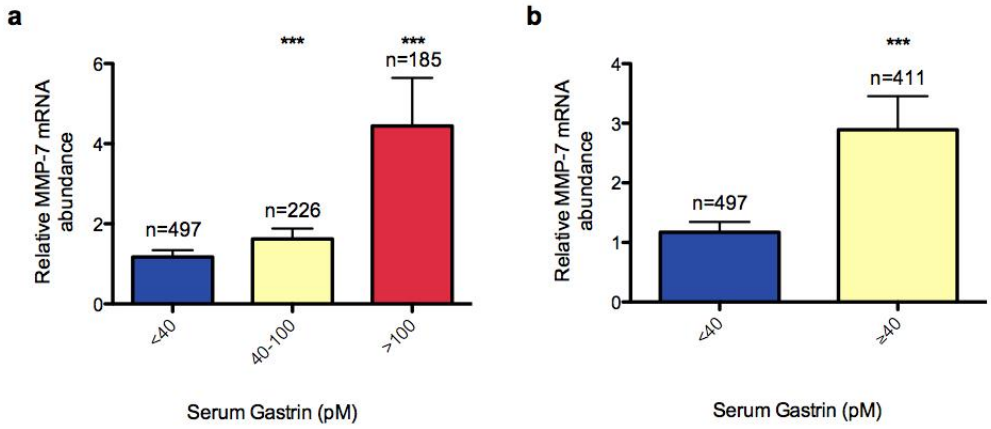

Figure 4-15 Mean relative abundance of MMP-7 in a) normal, moderate and severe hypergastrinaemia; and in b) normal and high serum gastrin groups (two-tailed Mann-Whitney tests).

These changes may well reflect the underlying mucosal pathology and so we compared hypergastrinaemic with normogastrinaemic subjects in those with histologically normal gastric mucosa (n=261). The data are shown in Figure 4-16. In this cohort of patients, we observed no significant difference between normogastrinaemic subjects and those with moderate hypergastrinaemia. 'Severe' hypergastrinaemia however conferred a fold change in the mean relative mRNA abundance of MMP-7 of 7.42 compared with normogastrinaemic subjects (p<0.0001,

two-tailed Mann-Whitney test). Combining hypergastrinaemic groups gave a fold change in mean relative mRNA abundance of MMP-7 of 3.67 (p=0.0016, two-tailed Mann-Whitney test). These data suggest an independent effect of fasting serum gastrin concentration on gastric mucosal MMP-7 mRNA abundance in keeping with previous observations.

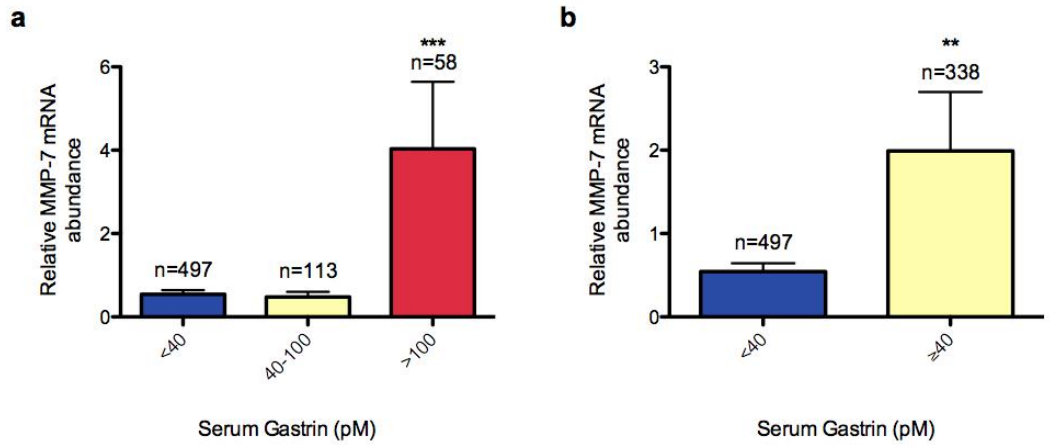

Figure 4-16 Mean relative mRNA abundance of MMP-7 in subjects with normal gastric mucosal histology and a) normal, moderate and severe hypergastrinaemia; and in b) normal and high serum gastrin groups (two-tailed Mann-Whitney tests).

4.5.7 The effect on mucosal expression of MMP-7 of commonly used drugs

At enrolment, we collected information about prescription or over-the-counter (OTC) medication use from each study subject. In particular, we recorded the recent use of common drugs known to affect the gastric mucosa: proton pump inhibitors (PPIs), non-steroidal anti-inflammatory drugs (NSAIDs) and aspirin. In the group reported to have normal gastric mucosal histology, we compared the relative mRNA abundance of MMP-7 in those who reported recent use of these drugs with those who denied it. The results are depicted in Figure 4-17. There was no significant change in the relative mRNA abundance of MMP-7 conferred by either PPI or aspirin use. In contrast, subjects who reported recent NSAID use exhibited a fold change in the mean relative mRNA abundance of 5.92 (p=0.0252, two-tailed Mann-Whitney test).

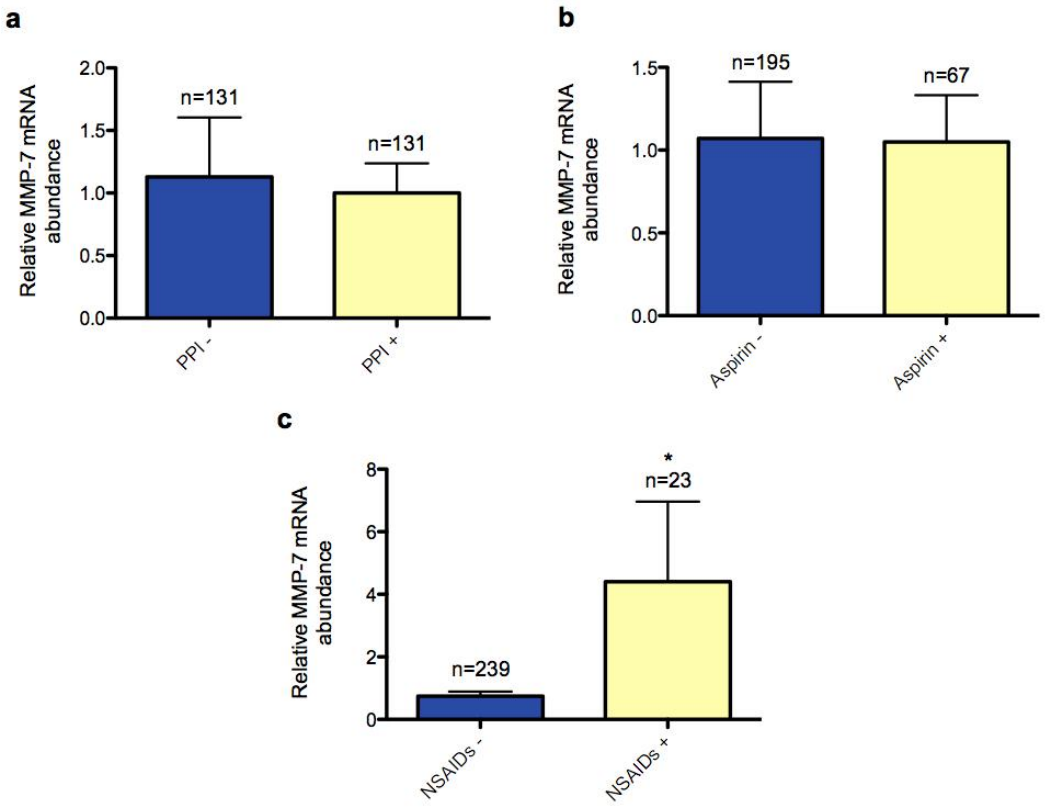

Figure 4-17 Mean relative mRNA abundance of MMP-7 in users and non-users of a) PPIs, b) aspirin and c) NSAIDs (two-tailed Mann-Whitney test).

These data suggest an independent role of NSAIDs in inducing gastric MMP-7 expression. We previously published data from this study cohort asserting that other extracellular proteolytic protein systems might be implicated in the gastric mucosal response to NSAID induced injury(299) and these data would mirror our earlier findings.

4.6 Serum concentration of MMP-7

As described above, we determined MMP-7 concentrations in the sera of selected subjects by ELISA. We obtained data for 606 patients whose mucosal MMP-7 relative mRNA abundance had previously been determined by qPCR. The numbers of patients selected to represent the various 'pathology' groups are listed in Table 4-7.

4.6.1 Correlation between mucosal mRNA abundance and serum concentration of MMP-7

We determined the correlation between the relative abundance of gastric mucosal MMP-7 mRNA and serum MMP-7 concentration by comparing these data from individual subjects. The data are illustrated in Figure 4-18 and suggest no significant correlation between the two sets.

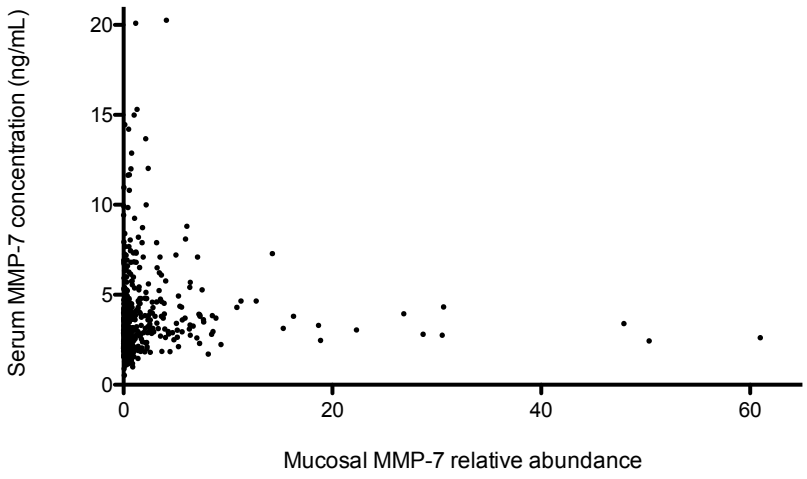

Figure 4-18 Correlation between serum concentration and mucosal relative abundance of MMP-7 mRNA.

Table 4-7 Gastric pathology groups and numbers of patients included for serum MMP-7 assay.

Gastric histopathology group	Total in group	Number assayed	Proportion assayed (of group)	Proportion assayed (of total cohort)
	n	**n**	**%**	**%**
Normal gastric histology	444	129	29.1	9.2
Normal histology but *H. pylori* serology+	140	9	6.4	0.6
Normal histology but *H. pylori* histology+	21	15	71.4	1.1
Gastritis B - Antrum	23	16	69.6	1.1
Gastritis B - Corpus	30	0	0.0	0.0
Gastritis B - Pangastric	79	64	81.0	4.6
Gastritis C - Antrum	39	7	17.9	0.5
Gastritis C - Corpus	5	0	0.0	0.0
Gastritis C - Pangastric	7	2	28.6	0.1
FH - Antrum	165	85	51.5	6.1
FH - Corpus	7	3	42.9	0.2
FH - Pangastric	7	3	42.9	0.2
Atrophy - Antrum	19	12	63.2	0.9
Atrophy - Corpus	55	52	94.5	3.7
Atrophy - Pangastric	5	5	100.0	0.4
IM - Antrum	111	43	38.7	3.1
IM - Corpus	16	10	62.5	0.7
IM - Pangastric	19	24	126.3	1.7
IM AND Atrophy - Antrum	24	8	33.3	0.6
IM AND Atrophy - Corpus	44	44	100.0	3.1
IM AND Atrophy - Pangastric	5	5	100.0	0.4
LGD - Antrum	6	3	50.0	0.2
LGD - Corpus	5	5	100.0	0.4
LGD - Pangastric	0	0	0.0	0.0
HGD - Antrum	0	0	0.0	0.0
HGD - Corpus	1	0	0.0	0.0
HGD - Pangastric	2	0	0.0	0.0
Barrett's oesophagus	54	39	72.2	2.8
Endoscopy report of Barrett's oesophagus	12	5	41.7	0.4
Oesophageal Cancer	11	6	54.5	0.4
Gastric Cancer	12	7	58.3	0.5
Gastric Ulcer only	10	2	20.0	0.1
Antrum MALT only	3	3	100.0	0.2

4.6.2 Control group

The cohort described here included 18 subjects from the control group described above. In this cohort however, there was no significant difference in mean serum MMP-7 concentration (Figure 4-19) between the 'control' group (3.627 ng/ml, median 2.830, SEM 0,5686) and the 'normal histology, *H. pylori* negative' group (n=129, 3.520 ng/ml, median 3.03 ng/ml, SEM 0.1645). For subsequent analyses, we have therefore compared 'pathology' groups with the 'normal histology, *H. pylori* negative' group, hereafter referred to as 'normal histology'.

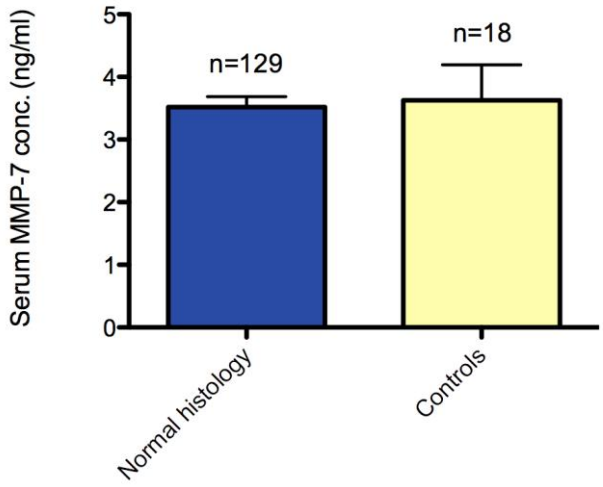

Figure 4-19 Mean serum MMP-7 concentration - comparison between subjects with normal gastric histology and the previously defined control subjects.

4.6.3 Serum MMP-7 concentration and gastric mucosal pathology

Using this commercially available ELISA kit, we set out to determine the effect of gastric mucosal pathology on the measured fasting serum concentration of MMP-7. Although we did not anticipate as pronounced an effect as that seen with the mucosal mRNA abundance PCR assays, we made the same comparisons to those described above with similar aims, namely: to determine the effect of presence of gastric preneoplasia on the serum concentration of MMP-7; to establish the usefulness of serum MMP-7 concentration for distinguishing between preneoplastic and 'benign' gastric mucosal disease and to determine how the serum concentration of MMP-7 varied with 'severity' of preneoplasia.

We compared the mean serum MMP-7 concentrations in the various pathology groups. The data are illustrated in Figure 4-20.

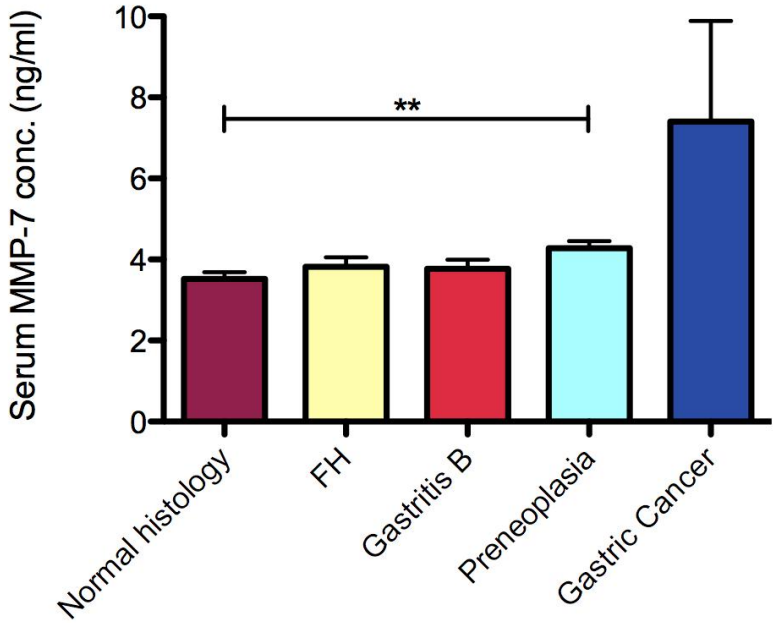

Figure 4-20 Mean serum MMP-7 concentration comparison between subjects with normal histology (n=129) various pathology groups: foveolar hyperplasia (n=91); gastritis B (n=87); preneoplasia (n=211) and gastric cancer (n=7) (Kruskal-Wallis 1-way ANOVA).

The mean serum concentration of MMP-7 in the 'normal histology' group was 3.520 ng/ml (median 3.03 ng/ml, SEM 0.1645). There was no significant increase observed in any of the other groups (Gastritis B, foveolar hyperplasia or gastric cancer) except for preneoplasia ($p<0.01$, Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test) where the mean was 4.279 ng/ml (median 3.700 ng/ml, SEM 0.1730). Again, our study was inadequately powered to determine the role of MMP-7 in gastric cancer. For these analyses there were only 7 cases included in the gastric cancer group where the mean serum concentration of MMP-7 was 7.406 ng/ml (median 4.200 ng/ml, SEM 2.483) and the difference from that of the 'normal' group was not statistically significant (Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test).

Furthermore, the same statistical interpretation of the difference between the preneoplasia group and the included 'benign' gastric disease groups. There were no significant differences between the preneoplasia group and either 'gastritis B' or 'foveolar hyperplasia'. This observation would suggest that the use of this assay for the determination of serum MMP-7 concentration would not be useful in discriminating preneoplastic from benign gastric mucosal diseases.

4.6.4 Serum concentration of MMP-7 and severity of preneoplasia

In section 4.3.2, we described the variation in mucosal expression of MMP-7 with increasing 'severity' of gastric preneoplasia. We therefore repeated this comparison for serum MMP-7 concentration. The results are summarised in Figure 4-21. Although there was a small observed increase in all preneoplasia pathology groups compared with 'normal' subjects, none were statistically significant with the exception of the 'intestinal metaplasia and atrophy' group, in which the mean serum concentration of MMP-7 was 4.658 ng/ml (median 3.920 ng/ml, SEM 0.4161, $p<0.05$ Kruskal-Wallis 1-way ANOVA/Dunn's multiple comparison test).

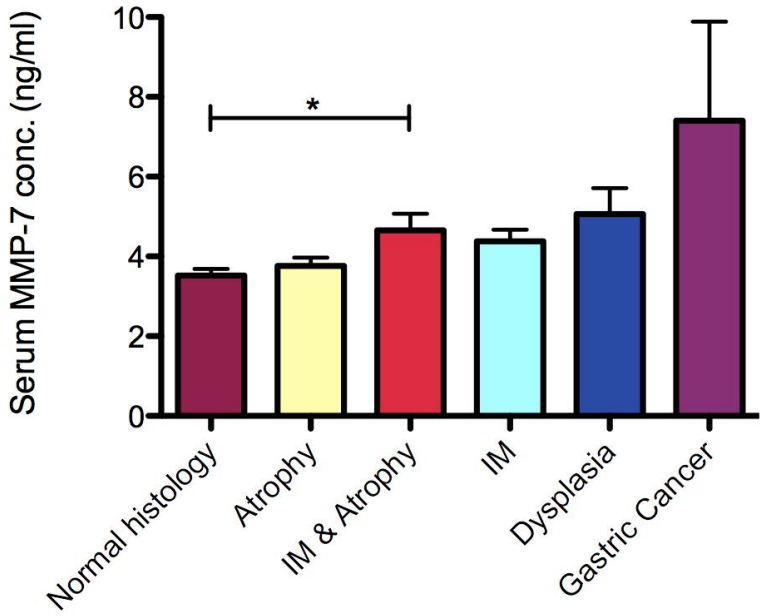

Figure 4-21 Mean serum MMP-7 concentration - comparison between subjects with normal gastric histology (n=129) and groups of varying preneoplastic 'severity': atrophy (n=69); IM & atrophy (n=57); IM only (n=77); dysplasia (n=8) and gastric cancer (n=7) (Kruskal-Wallis 1-way ANOVA).

4.6.5 Serum concentration of MMP-7 and Barrett's oesophagus

We compared the major histology groups with the group comprising cases of Barrett's oesophagus (Figure 4-22). There was a small increase in serum concentration of MMP-7 in the Barrett's oesophagus group (mean 4.074 ng/ml, median 3.470 ng/ml, SEM 0.3379) compared with the normal histology group but this was not statistically significant. Similarly, the relationship between serum MMP-7 concentration in the preneoplasia and Barrett's oesophagus groups echoed that seen in the mucosal MMP-7 mRNA abundance assays though the difference in this analysis was not statistically significant.

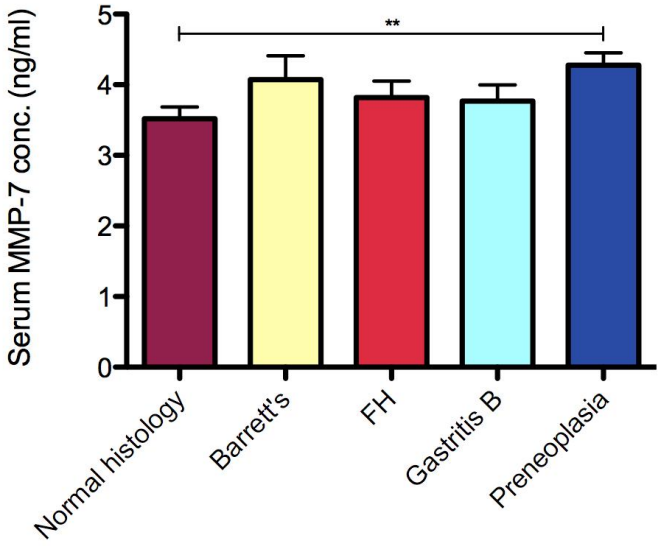

Figure 4-22 Mean serum MMP-7 concentration - comparison between subjects with normal gastric histology (n=129), major gastric histology groups: foveolar hyperplasia (n=91); gastritis B (n=87) and preneoplasia (n=211); and Barrett's oesophagus (n=39) (Kruskal-Wallis 1-way ANOVA).

4.6.6 Serum concentration of MMP-7 as part of a 'diagnostic test'

In section 3.5, we described the performance of conventional non-invasive biomarkers in identifying or excluding gastric mucosal preneoplasia in our study population. We concluded that a 'test' panel comprising serum pepsinogen 1/2 ratio, fasting serum gastrin concentration and *H. pylori* serology identified or excluded preneoplasia with high sensitivity (90.0%), specificity (74.4%) and positive/negative predictive values of 52.1% and 96.0% respectively. It might be expected that adding serum MMP-7 concentration might enhance the discriminative ability of this combined test.

Alone, serum concentration of MMP-7 performs poorly as a diagnostic 'test' for gastric preneoplasia. The ROC curve is shown in Figure 4-23. The AUC was 0.61 (p=0.0005, 95% CI: 0.5506 - 0.6747). Finding the approximate optimal cutoff in our study by drawing the tangent to the curve in parallel with its axis gives a serum MMP-7 concentration cutoff of 3.0ng/ml with a sensitivity and specificity of 66.4% and 49.6% respectively.

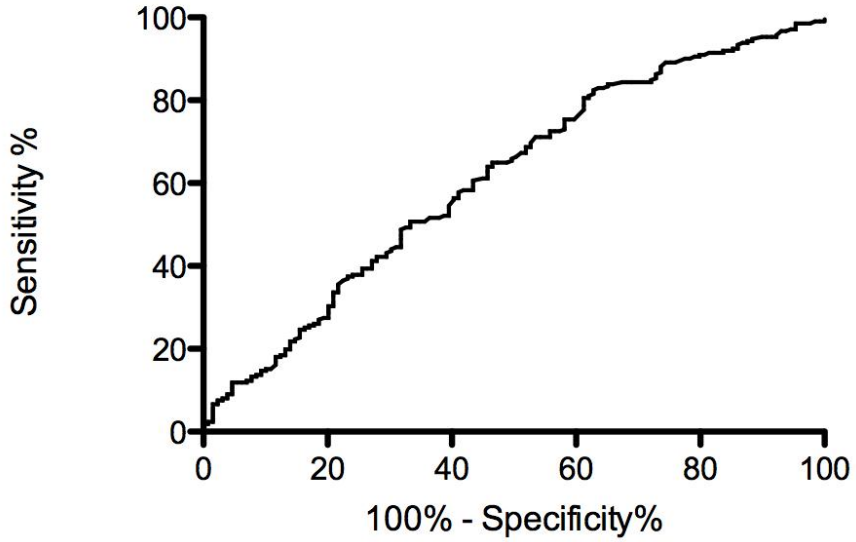

Figure 4-23 ROC curve for serum MMP-7 concentration for the detection of gastric preneoplasia.

For completeness, we combined this with the previously evaluated biomarkers listed above. For any given study subject, a 'test' was regarded as 'positive' if the serum PG 1/2 ratio was <8.8 and/or the fasting serum gastrin ≥100pM and/or the *H. pylori* serology was positive and/or the serum concentration of MMP-7 was ≥3.0ng/ml. The results are summarised in Table 4-8. Sensitivity and negative predictive value were both substantially increased over the previous combination, but at the expense of specificity and positive predictive value.

Table 4-8 Diagnostic performance serum MMP-7 concentration in combination with pepsinogen 1/2 ratio, *H. pylori* serology, histology and CagA serology. PPV = positive predictive value; NPV = negative predictive value.

	Combined test	
	Test +	Test -
Preneoplasia +	209	4
Preneoplasia -	41	28
Sensitivity %	98.1	
Specificity %	40.6	
PPV %	33.8	
NPV %	98.6	

4.6.7 Mucosal abundance of MMP-7 mRNA as part of a 'diagnostic test'

Given the suboptimal performance of the 'off-the-shelf' MMP-7 ELISA as a diagnostic or screening test described in section 4.6.6, we hypothesised that a technique for determining serum MMP-7 concentration which better reflects gastric mucosal expression might substantially improve the performance of said test. The development of such techniques is dependent on future study, but to assess the potential influence of an accurate assay on the combined diagnostic test, we used gastric mucosal MMP-7 relative mRNA abundance as a surrogate.

Alone, the gastric mucosal MMP-7 relative mRNA abundance outperforms the serum concentration as determined by the ELISA described earlier. The ROC curve is shown in Figure 4-24. The AUC was 0.76 ($p<0.0001$, 95% CI: 0.7135 - 0.8004).

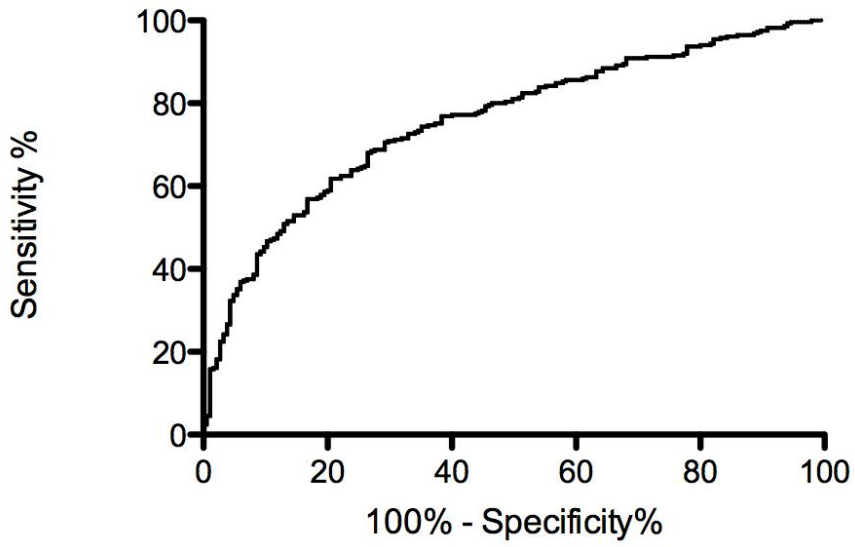

Figure 4-24 ROC curve for gastric mucosal MMP-7 relative mRNA abundance for the detection of gastric preneoplasia.

The optimal cut-off for MMP-7 relative mRNA abundance was determined as being ≥0.33 (giving sensitivity and specificity values of 67.7% and 73.5% respectively) and we combined this as before with *H. pylori* serology, fasting serum gastrin

concentration and serum pepsinogen 1/2 ratio. The data are summarised in Table 4-9.

Table 4-9 Diagnostic performance gastric mucosal MMP-7 relative mRNA abundance in combination with pepsinogen 1/2 ratio, *H. pylori* serology, histology and CagA serology. PPV = positive predictive value; NPV = negative predictive value.

	Combined test	
	Test +	Test -
Preneoplasia +	195	7
Preneoplasia -	10	29
Sensitivity %	96.5	
Specificity %	68.8	
PPV %	48.8	
NPV %	98.5	

In comparison with the combined test described in section 3.7 (incorporating *H. pylori* serology, fasting serum gastrin concentration and serum pepsinogen 1/2 ratio), the addition of mucosal MMP-7 relative mRNA abundance improves both sensitivity and NPV at the expense of specificity and PPV. Compared to the test incorporating serum MMP-7 concentration (described in section 4.6.6), sensitivity and NPV are similar but specificity and PPV are considerably better.

4.7 Discussion

At the inception of this study, we identified a group of 12 proteins, whose expression we hypothesised might be implicated in the development of gastric mucosal preneoplasia and its subsequent progression to adenocarcinoma. This preliminary selection was based on previous work published by our own research group and others as discussed in chapter 1.

I have outlined the process by which we identified one of these genes – MMP-7 – as the most promising candidate for further study. Of the 12 candidate genes, only 3 MMP-7, MMP-1 and TIMP-1) exhibited the characteristics desired from a potential biomarker, namely a significant difference in expression between the disease state of interest (gastric preneoplasia) and other pathology or normal controls. Of these three genes, MMP-7 exhibited the greatest and most statistically significant difference between the relevant groups and so was selected for further study. There is a sound rationale for further studying the roles of MMP-1 and TIMP-1 in the development of gastric adenocarcinoma but the scale of the present study precluded additional analyses at this time.

The large number of patients recruited to our study also enabled us to identify 'control' subjects in whom factors likely to affect MMP-7 expression could be excluded. We identified 36 such individuals as described in section 4.2.1 and demonstrated that the mean mucosal relative mRNA abundance in this groups was significantly lower than in the group comprising subjects with 'normal histology' but other variables unfiltered.

We observed that *H. pylori* associated mucosal inflammation (gastritis B) was associated with increased MMP-7 expression compared with a) controls and b) gastritis of alternative aetiologies. This corroborates earlier work(51,300,301) and supports the first tenet of our thesis – that MMP-7 participates in mucosal remodelling following *H. pylori* infection. Recent studies in murine models(302,303) suggest that MMP-7 acts to 'restrain' gastric inflammation and preneoplastic remodelling after *H. pylori* infection. In this study, the authors report that mmp-$7^{-/-}$ mice exhibit augmented gastric mucosal inflammation and increased epithelial cell proliferation and apoptosis compared to wild type mice following colonisation with *H. pylori*.

Upregulated MMP-7 expression has previously been demonstrated in gastric adenocarcinoma(304–307) but our observation that MMP-7 expression is increased in the presence of gastric mucosal preneoplastic lesions is novel. The present study

was not intended to study the effects on mucosal MMP-7 mRNA abundance of gastric cancer and includes only 12 cases. There was an increase in mucosal MMP-7 mRNA abundance in this group but the difference between this and controls was statistically insignificant.

The response in mucosal MMP-7 expression to *H. pylori* mediated inflammation might have led us to predict that ongoing mucosal remodelling (as seen in preneoplasia) would also require increased MMP-7 activity. We did not however predict the attenuation of this effect that we observed in the 'intestinal metaplasia only' group. In this group, subjects whose gastric histology reports included atrophy were excluded. The implication of this is that the gastropathy described is further along the 'pathway' towards carcinogenesis than either the 'atrophy' or 'intestinal metaplasia & atrophy' groups that conceptually precede it. Though the difference in mucosal MMP-7 mRNA abundance between this group and controls was significant, the abundance was significantly less than in other the other two major preneoplasia groups.

Further studies are required to clarify the mechanism responsible for this effect. We might speculate that 'atrophy-inclusive' gastric preneoplasia represents a disease state in which mucosal remodelling is proceeding at greater intensity. In multifocal atrophic gastritis, chronic, active inflammation leads to loss of mucosal cells (particularly chief and parietal cells) and their replacement by fibrosis or metaplasia. It might be suggested that once chronic atrophic gastritis gives way to intestinal metaplasia, mucosal inflammation is attenuated and cellular/extracellular matrix turnover is diminished.

The present study examined mucosal MMP-7 mRNA abundance in biopsy specimens obtained from the gastric corpus only. As outlined in chapter 1, the disease phenotype associated with gastric preneoplasia following *H. pylori* infection is one of pangastric inflammation followed by multifocal atrophic gastritis (MAG). Antrum-restricted atrophic gastritis is thought of as a distinct disease phenotype with a lower risk of preneoplasia and subsequent cancer formation.

We have shown that mucosal MMP-7 mRNA abundance in corpus mucosa was significantly increased in the presence of gastric preneoplasia irrespective of disease distribution. As might be expected however, abundance was greatest in cases of corpus predominant disease than in antrum predominant preneoplasia. We were surprised however, to find that in cases of pangastric preneoplasia (i.e. where preneoplastic lesions were reported in both antrum and corpus specimens), the mucosal abundance of MMP-7 mRNA was significantly diminished compared with corpus predominant cases and in fact were comparable to antrum predominant cases. The mechanism responsible for this effect is unclear and further studies will be required to characterise this.

When considering MMP-7 as a potential biomarker, the assessment of mucosal mRNA abundance is rather moot, as upper GI endoscopy is required to obtain tissue. We therefore examined the performance of circulating MMP-7 concentration as a surrogate biomarker.

We determined serum MMP-7 concentration using a commercially available ELISA kit. We anticipated that the specificity of such assays for gastric MMP-7 might be poor and found this to be the case. Gratifyingly, we observed increases in serum MMP-7 concentration in the same groups in which mucosal abundance of the gene transcript was increased. However, the only group in which the observed increase was significant was the gastric preneoplasia group. The bulk of this effect was attributed to the 'intestinal metaplasia & atrophy' group in which we also observed the greatest increase in MMP-7 mRNA mucosal abundance. This pattern suggests that the concentration of MMP-7 in the circulation is influenced by gastric mucosal expression but that the assay used in neither sensitive nor specific enough to be considered diagnostically accurate. The development of a more accurate assay (ELISA or otherwise) is required before the performance of serum MMP-7 as a biomarker for gastric preneoplasia can be determined.

5 The Effect of Single Nucleotide Polymorphisms of *MMP-7* on Gastric Mucosal Preneoplastic Remodelling and MMP-7 expression

5.1 Introduction

As outlined in chapter 1, matrix metalloproteinase (MMP)-7 is one of a family of proteolytic enzymes responsible for extracellular proteolysis during tissue homeostasis, healing and host defence. The expression and activity of MMPs and their inhibitors (tissue inhibitors of metalloproteinase (TIMPs)) is carefully regulated in normal physiology and their dysregulation has been implicated in pathological inflammation and cancer formation(308–310). Single nucleotide polymorphisms (SNPs) are genetic variations of a single nucleotide in the DNA sequence, which occur commonly within a given population. SNPs in various genes are reported to affect gene expression, gene product structure or function and have been implicated in disease susceptibility.

MMP-7 gene SNPs have been shown to confer increased risk for the development of a variety of gastrointestinal cancers(311–316) including gastric adenocarcinoma, oesophageal squamous cell carcinoma, gallbladder adenocarcinoma and colorectal carcinoma. We selected *MMP-7* SNPs for genotyping in our study population to determine a) whether *MMP-7* SNPs influence susceptibility towards developing gastric mucosal preneoplasia and b) if *MMP-7* SNPs affect gastric mucosal MMP-7 expression.

5.2 Materials & Methods

Detailed materials and methods are described in chapter 2 and are summarised here for convenience.

Symptomatic adults referred to the Royal Liverpool University Hospital for upper gastrointestinal endoscopy were recruited to the study as described in chapter 3. We

recorded demographic details, drug history, relevant family history and medical history including specific details of any pulmonary, cardiovascular or gastrointestinal disease. During upper GI endoscopy, gastric biopsies were taken to enable identification of mucosal pathology including preneoplasia. Gastric biopsies were also used to obtain RNA and cDNA by subsequent reverse transcription. The mucosal mRNA abundance of putative biomarkers of preneoplasia was determined by qPCR and the results have been described in chapter 4. Whole blood was also taken and stored at -20°C before subsequent DNA extraction. We used the Chemagen magnetic bead separation method to extract genomic DNA and this was subsequently stored at 4°C before genotyping. Single nucleotide polymorphisms in the MMP-7 gene were selected for genotyping (as described in chapter 2) and this was performed using off-the-shelf TaqMan allelic discrimination assays and the Applied Biosystems HT7900 real-time PCR system with supplied SDS software.

5.3 MMP-7 single nucleotide polymorphisms in the study population

Table 5-1 describes the *MMP-7* SNPs, genotype and allelic frequencies in the study population. Eight of the nine SNPs selected were polymorphic and met the expectation that the minor allele frequency (MAF) should be greater than 0.05. The ninth SNP (rs11568819) was also polymorphic but only 3 cases (0.22%) of the 1349 genotyped were homozygous for the minor allele. Although the MAF for rs11568819 was found to be marginally below the 0.05 frequency threshold (0.049) it was still included in further analyses. Two of the SNP assays (for rs10502001 and rs10750646) were less reliable than the remainder and genotyping was only obtained for 1034 and 999 subjects respectively. All SNPs were found to be distributed in line with Hardy-Weinberg equilibrium (HWE) (Chi-squared tests).

Table 5-1 Description of SNPs, genotype and allelic frequencies in all subjects genotyped. AA = major allele homozygote; AB = heterozygote; BB = minor allele homozygote.

SNP	Number of patients successfully genotyped	Gene region	Major>minor allele	MAF (%)	Genotypes (%)			
					AA	AB	BB	HWE p
rs11568818	1352	promoter	A>G	43.5	31.1	50.8	18.1	0.217
rs17352054	1356	intron	A>C	22.4	60.7	33.8	5.5	0.343
rs12285347	1349	intron	T>C	43.3	31.3	50.8	17.9	0.212
rs11225297	1357	3' FR	A>T	11.4	78.0	21.1	0.8	0.076
rs11225307	1350	intron	A>G	22.7	60.1	34.4	5.5	0.471
rs17098318	1341	promoter	G>A	31.7	46.2	44.3	9.5	0.398
rs10502001	1034	exon	C>T	23.5	58.6	35.7	5.7	0.775
rs10750646	999	intron	A>G	74.9	55.7	38.4	5.9	0.494
rs11568819	1349	promoter	G>A	4.9	90.4	9.4	0.2	0.872

The genotype frequencies observed in our study broadly matched those reported in previous series. As a reference, we used the data from the largest reported European genome database – (HapMap-CEU). The comparisons are shown in Table 5-2.

Table 5-2 Comparison of study genotype frequencies with reference database (HapMap-CEU) frequencies. AA = major allele homozygote; AB = heterozygote; BB = minor allele homozygote.

SNP	Study genotype frequency (%)			Reference genotype frequency (%)		
	AA	AB	BB	AA	AB	BB
rs11568818	31.1	50.8	18.1	32.1	42.0	25.9
rs17352054	60.7	33.8	5.5	61.9	34.5	3.5
rs12285347	31.3	50.8	17.9	31.7	43.3	25.0
rs11225297	78.0	21.1	0.8	77.0	22.1	0.9
rs11225307	60.1	34.4	5.5	61.9	34.5	3.5
rs17098318	46.2	44.3	9.5	48.3	36.7	15.0
rs10502001	58.6	35.7	5.7	62.5	33.9	3.6
rs10750646	55.7	38.4	5.9	56.2	36.6	7.1
rs11568819	90.4	9.4	0.2	88.5	11.5	0.0

5.4 MMP-7 single nucleotide polymorphisms and the development of gastric mucosal preneoplastic pathology

We proceeded to examine the association between the described *MMP-7* gene SNPs and the presence of gastric preneoplastic pathology in our study population. To do

so, we compared affected individuals with those whose gastric biopsies were reported as normal and whose testing for current infection by *H. pylori* (i.e. rapid urease test and histology) had been negative. These individuals are referred to as "normal" for the purposes of the following analyses and the detailed description of this group can be found in section 4.4.1.

Genotype frequencies in the "pathology" group were compared with the corresponding values from the "normal" group. Table 5-3 lists the genotype frequencies in both groups for each SNP.

Table 5-3 Genotype frequencies for each SNP in the 'normal' histology group and in the preneoplastic 'pathology' group. AA = major allele homozygote, AB = heterozygote, BB = minor allele homozygote.

SNP	Genotype frequency (%) – Normal			Genotype frequency (%) – Pathology		
	AA	AB	BB	AA	AB	BB
rs11568818	30.9	51.5	17.6	35.2	45.7	19.1
rs17352054	63.4	31.0	5.6	56.3	33.4	10.2
rs12285347	31.0	51.6	17.4	31.8	48.1	20.1
rs11225297	79.4	19.4	1.2	76.6	22.7	0.6
rs11225307	63.4	31.0	5.6	59.1	35.3	5.6
rs17098318	45.1	46.3	8.6	41.3	48.2	10.6
rs10502001	62.5	32.2	5.3	57.0	37.4	5.7
rs10750646	52.6	39.4	8.0	57.1	38.5	4.4
rs11568819	91.8	8.0	0.2	88.9	10.8	0.3

We compared the genotype frequencies using χ^2 tests to generate odds ratios. These data are summarised in Table 5-4 and are illustrated in Figure 5-1.

Table 5-4 Association of gastric mucosal preneoplasia with *MMP-7* SNPs comparing the reference genotype (major allele homozygote, AA) with AB (heterozygote) and BB (minor allele homozygote) genotypes. Significant association shown in bold.

SNP	AB genotype		BB genotype	
	OR (95% CI)	p-value	OR (95% CI)	p-value
rs11568818	0.7778 (0.5617 to 1.077)	0.1297	1.051 (0.6914 to 1.597)	0.8168
rs17352054	1.213 (0.8853 to 1.663)	0.2289	**2.07 (1.184 to 3.618)**	**0.0095****
rs12285347	0.9061 (0.6486 to 1.266)	0.5632	0.8861 (0.5782 to 1.358)	0.5787
rs11225297	1.211 (0.8467 to 1.732)	0.2937	0.5814 (0.1118 to 3.023)	0.514
rs11225307	1.222 (0.8908 to 1.677)	0.2133	1.084 (0.5664 to 2.076)	0.807
rs17098318	1.139 (0.8350 to 1.553)	0.4117	0.745 (0.4412 to 1.258)	0.2698
rs10502001	1.275 (0.8882 to 1.830)	0.1875	1.167 (0.5486 to 2.484)	0.6874
rs10750646	0.8992 (0.6245 to 1.295)	0.5679	0.5123 (0.2351 to 1.116)	0.0878
rs11568819	1.399 (0.8455 to 2.314)	0.1897	0.6939 (0.04318 to 11.15)	0.7953

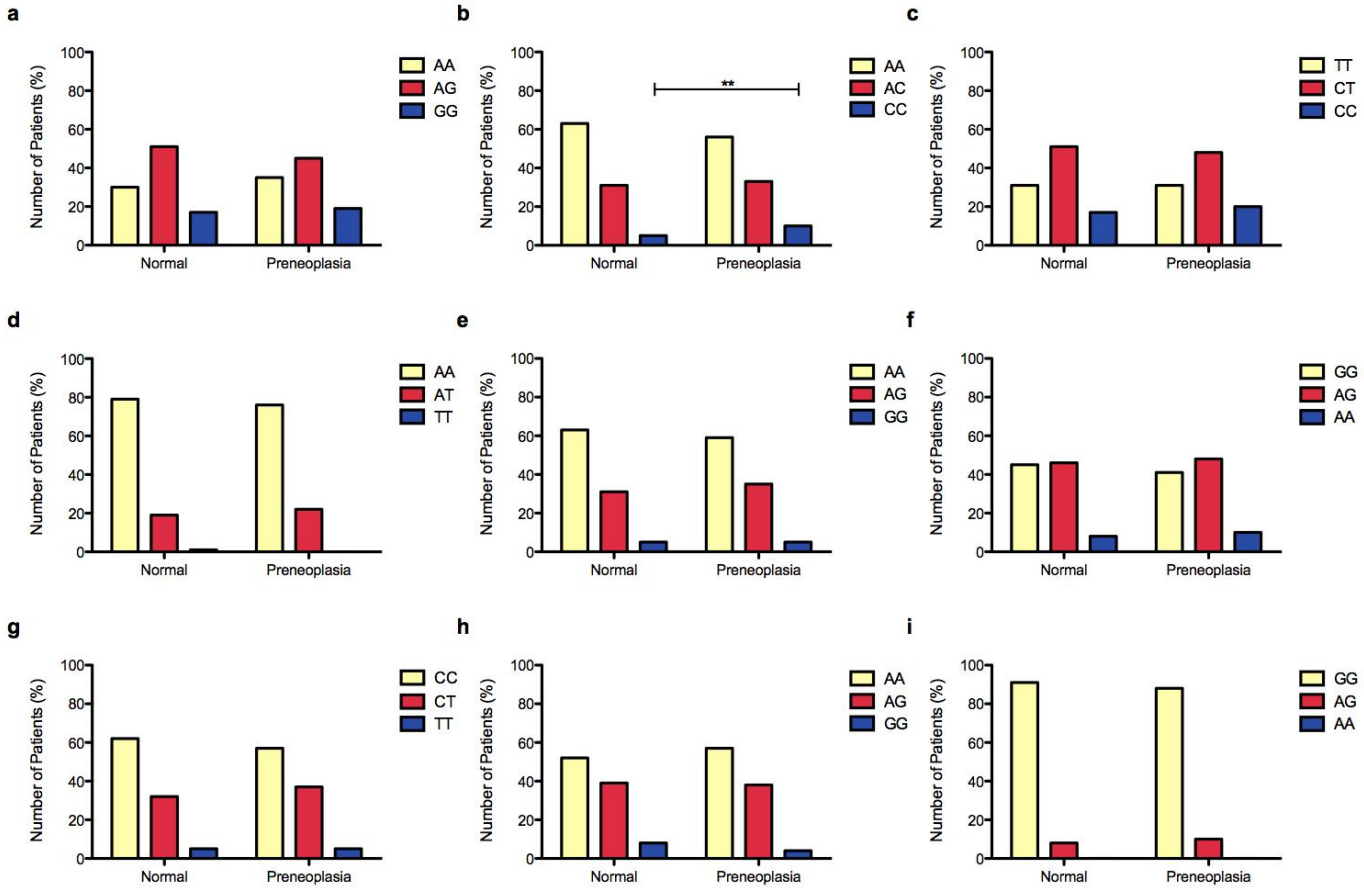

Figure 5-1 Relative genotype frequencies (shown as percentages) in subjects with normal gastric mucosal histology compared to those in subjects with gastric mucosal preneoplasia in the *MMP-7* SNPs a) rs11568818; b) rs17352054; c) rs12285347; d) rs11225297; e) rs11225307; f) rs17098318; g) rs10502001; h) rs10750646; and i) rs11568819.

The relative genotype frequency differed significantly between normal and preneoplasia groups for only one *MMP-7* SNP genotype - rs17352054 CC (minor allele homozygote). For this genotype, the minor allele homozygote genotype represented 10.2% of the preneoplasia pathology group as compared with only 5.6% of the normal histology group. Comparison using the χ^2 test gave an odds ratio of 2.07 (95% CI = 1.184 to 3.618).

5.4.1 Gastric preneoplasia following *H. pylori* infection

One of the objectives of this study was to determine some of the factors responsible for influencing the outcome of *H. pylori* infection in favour of preneoplasia development. We therefore repeated the analysis described above for patients with *H. pylori* seropositivity (implying current or previous infection). First we compared SNP genotype frequencies in preneoplasia groups compared with *H. pylori* seropositive individuals with normal histology *or* gastritis B (*H. pylori* associated gastritis). The genotype frequencies in this 'seropositive' group are shown compared with the genotype frequencies in the 'pathology' group in Table 5-5.

Table 5-5 Genotype frequencies for each SNP in the 'seropositive' group and in the preneoplastic 'pathology' group. AA = major allele homozygote, AB = heterozygote, BB = minor allele homozygote.

SNP	Genotype frequency (%) – Seropositive			Genotype frequency (%) – Pathology		
	AA	AB	BB	AA	AB	BB
rs11568818	14.7	29.0	56.3	19.0	30.2	50.7
rs17352054	57.3	36.8	5.9	60.0	36.6	3.4
rs12285347	30.2	56.2	13.6	30.6	50.0	19.4
rs11225297	71.7	27.5	0.8	77.7	22.3	0.0
rs11225307	56.3	37.7	6.0	60.3	36.3	3.4
rs17098318	45.8	45.4	8.8	47.8	42.9	9.4
rs10502001	53.4	39.8	6.8	57.4	39.4	3.2
rs10750646	56.9	38.7	4.4	56.0	38.7	5.3
rs11568819	93.2	6.8	0.0	89.3	10.2	0.5

Again, we compared the genotype frequencies using χ^2 tests to generate odds ratios. These data are summarised in Table 5-6 and illustrated in Figure 5-2.

Table 5-6 Association of gastric mucosal preneoplasia with *MMP-7* SNPs comparing the reference genotype (major allele homozygote, AA) with AB (heterozygote) and BB (minor allele homozygote) genotypes from contingency of *H. pylori* seropositive individuals with normal gastric histology *or* gastritis B, and preneoplasia groups.

SNP	AB genotype		BB genotype	
	OR (95% CI)	p-value	OR (95% CI)	p-value
rs11568818	0.8623 (0.5651 to 1.316)	0.4920	0.8058 (0.4588 to 1.415)	0.4518
rs17352054	0.9507 (0.6451 to 1.401)	0.7982	0.5501 (0.2173 to 1.393)	0.2018
rs12285347	0.8795 (0.5794 to 1.335)	0.5461	0.7067 (0.4028 to 1.240)	0.2255
rs11225297	0.7491 (0.4887 to 1.148)	0.1843	0.2312 (0.0110 to 4.854)	0.1895
rs11225307	0.8993 (0.6099 to 1.326)	0.5918	0.5388 (0.2127 to 1.365)	0.1863
rs17098318	0.9048 (0.6131 to 1.335)	0.6145	0.9852 (0.5036 to 1.927)	0.9653
rs10502001	0.9199 (0.5919 to 1.430)	0.7104	0.4408 (0.1512 to 1.285)	0.1250
rs10750646	1.016 (0.6466 to 1.596)	0.9451	1.226 (0.4414 to 3.406)	0.6925
rs11568819	1.573 (0.8062 to 3.068)	0.1811	0.2620 (0.0106 to 6.473)	0.2599

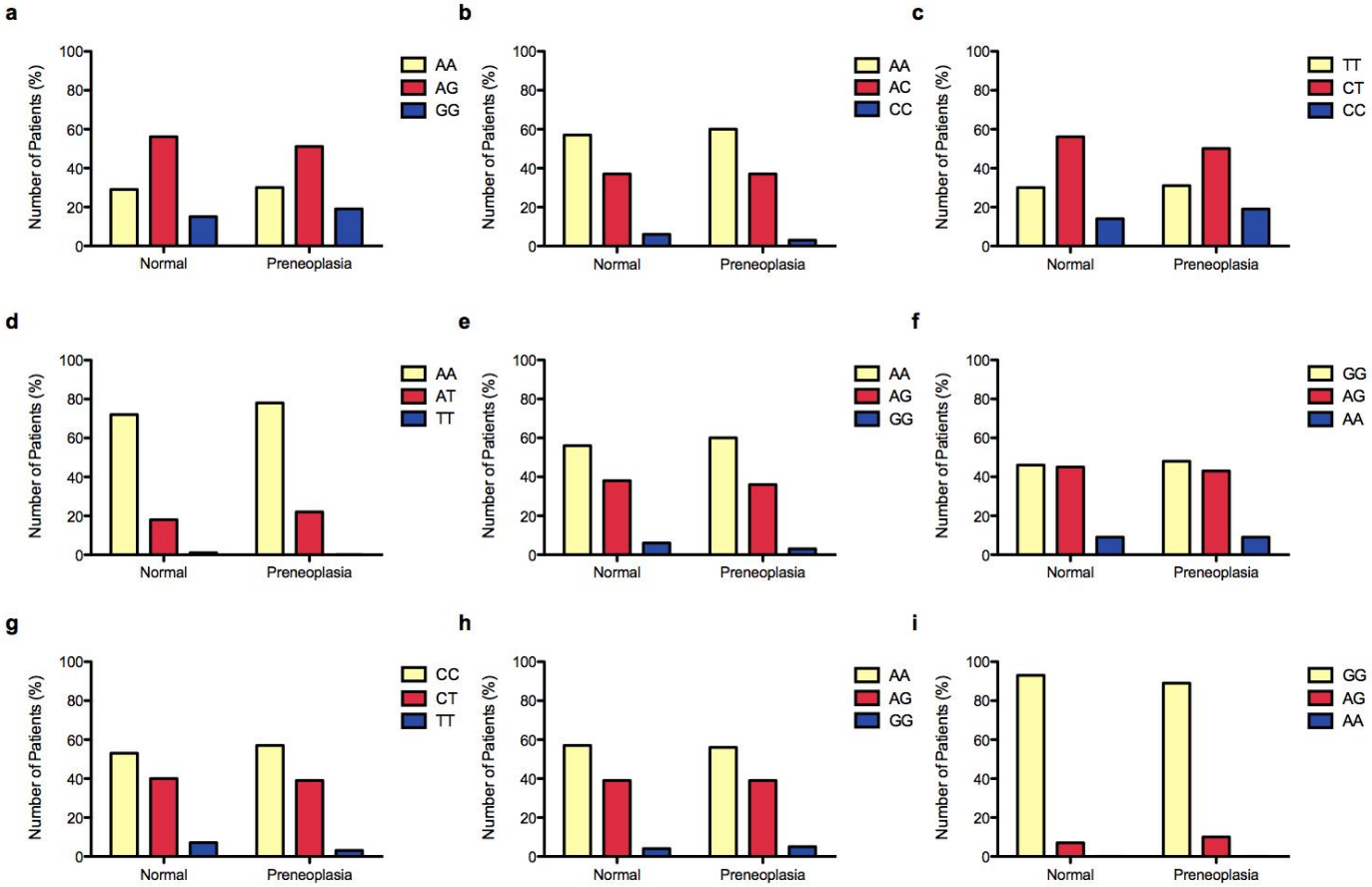

Figure 5-2 Relative genotype frequencies (shown as percentages) in subjects with *H. pylori* seropositivity and normal histology *or* gastritis B, compared to those in subjects with gastric mucosal preneoplasia in the *MMP-7* SNPs a) rs11568818; b) rs17352054; c) rs12285347; d) rs11225297; e) rs11225307; f) rs17098318; g) rs10502001; h) rs10750646; and i) rs1156881

In this comparison, there were no significant differences between the genotype frequencies observed in *H. pylori* seropositive patients with normal gastric histology or gastritis B and those with gastric preneoplasia.

To mitigate the effects of active *H. pylori* infection on this analysis, it was repeated, this time comparing SNP genotype frequencies in preneoplasia groups with those in *H. pylori* seropositive individuals with normal gastric histology only. The genotype frequencies in this '*H. pylori*' group are shown compared with the genotype frequencies in the 'pathology' group in Table 5-7.

Table 5-7 Genotype frequencies for each SNP in the '*H. pylori*' group and in the preneoplastic 'pathology' group. AA = major allele homozygote, AB = heterozygote, BB = minor allele homozygote.

SNP	Genotype frequency (%) – *H. pylori*			Genotype frequency (%) – Pathology		
	AA	AB	BB	AA	AB	BB
rs11568818	16.8	26.9	56.3	19.0	30.2	50.7
rs17352054	53.3	42.2	4.4	60.0	36.6	3.4
rs12285347	29.9	56.0	14.2	30.6	50.0	19.4
rs11225297	66.2	32.4	1.5	77.7	22.3	0.0
rs11225307	51.9	43.7	4.4	60.3	36.3	3.4
rs17098318	49.2	41.7	9.1	47.8	42.9	9.4
rs10502001	49.5	45.7	4.8	57.4	39.4	3.2
rs10750646	57.6	37.6	4.7	56.0	38.7	5.3
rs11568819	93.3	6.7	0.0	89.3	10.2	0.5

Once again, we compared the genotype frequencies using χ^2 tests to generate odds ratios. These data are summarised in Table 5-8and illustrated in Figure 5-3.

Table 5-8 Association of gastric mucosal preneoplasia with *MMP-7* SNPs comparing the reference genotype (major allele homozygote, AA) with AB (heterozygote) and BB (minor allele homozygote) genotypes from contingency of *H. pylori* seropositive individuals with normal histology and those with gastric preneoplasia. Significant association shown in bold.

SNP	AB genotype		BB genotype	
	OR (95% CI)	p-value	OR (95% CI)	p-value
rs11568818	0.8018 (0.4736 to 1.355)	0.4081	0.9936 (0.4996 to 1.976)	0.9854
rs17352054	0.7702 (0.4907 to 1.209)	0.2559	0.6829 (0.2209 to 2.112)	0.5056
rs12285347	0.8720 (0.5312 to 1.431)	0.5878	0.7481 (0.3810 to 1.496)	0.3986
rs11225297	**0.5881 (0.3612 to 0.9575)**	**0.0319***	0.1128 (0.0054 to 2.377	0.0611
rs11225307	0.7138 (0.4548 to 1.120)	0.1420	0.6640 (0.2146 to 2.054)	0.4747
rs17098318	1.060 (0.6683 to 1.681)	0.8045	0.9425 (0.4285 to 2.073)	0.8829
rs10502001	0.7425 (0.4458 to 1.237)	0.2520	0.5843 (0.1614 to 2.115)	0.4082
rs10750646	1.057 (0.6056 to 1.846)	0.8446	1.167 (0.3339 to 4.077)	0.8090
rs11568819	1.607 (0.7123 to 3.624)	0.2497	0.4835 (0.0195 to 11.97)	0.4072

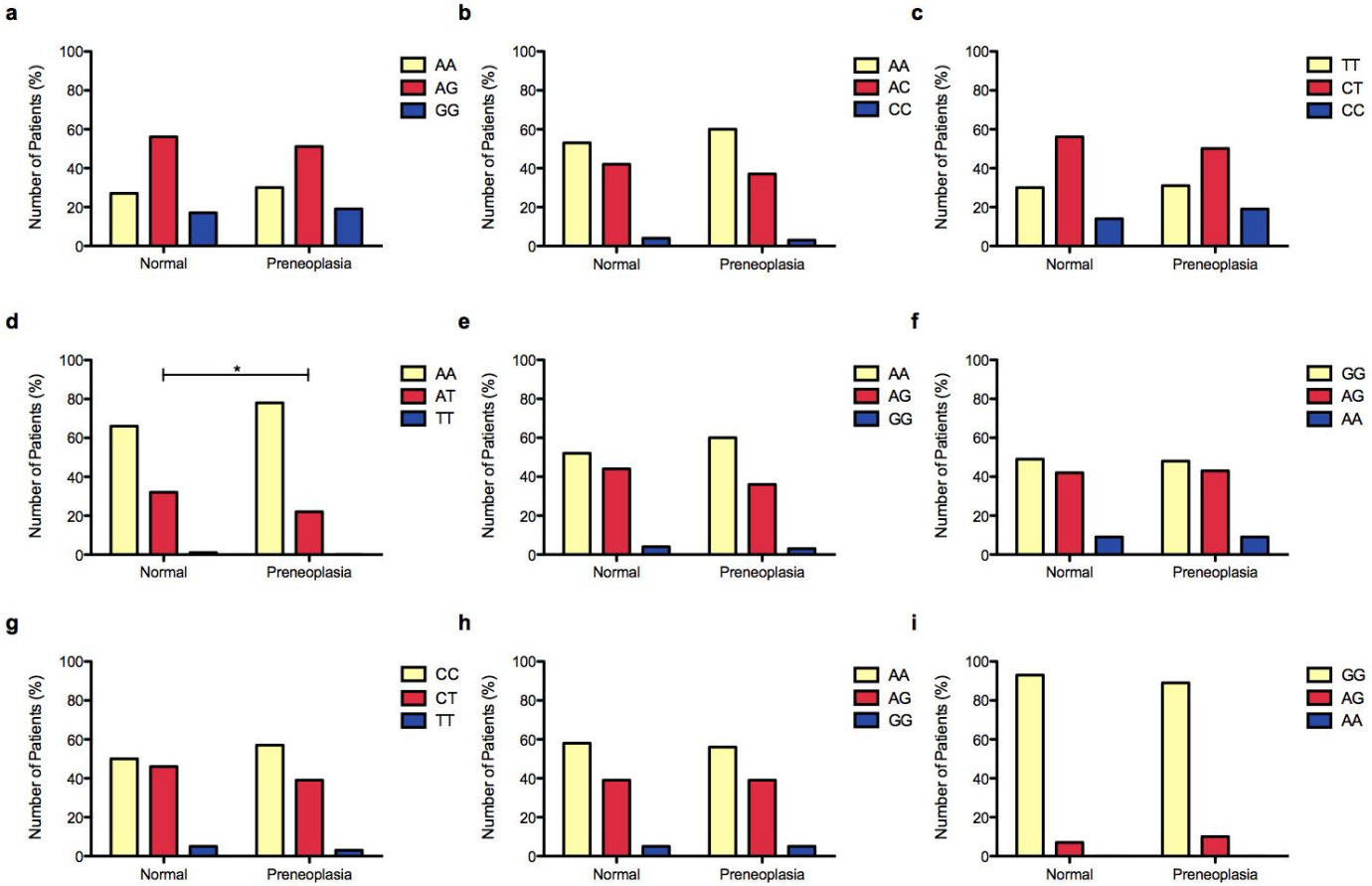

Figure 5-3 Relative genotype frequencies (shown as percentages) in subjects with *H. pylori* seropositivity and normal histology compared to those in subjects with gastric mucosal preneoplasia in the *MMP-7* SNPs a) rs11568818; b) rs17352054; c) rs12285347; d) rs11225297; e) rs11225307; f) rs17098318; g) rs10502001; h) rs10750646; and i) rs11568819

In this series, we found a small but significant difference in genotype frequency for one of the 9 SNPs between *H. pylori* seropositive individuals with normal gastric histology and those with preneoplasia. Heterozygotes for SNP rs11225297 (AT) were less frequent in the preneoplasia group (22.3%) than in *H. pylori* seropositive but normal histology group (32.4%) implying a negative association between the minor allele and gastric preneoplasia. Using the χ^2 test gave an odds ratio of 0.59 (p=0.03, 95% CI = 0.361-0.968). In such cases, we would anticipate seeing a similar if not greater effect in homozygotes for the minor allele but the frequency of that genotype was very low in our study cohort (0.8%) and so we were unable to detect an effect in that group.

5.5 MMP-7 single nucleotide polymorphisms and gastric mucosal MMP-7 mRNA abundance

We also examined the association between *MMP-7* SNPs genotypes and gastric mucosal expression of MMP-7 as evidenced by the relative mRNA abundance determined by qPCR. Here we compared the relative mucosal mRNA abundance of MMP-7 in individuals with the reference (major allele homozygote) genotype to that of individuals with the heterozygote genotype and of those with the minor allele homozygote genotype. Mean relative abundances were compared using the Kruskal Wallis ANOVA with Dunn's multiple comparison tests. The data are summarised in .

Table 5-9 and are illustrated in Figure 5-4.

Table 5-9 Comparison between mean relative (rel.) abundance of gastric mucosal MMP-7 mRNA in the reference, major allele homozygote (AA) genotype with that in the heterozygote (AB) and minor allele homozygote (BB) genotypes for MMP-7 SNPs. *** = p<0.001; *** = p<0.01.

SNP	AA genotype	AB genotype		BB genotype	
	Mean MMP-7 rel. abundance (SEM)	Mean MMP-7 rel. abundance (SEM)	Fold-change rel. to AA	Mean MMP-7 rel. abundance (SEM)	Fold-change rel. to AA
rs11568818	0.61 (0.15)	1.79 (0.24)	2.9***	3.06 (0.82)	5.0***
rs17352054	1.24 (0.14)	3.31 (0.79)	2.7***	1.97 (0.54)	1.6
rs12285347	3.02 (0.81)	1.80 (0.24)	-1.7**	0.67 (0.16)	-4.5***
rs11225297	1.82 (0.32)	2.56 (0.57)	1.4	0.71 (0.24)	0.4
rs11225307	1.26 (0.14)	3.28 (0.79)	2.6**	1.73 (0.41)	1.4
rs17098318	2.40 (0.56)	1.82 (0.28)	-1.3	0.80 (0.27)	-3.0***
rs10502001	1.11 (0.14)	3.29 (0.98)	3.0***	1.86 (0.63)	1.7
rs10750646	1.95 (0.30)	1.75 (0.29)	0.9	1.54 (0.38)	0.8
rs11568819	1.84 (0.29)	3.11 (0.89)	1.7	1.37 (1.22)	0.7

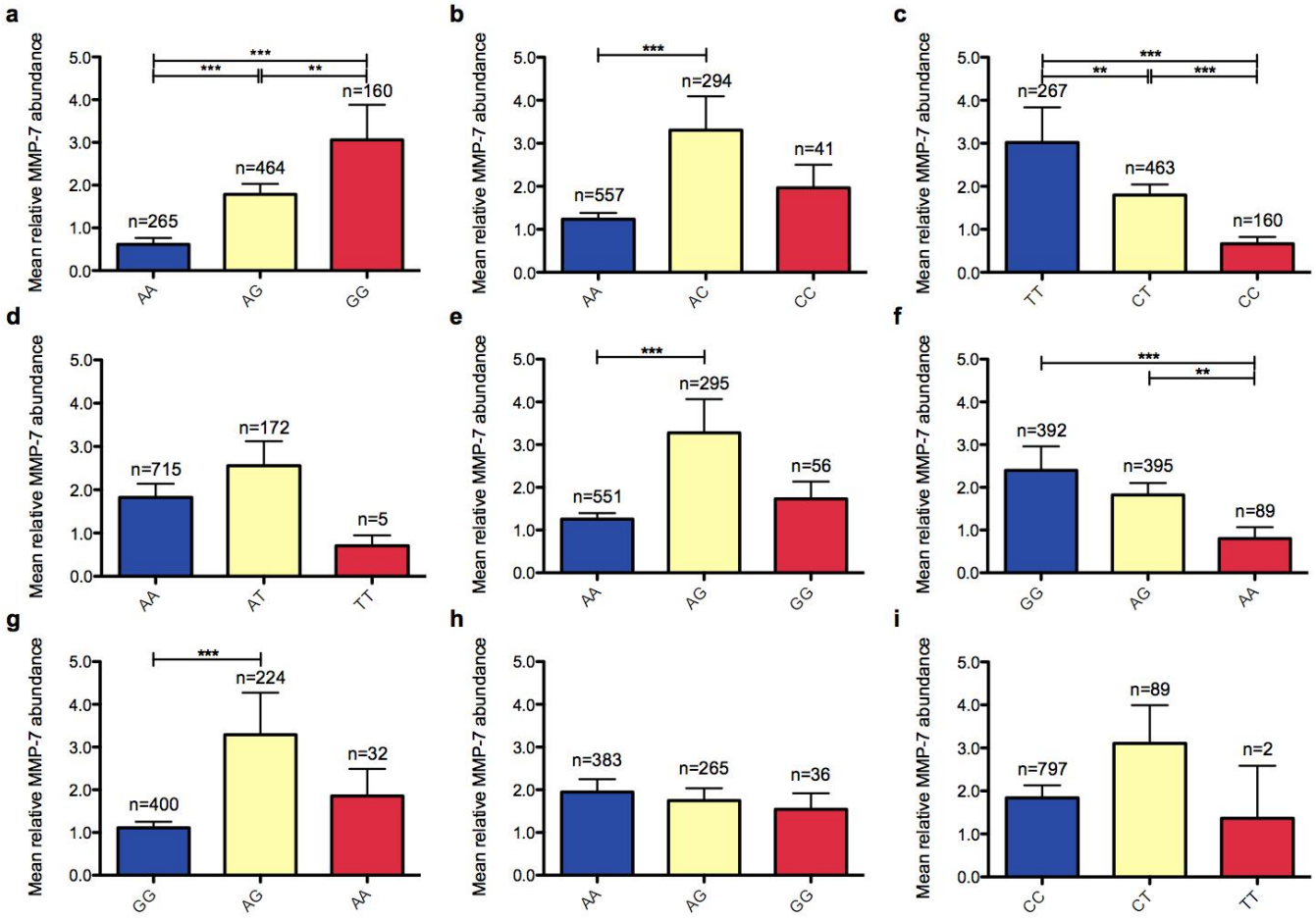

Figure 5-4 Comparison of mean relative abundance of gastric mucosal MMP-7 mRNA in different genotypes for SNPs: a) rs11568818; b) rs17352054; c) rs12285347; d) rs11225297; e) rs11225307; f) rs17098318; g) rs10502001; h) rs10750646; and i) rs11568819. n=number of patients.

As can be seen, there was a significant difference in mucosal MMP-7 relative mRNA abundance between genotypes for six of the nine SNPs: rs11568818; rs17352054; rs12285347; rs11225307; rs17098318; and rs10502001 (labelled ‘a’, ‘b’, ‘c’, ‘e’, ‘f’ and ‘g’ respectively in Figure 5-4).

For SNPs rs11568818; rs17352054; rs11225307 and rs10502001 (labelled ‘a’, ‘b’, ‘e’ and ‘g’ in the figure), we observed an increased relative abundance of MMP-7 mRNA in the presence of the minor allele compared with the reference genotype. In the case of SNPs rs17352054; rs11225307 and rs10502001 (labelled ‘b’, ‘e’ and ‘g’ in the figure), this phenomenon was only true for the heterozygote genotype and the minor allele homozygote was not significantly different. This might be attributable to the comparatively low numbers of minor allele heterozygotes included for these SNPs.

SNP rs11568818 was associated with a significant increase in mean MMP-7 relative mRNA abundance in the presence of both the heterozygote (2.9-fold) and the minor allele homozygote (5-fold) genotypes compared with the reference genotype. There was also a significantly greater mean relative abundance in the minor allele group compared with the heterozygote group (1.7-fold).

In contrast to these SNPs, presence of the minor allele in rs12285347 and rs17098318 (labelled “c” and “f” respectively in the figure) was associated with a *decrease* in the relative abundance of MMP-7 mRNA compared with the reference genotype.

The heterozygote and minor allele homozygote genotypes for rs12285347 were associated with statistically significant 1.7-fold and 4.5 fold decreases in mean MMP-7 relative mRNA abundance compared with the reference genotype respectively. For rs17098318, the observed difference between the heterozygote and reference genotypes was not statistically significant but there was a highly significant 3.0-fold decrease in the relative mRNA abundance of MMP-7 between homozygote genotypes. For both of these SNPs, there was a significant decrease in MMP-7

relative mRNA abundance between heterozygote and minor allele homozygote genotypes.

5.6 Discussion

The studies conducted to-date on *MMP-7* polymorphisms have largely focused on epithelial cancers and inflammatory diseases. For gastric cancer in particular, there is a lack of studies reporting on MMP-7 polymorphisms and their functional effects in European populations. Additionally, studies examining such polymorphisms have largely ignored the question of influence on the development of preneoplastic lesions.

The best studied SNPs with regards gastric cancers are rs11568818 and rs11568819. Both have been shown to be associated with increased frequencies in gastric cancer and have also been associated with more 'aggressive' tumour behaviour including enhanced invasiveness, metastasis and lymph node invlovement.

In this study, we found no associations between these polymorphisms and gastric preneoplasia, though both exhibited an increased expression of mucosal MMP-7 with carriage of the minor allele. For rs11568819, this was not statistically significant and there were insufficent subjects with the minor allele homozygote genotype to adequately characterise its effect. For rs11568818 however, there was a significant and stepwise increase in mucosal MMP-7 expression between major allele homozygote, heterozygote and minor allele homozygote. The converse was true for rs17098318, where there was a significant and stepwise decreease in mucosal MMP-7 mRNA abundance in the same direction, though this too was not translated into a measurable effect on the risk of preneoplasia.

We have however identified two other *MMP-7* SNPs whose genotypes seem to be associated with the presence of gastric preneoplasia. In the comparision between all individuals with gastric preneoplasia and all those with normal stomach histology,

rs17352054 minor allele homozygosity was slightly but signnificantly more frequent in the preneoplasia group. When comparing individuals with gastric preneoplasia to those with normal stomach histology but evidence of *H. pylori* infection, rs11225297 heterozygosity was negatively associated with preneoplasia. The minor allele homozygote is insufficiently frequent in Europeans for us to have been able to measure an effect. Neither SNPs have previously been described in association with GI cancer, though both have been studied for their effect on breast cancer survival and susceptibility and rs11225297 has previously been associated with improved breast cancer survival in a Chinese study (311,317). Neither SNP has previously been reported as having disease-associations in a European population.These SNPs therefore warrant further investigation in other European cohorts with gastric preneoplasia and cancer.

6 A pilot study of the novel gastrin antagonist netazepide (YF476) for the treatment of type 1 gastric neuroendocrine tumours

6.1 Introduction

Autoimmune chronic gastritis is a condition characterised by the autoimmune-mediated loss of oxyntic glands from the corpus mucosa(243). The resulting hypochlorhydria leads to unopposed gastrin secretion by antral G-cells. Gastrin exerts both stimulatory and trophic effects on ECL-cells. In some cases, gastrin-dependent ECL-cell hyperplasia can progress to dysplasia and ECL-cell tumour (gastric neuroendocrine tumour) formation. Gastric neuroendocrine tumours of this aetiology are categorized as type 1 NETs. They are typically multiple, small and located in the gastric corpus and fundus(259,258).

Although increasing in incidence, type 1 gastric NETs are rarely diagnosed and so evidence to guide their management is limited. Small tumours (smaller than 10mm) typically exhibit an indolent natural history. They grow slowly, rarely metastasise and are generally associated with a favourable prognosis. Conservative treatment and surveillance is the widely accepted strategy for managing this group of patients(265,318–320). Larger tumours are regarded as having the potential to grow more rapidly and metastasise. Surgical antrectomy can induce tumour regression by removing the source of gastrin but is not universally effective and carries the associated risks of surgery. Long acting somatostatin analogues such as octreotide have been shown to reduce tumour size, number and circulating biomarkers but require parenteral administration and are not always well tolerated.

Many gastrin/CCK-2 receptor antagonists have been described, but they have proven limited in terms of their potency, selectivity and bioavailability. None have been developed as a medicine(11). Netazepide is a potent, orally bioavailable and highly selective competitive gastrin/CCK-2 receptor antagonist.

The present study describes the first clinical use of netazepide. Our objectives were to assess the safety, tolerability and efficacy of netazepide in patients with autoimmune chronic atrophic gastritis and type 1 gastric NETs.

6.2 Study design

The study methodology is described in detail in chapter 2 and is summarised here for convenience. Patient recruitment took place over a 12-month period. Subjects who satisfied the inclusion criteria were treated in two stages.

Stage 1 comprised 12 weeks of treatment with netazepide 50mg daily and a 12-week follow-up period (after discontinuing netazepide treatment). There were 6 study visits. At baseline (0 weeks), 3, 6, 9, 12 and 24 weeks we collected blood for determination of fasting serum gastrin and chromogranin A concentrations along with routine 'safety' parameters (full blood count, urea and electrolytes, liver function tests, lipid profile and fasting glucose concentration). Patients underwent gastroscopy at baseline (0 weeks) and at 6, 12 and 24 weeks to measure and photograph gastric lesions and to obtain biopsies of gastric mucosa and visible tumours. Assays for plasma netazepide concentration were performed on blood samples taken for trough (prior to dosing) and peak levels (1 hour after dosing) at each visit.

Stage 2 comprised 52 weeks of treatment with netazepide. Patients were eligible for inclusion in the second stage if they were considered to have benefited from the primary treatment course. All eight patients from stage 1 were included. There were 5 study visits. At 0, 12, 24, 36 and 52 weeks, we collected blood for gastrin, CgA, and netazepide assays along with 'safety' monitoring as before. We performed gastroscopy at baseline (0 weeks), 24 and 52 weeks as before. There was no follow-up study visit after this stage and patients were returned to routine clinic follow-up. The investigations performed at each study visit are summarised in Table 6-1.

There was considerable variation across the study cohort in the time interval between completion of stage 1 (including the 12 week follow-up period) and commencement of stage 2 (range 22-66 weeks, mean 46 weeks, median 47 weeks). For one patient (patient 8), this interval was less than 6 months and so repeat endoscopy prior to restarting netazepide was considered unnecessary.

Table 6-1 Investigations performed at each study visit.

Stage 1

Weeks	0	3	6	9	12	24
On treatment		●	●	●	●	
Blood tests	●	●	●	●	●	●
Endoscopy	●		●		●	●

Stage 2

Weeks	0	12	24	36	52
On treatment		●	●	●	●
Blood tests	●	●	●	●	●
Endoscopy	●		●		●

6.3 Patient characteristics

Patients were recruited to the present study from the professorial tertiary referral neuroendocrine tumour outpatient clinic at the Royal Liverpool University Hospital as described in chapter 2. We enrolled 8 patients to the trial over a 12-month period. 4 were female. The mean age was 66 years (range 56-76 years). Patient characteristics are summarised in Table 6-2. Prior to enrolment, all patients had undergone baseline investigations and had been shown to have a) histologically proven type 1 gastric neuroendocrine tumour(s); b) chronic atrophic gastritis and vitamin B12 deficiency and c) no evidence of metastatic disease either by computed tomography or somatostatin-receptor scintigraphy (^{111}In-octreotide scan).

All patients were found to have anti-parietal cell antibodies and two also had anti-intrinsic factor antibodies. There were a variety of well-controlled chronic comorbidities reported by study subjects and careful records of medication use were made prior to and during the trial.

Table 6-2 Patient characteristics. AG = atrophic gastritis, IM = intestinal metaplasia, ECL-L = linear ECL-cell hyperplasia, ECL-M = micronodular ECL-cell hyperplasia, ECL-D = ECL-cell dysplasia, NET = neuroendocrine tumour.

	Patient number							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Age	60	64	67	69	76	67	55	66
Gender	Female	Female	Male	Male	Female	Female	Male	Male
Number of gastric polyps	8	8	4	9	30	10	12	10
Size of largest polyp (mm)	6	15	3	5	7	8	10	10
Histology of tumour	Low grade NET	Low grade NET	ECL-M	Low grade NET	Low grade NET	Low grade NET	Low grade NET	Low grade NET
Gastric corpus histology	AG, IM, ECL-M	AG, IM, ECL-M	AG, IM, ECL-L	AG, IM, ECL-M	AG, IM, low grade NET	AG, IM, ECL-M	AG, IM, ECL-D	AG, IM, ECL-D
Serum gastrin by RIA (pmol/L)	800	800	580	960	1050	470	1750	520
Serum gastrin by ELISA (pmol/L)	531	494	414	645	655	332	953	415
Serum CgA (U/L)	25.2	52.6	54	33	93	56	128	64
***H. pylori* histology**	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
***H. pylori* serology**	Negative	Negative	Negative	Not done	Negative	Positive	Negative	Positive
Vitamin B12 deficiency	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Anti-parietal cell antibody	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Anti-intrinsic factor antibody	Negative	Positive	Positive	Negative	Negative	Negative	Negative	Negative

6.3.1 Tumour number and size

At baseline gastroscopy, all 8 patients had visible gastric tumours. The median number was 10 (range 4–30), and the mean diameter of the largest tumour was 6.75 mm (range 3–15 mm).

6.3.1.1 Stage 1

After 6 weeks of treatment, five of the patients had fewer tumours and in two of these, a further reduction in number was observed after 12 weeks of treatment. Two patients exhibited no change in tumour number and we recorded a slight increase in the number of visible tumours for the remaining patient. After 6 and 12 weeks' treatment, the mean decrease in the number of tumours relative to baseline was 24 and 30%, respectively (p=0.041 and 0.046 respectively, Wilcoxon signed ranks test). At 24 weeks, 12 weeks after completion of treatment, findings were similar to those at 12 weeks. The mean decrease relative to baseline was 29% (p=0.092, Wilcoxon signed ranks test).

All but one of the patients (patient 7) had a decrease in the diameter of their largest tumour after 6 and/or 12 weeks' treatment. The mean decrease relative to baseline was 20 and 33%, respectively (p=0.026 and 0.018 respectively, Wilcoxon signed ranks test). None of the largest tumours had increased in size at 12 weeks after stopping treatment, and 2 of them were slightly smaller. The mean decrease relative to baseline was 40% (p=0.017, Wilcoxon signed ranks test). The endoscopic findings are summarised in Figure 6-1.

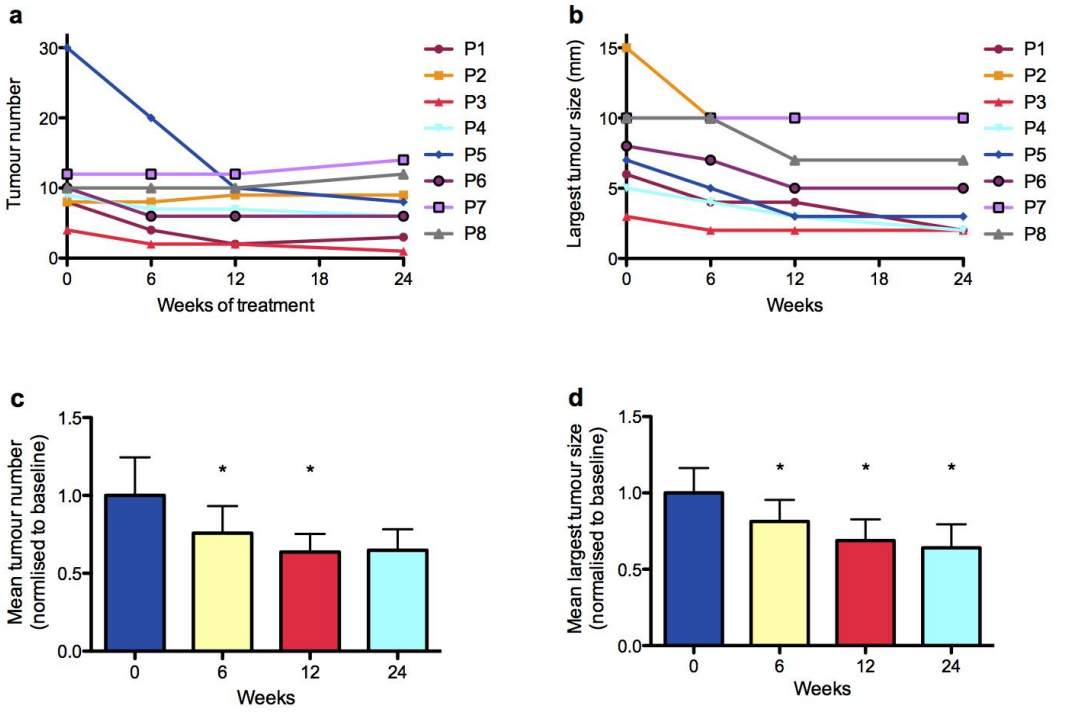

Figure 6-1 Stage 1 endoscopic tumour characteristics: (a) number of tumours; (b) size of largest tumour, and (c,d) % change from baseline after 6 and 12 weeks' netazepide treatment, and at follow-up at 24 weeks, 12 weeks after end of treatment. *Note image 'p' in right panel recorded in greyscale due to a technical fault.*

Illustrative endoscopic images taken at baseline and at week 24 (12 weeks after completing 12 weeks treatment with netazepide) are shown in Figure 6-2 (note image 'p' in right panel was recorded in greyscale due to a technical fault with the endoscopy equipment at the time of endoscopy.)

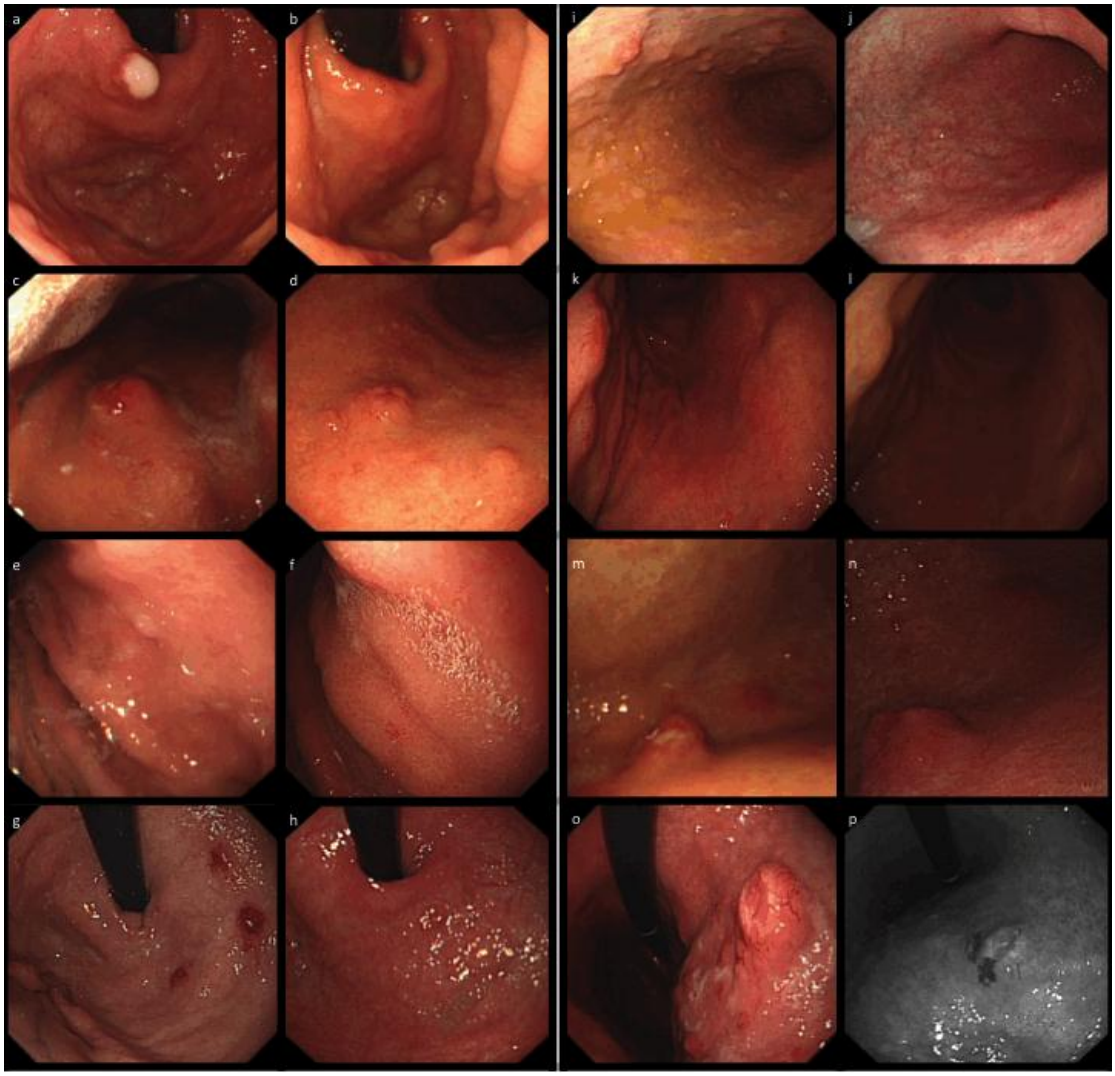

Figure 6-2 Illustrative endoscopic photographs taken at gastroscopy from patients 1 (a, b); 2 (c, d); 3 (e, f) and 4 (g, h) in left panel and patients 5 (i, j); 6 (k, l); 7 (m, n) and 8 (o, p) (in right panel) at baseline and 24 weeks respectively.

6.3.1.2 Stage 2

In the interval between completing stage 1 and beginning stage 2, we observed small but significant increases in tumour number and size in most subjects. As described above, patient 8 did not undergo a repeat gastroscopy prior to starting stage 2. Of the remaining 7 subjects, 6 were reported to have more visible tumours at the start of stage 2 than on completion of stage 1 (Figure 6-3a). One patient (patient 7) had fewer visible tumours. The mean increase in visible tumour number was 55% (p=0.034, Wilcoxon signed ranks test). Of the same 7 patients, 6 developed an increase in the size of their largest visible tumour in the interval period and 1

(patient 1) was unchanged (Figure 6-3b). The mean increase in the diameter of the largest tumour was 38% (p=0.023, Wilcoxon signed ranks test).

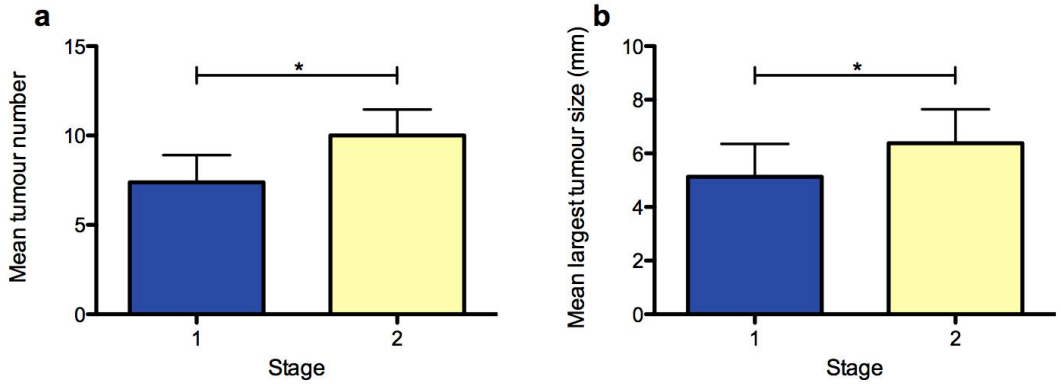

Figure 6-3 a) Mean tumour number and b) Mean size of largest tumour at completion of stage 1 compared with the start of stage 2.

After 24 weeks of treatment with netazepide in stage 2, 6 patients had fewer tumours and in 2, the numbers were unchanged. After 52 weeks of treatment, there had been an increase in tumour number in 3 patients, including 2 in whom the number had initially decreased. One patient (patient 1) exhibited a complete endoscopic response with no visible tumours after 24 weeks and no recurrence after 52 weeks. Patient 3 had started stage 1 with a small number (4) of diminutive tumours (largest 3mm). At the start of stage 2, we recorded 2 tumours with the largest being 4mm. We recorded no visible tumours after 24 weeks of treatment but after 52 weeks reported 1 tumour, 1mm in diameter. In retrospect, it seems possible that this tumour was present but not seen at the 24 week examination. After 24 and 52 weeks of treatment, the mean decreases in tumour number were 36% (p=0.026) and 32% (ns p=0.127, Wilcoxon signed rank tests) respectively.

All but one of the patients (patient 5) exhibited a decrease in the size of their largest tumour after 24 weeks of treatment. After 52 weeks, the size of the largest tumour had decreased in all 8 patients compared with the endoscopy performed at the start of stage 2. Interestingly, the patient whose macroscopic response had been negligible in stage 1 actually showed a small decrease in visible tumour number and

a significant decrease in the size of his largest tumour in stage 2. The mean decreases in the size of the largest tumour after 24 and 52 weeks of treatment were 50% (p=0.017) and 57% (p=0.011, Wilcoxon signed rank tests) respectively. The endoscopic findings from stage 2 of the study are summarised in Figure 6-4.

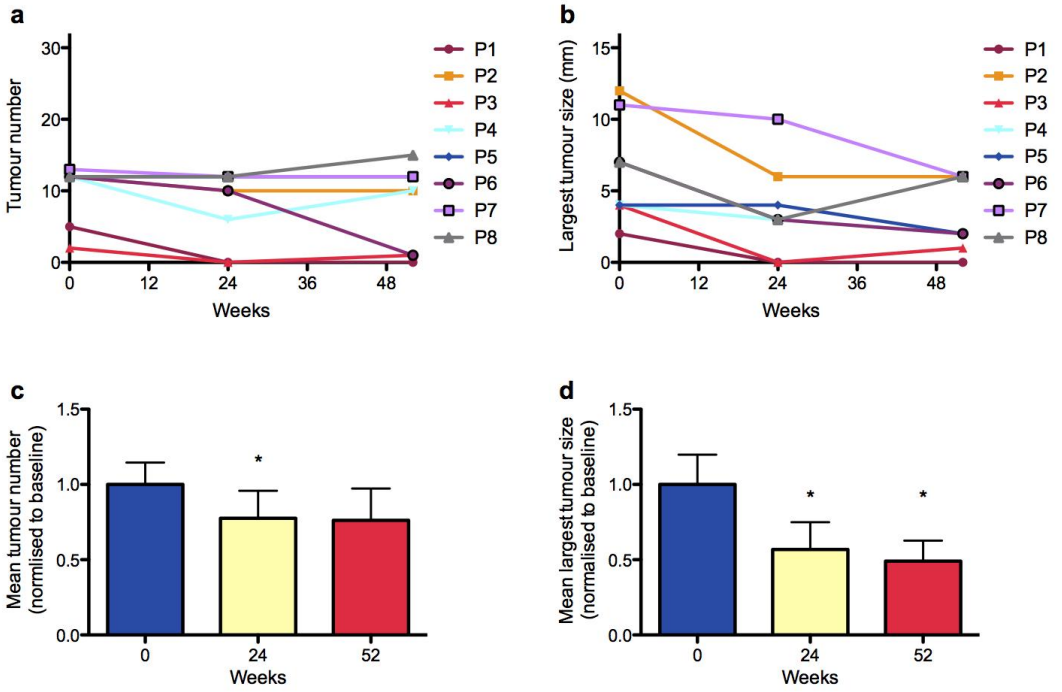

Figure 6-4 Stage 2 endoscopic tumour characteristics: (a) number of tumours; (b) size of largest tumour, and (c,d) % change from pre-treatment measurements after 24 and 52 weeks' netazepide treatment.

Illustrative endoscopic images taken at the start of stage 2 and after 52 weeks of treatment with netazepide are shown in Figure 6-5

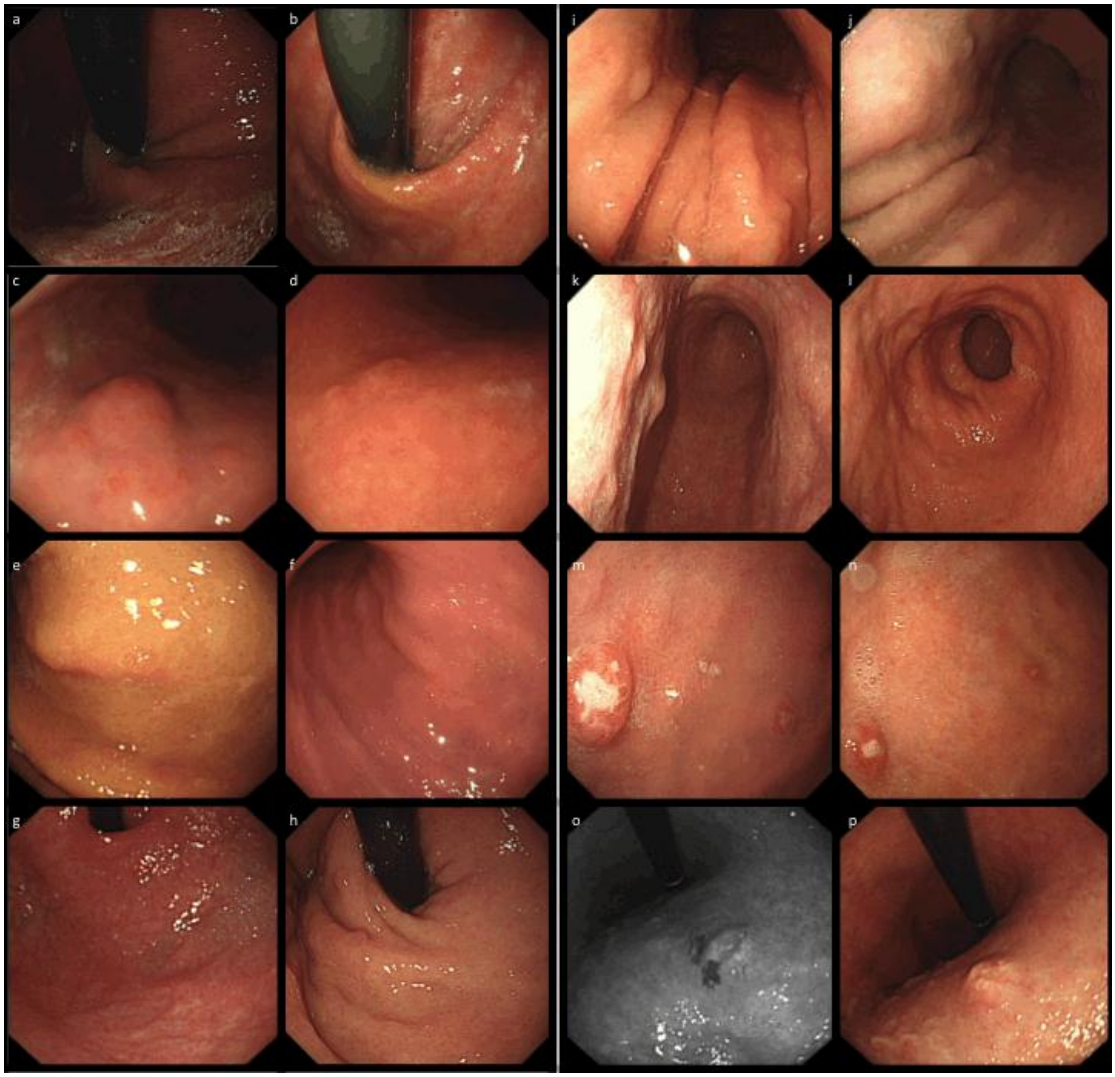

Figure 6-5 Illustrative endoscopic photographs taken at gastroscopy from patients 1 (a, b); 2 (c, d); 3 (e, f) and 4 (g, h) (in left panel) and patients 5 (i, j); 6 (k, l); 7 (m, n) and 8 (o, p) (in right panel) at the start of stage 2 and after 52 weeks of netazepide treatment respectively.

6.3.2 12 month follow-up

On completion of stage 2, all 8 subjects were returned to routine follow-up care in the regional neuroendocrine tumour outpatient clinic. As part of their routine surveillance, all 8 underwent repeat upper GI endoscopy approximately 12 months after the final study visit. Though not included in the study protocol, we report the results for completeness.

In the 12-month period between completing the study and the repeat endoscopy, three patients (patients 2, 3 and 4) were reported to have fewer tumours and

another three (patients 1, 7 and 8) exhibited no change. Patient 6 who had concluded the study with only one visible polyp, 2mm in diameter was reported to have 2 visible tumours at follow-up, the largest estimated to be 3mm. Only patient 5 who had 12 diminutive tumours at the end of stage 2 (largest 2mm) really seemed to have a significant increase in tumour number. At her follow-up endoscopy, she was reported to have 20 tumours (largest 2mm). Patient 1, in whom we reported a complete response to netazepide treatment, was still free of visible tumours. Patient 3 had been reported to have a single; 1mm polyp at the end of stage 2 had no visible tumours reported at follow-up.

At follow up endoscopy, four patients (patients 4, 5 and 8) exhibited no increase in the size of their largest tumour. Patients 1 and 3 had no visible tumour. There were three patients in whom the size of the largest tumour was reportedly increased. Patients 6 and 7 had an increase in tumour size of 1 and 2mm respectively. Only patient 2 exhibited a clinically significant change as her largest tumour had increased in size from 6mm to 10mm.

6.3.3 Histopathology

All 8 patients had low-grade type 1 gastric NETs prior to enrolment. Seven still had low-grade NETs at baseline; the other had micronodular ECL-cell hyperplasia throughout the study. All gastric corpus mucosal biopsies showed ECL-cell hyperplasia throughout the study, but there were no further morphological or histopathological changes. Histological findings from endoscopic biopsies are summarised in Table 6-3.

Table 6-3 Histological findings of endoscopic biopsies. NET - neuroendocrine tumour, ECL-L - linear ECL cell hyperplasia, ECL-M - micronodular ECL-cell hyperplasia, ECL-D - ECL-cell dysplasia.

	Stage 1				Stage 2		
	Week 0	Week 6	Week 12	Week 24	Week 0	Week 24	Week 52
P1	NET	ECL-M	ECL-M	ECL-M	NET	ECL-M	ECL-M
P2	NET	NET	NET	ECL-M	ECL-D	ECL-M	ECL-M
P3	ECL-M	ECL-M	ECL-M	ECL-M	ECL-M	ECL-M	ECL-L
P4	NET	ECL-M	ECL-M	NET	ECL-M	ECL-M	ECL-M
P5	NET	NET	NET	NET	NET	ECL-M	NET
P6	NET	NET	ECL-M	NET	NET	NET	NET
P7	NET	NET	NET	NET	NET	NET	NET
P8	NET	NET	NET	ECL-M	n/a	ECL-M	ECL-M

6.3.4 Plasma chromogranin A and fasting serum gastrin concentrations

6.3.4.1 Stage 1

CgA and gastrin concentrations at baseline, 3, 6, 9 and 12 weeks and at follow-up at 24 weeks are shown in Figure 6-6 and Figure 6-7 respectively.

After 3 weeks of netazepide treatment, plasma CgA concentration had decreased in all subjects (Figure 6-6); mean decrease relative to baseline was 70% ($p=0.001$ two-tailed Mann-Whitney test). The response was sustained throughout treatment; mean decrease at 12 weeks relative to baseline was 69% ($p=0.002$ two-tailed Mann-Whitney test). Patient 5 appeared to respond less favourably and her diary card entries and capsule counts showed erratic treatment compliance. At follow-up, 12 weeks after treatment cessation, plasma CgA was elevated again in all patients (mean = 82% of baseline). In two patients (patients 1 and 2) plasma CgA concentration was higher than at baseline but for the remaining 6 patients, it was lower. The difference between baseline and follow-up (week 24) concentrations was not significant.

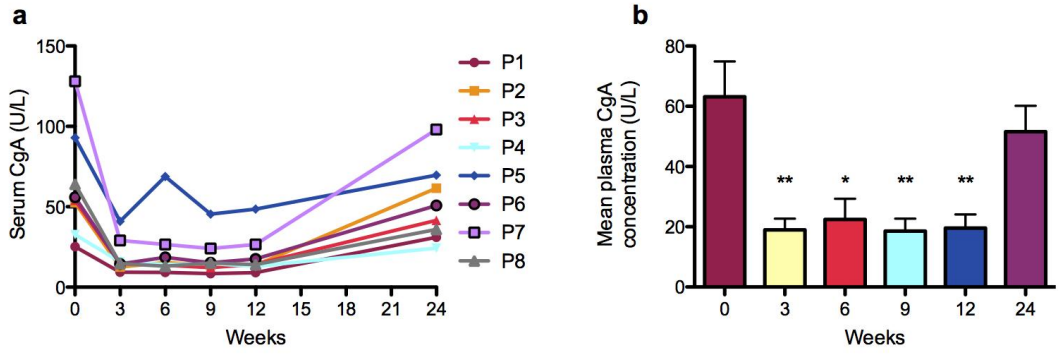

Figure 6-6 a) Individual and b) mean fasting plasma chromogranin A (U/L) concentration at baseline, after 3, 6, 9 and 12 weeks' netazepide treatment, and at follow-up at 24 weeks, 12 weeks after end of treatment.

All patients had a high serum gastrin concentrations at baseline (Table 6-2); mean (range) was 866 pM (490–1010 pM) by RIA (Figure 6-7a), and 555 pM (331–953 pM) by ELISA (Figure 6-7b). There were no significant changes during treatment.

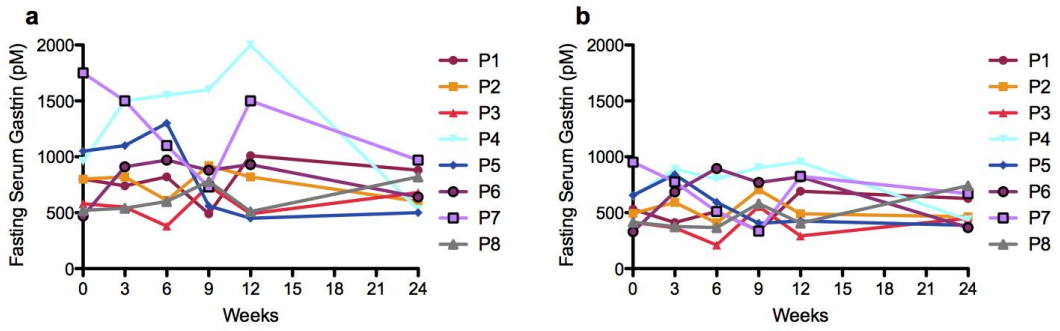

Figure 6-7 Fasting serum gastrin (pmol/L) concentrations measured by RIA (a) and ELISA (b) at baseline, after 3, 6, 9 and 12 weeks' netazepide treatment, and at follow-up at 24 weeks, 12 weeks after end of treatment.

6.3.4.2 Stage 2

CgA and gastrin concentrations at the start of stage 2 (0 weeks) and after 12, 24, 36 and 52 weeks of treatment with netazepide are shown in Figure 6-8 and Figure 6-9 respectively.

In keeping with our observations from stage 1, plasma CgA concentrations diminished significantly during treatment with netazepide. After 12 weeks of

treatment in stage 2, the mean decrease in plasma CgA was 64%. There appeared to be a modest attenuation of this effect as the course of treatment proceeded. The mean decrease in plasma CgA concentration relative to week 0 at 12, 24, 36 and 52 weeks was 64%, 64%, 58% and 54% respectively (p=0.019, 0.011, 0.047, and 0.070 respectively, two-tailed Mann-Whitney tests). This stage of the study did not include a formal follow-up study visit to determine plasma CgA concentrations after cessation of prolonged netazepide treatment.

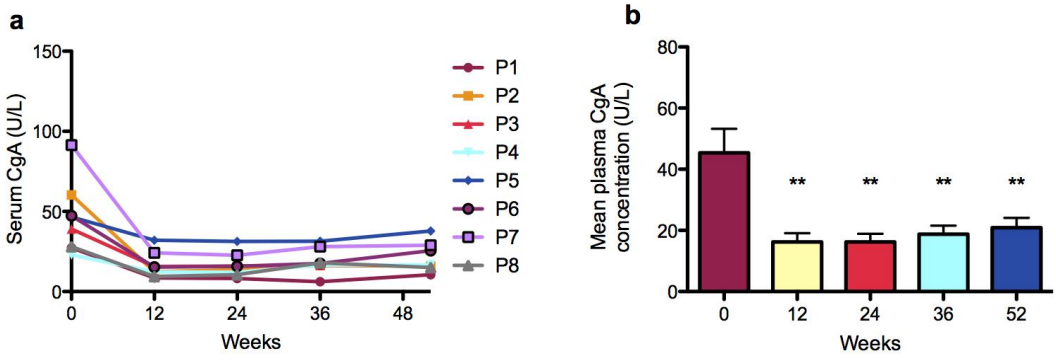

Figure 6-8 a) Individual and b) mean fasting plasma chromogranin A (U/L) concentration before restarting netazepide and after 12, 24, 36 and 52 weeks' of treatment.

Given the results obtained in the first stage, we did not anticipate a significant change in fasting serum gastrin in these patients during treatment with netazepide. Therefore, for convenience, serum gastrin concentration was determined by ELISA alone. There was no significant change in fasting serum gastrin concentration during netazepide treatment in the second stage of the study.

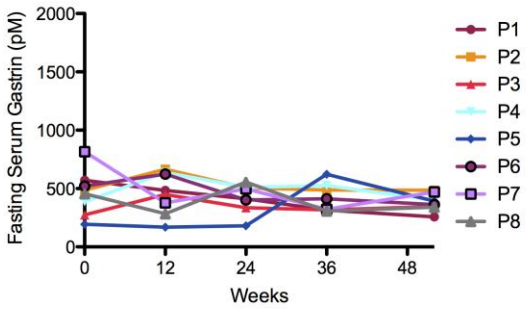

Figure 6-9 Fasting serum gastrin (pmol/L) concentrations measured by ELISA before restarting netazepide and after 12, 24, 36 and 52 weeks' of treatment.

6.3.5 Gastric mucosal biomarkers

6.3.5.1 Stage 1

Real-time PCR mRNA abundances of the ECL-cell constituents CgA and HDC, normalised for the housekeeper gene GAPDH, decreased relative to baseline in all patients during netazepide treatment, and increased again after treatment cessation. Mean real-time PCR abundance of CgA mRNA relative to baseline was 31% at 6 weeks (p=0.012) and 35% at 12 weeks (p=0.012). At 24 weeks, it was 138% of baseline (p=0.779, Wilcoxon signed ranks tests). Mean real-time PCR abundance of HDC mRNA relative to baseline was 38% at 6 weeks (p=0.012) and 59% at 12 weeks (p=0.050). At follow-up, it was 179% relative to baseline (p=0.674, Wilcoxon signed ranks tests). Mean real-time PCR abundance of MMP-7 mRNA relative to baseline was 82% at 6 weeks (p=0.161) and 56% at 12 weeks (p=0.017). At follow-up, it was 116% relative to baseline p=0.779, Wilcoxon signed ranks tests). The real-time PCR abundances of PAI-1 and PAI-2 mRNA did not change significantly. The real-time PCR abundance data from stage 1 are illustrated in Figure 6-10.

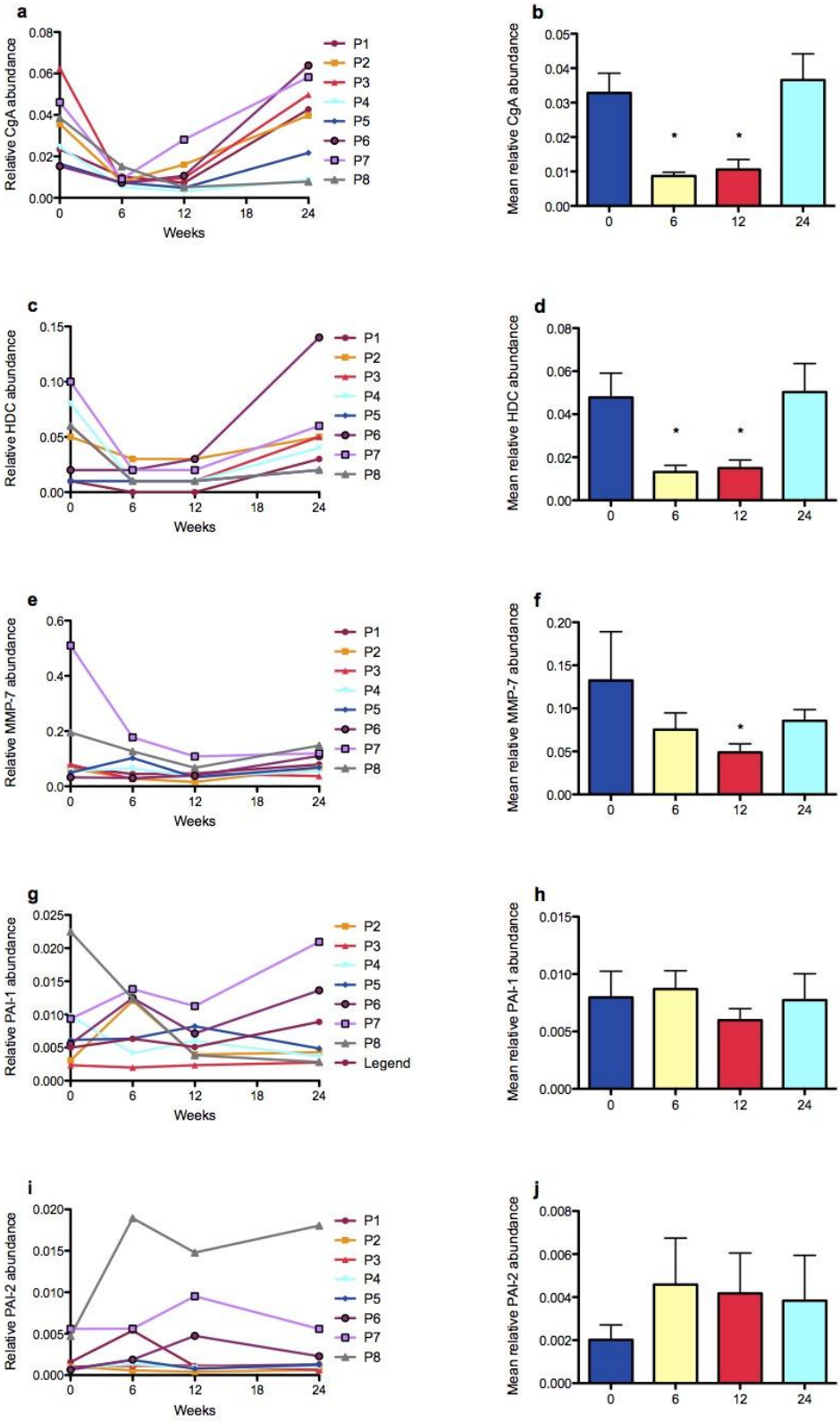

Figure 6-10 Individual and mean relative abundance of a,b) CgA; c,d) HDC; e,f) MMP-7; g,h) PAI-1 and i,j) PAI-2 at baseline, after 6 and 12 weeks' netazepide treatment, and at follow-up at 24 weeks, 12 weeks after end of treatment

6.3.5.2 Stage 2

Based on our observations in stage 1, we excluded PAI-1 and PAI-2 from the mucosal biomarker analyses in the second stage of this study. We therefore proceeded to analyse the mucosal abundance of CgA, HDC and MMP-7 at the specified treatment intervals. In this series, we also incorporated samples obtained from the baseline endoscopy (prior to starting netazepide treatment in stage 1).

In the interval between completion of stage 1 and restarting netazepide, we observed a small increase in the relative mucosal abundance of CgA and small decreases in the relative mucosal abundances of HDC and MMP-7 (Figure 6-11). None were statistically significant.

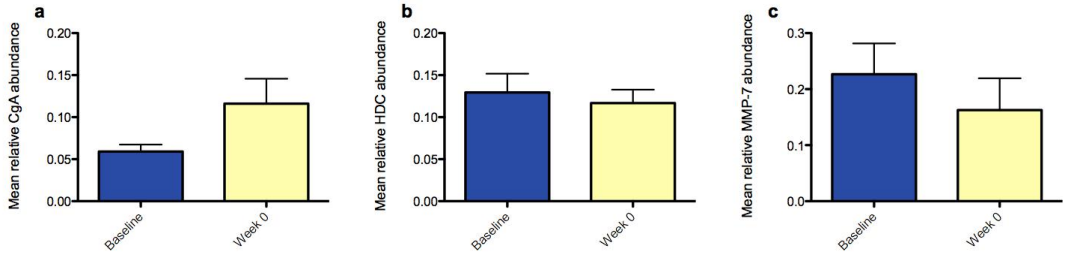

Figure 6-11 Mean relative abundance of a) CgA b) HDC and c) MMP-7 prior to netazepide treatment in stage 1 (baseline) and prior to treatment in stage 2 (week 0).

In keeping with the observations made during stage 1, the relative mucosal mRNA abundances of both CgA and HDC decreased in all but one patient following treatment with netazepide. Mucosal MMP-7 mRNA abundance did not change significantly during this phase of the study. These data are summarised in Figure 6-12.

7 of the patients showed a decrease in gastric mucosal CgA mRNA abundance after 24 weeks of treatment, which was sustained after 52 weeks. Patient 6 exhibited a small increase in the relative mRNA abundance of CgA at 24 weeks but the value at 52 weeks was lower than the corresponding measurement at both week 0 and week

24. After 24 and 52 weeks of treatment, the mean relative mucosal abundances of CgA mRNA were 17% (p=0.017) and 10% (p=0.012, Wilcoxon signed rank tests) of the abundance recorded prior to restarting netazepide (week 0).

All patients exhibited diminished relative abundance of HDC mRNA after 24 weeks and 52 weeks of netazepide treatment. The mean relative mRNA abundances after 24 and 52 weeks of treatment were 21% (p=0.012) and 28% respectively (p=0.012, Wilcoxon signed rank tests).

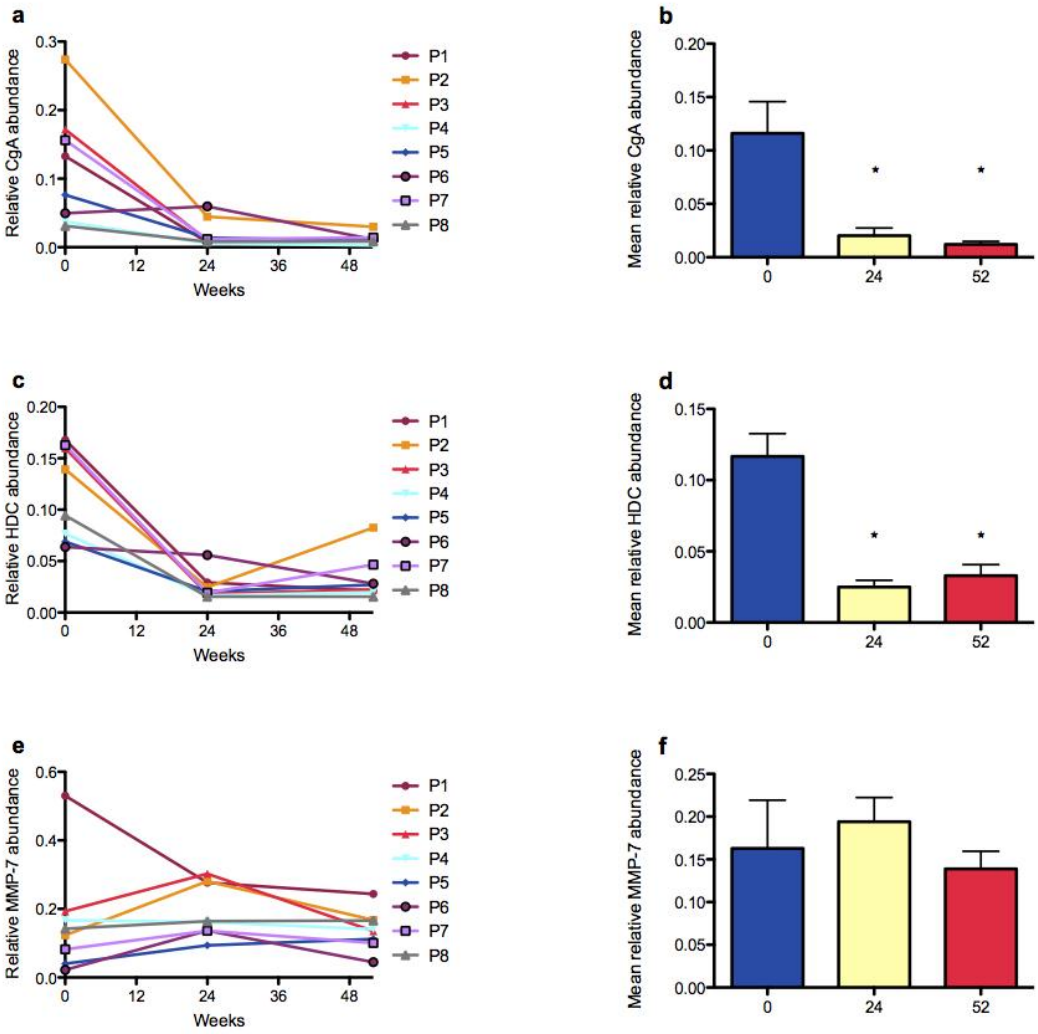

Figure 6-12 Individual and mean relative mRNA abundance of a,b) CgA; c,d) HDC and e,f) MMP-7 prior to restarting netazepide and after 24 and 52 weeks' netazepide treatment

6.3.6 Pharmacokinetics

Plasma netazepide trough and peak concentrations did not change significantly during treatment in either stage of the present study (data not shown).

6.3.7 Safety, tolerability and compliance

There were no adverse events that could be reasonably attributed to netazepide, and there were no clinically relevant changes in safety assessments. There was no evidence of a drug-drug interaction in those patients taking concomitant medication.

6.3.8 Discussion

In the present study, we report the first clinical trial of netazepide in two stages. In both stages, netazepide was safe, well tolerated and appeared efficacious.

The first outcome measures we described were the macroscopic changes reported at upper GI endoscopy. We observed a significant reduction in both the size of the largest gastric tumour and in overall tumour number. We strove to limit the subjectivity of these observations by having a single endoscopist and assistant perform all study procedures and conducting these according to a strict routine. During each procedure, standard views of the gastric mucosa were photographed and compared in real-time to images recorded at the preceding procedures. Estimation of lesion size at fibreoptic video endoscopy is notoriously unreliable, but we ensured that we measured largest tumour dimensions in a standardised fashion (by comparing their diameter to the open jaws of adjacent biopsy forceps). We recognise the limitations of recording endoscopic findings as described in this study. Firstly, estimation of tumour size and number are inherently subjective and open to observer bias(321). Secondly, we acknowledge that the effect on tumour size of taking biopsies at each procedure may have been a significant contributor to the observed decrease in dominant tumour size.

The second outcome measure reported above is the described effect of netazepide treatment on circulating levels of CgA. This ECL-cell product is a well-recognised biomarker of ECL-cell mass and activity(322). It has been suggested that chronic atrophic gastritis and hypergastrinaemia result in an increase in circulating CgA concentration with or without gastric neuroendocrine tumour development. In our patients, the circulating CgA can be attributed both to hyperstimulated ECL-cells in non-dysplastic gastric mucosa and to the ECL-cell tumours themselves. Netazepide acts to antagonise the effects of gastrin on ECL-cells irrespective of their location and the decrease in plasma CgA concentration probably reflects the effect of the drug on ECL-cells located both in non-dysplastic mucosa and in NETs. In the first stage of this study, we repeated our measurement of plasma CgA concentration 12 week after completing the initial 12-week treatment period. By this time, mean CgA concentration had risen, though not quite to pre-treatment levels. The difference between baseline and follow-up levels was not statistically significant though the finding that macroscopic features (tumour size and number) remained diminished would suggest that the follow-up period of 12 weeks was insufficient for the ECL-cell population to 'recover'.

Arguably the most compelling evidence for the efficacy of netazepide in this study is the effect on mucosal biomarkers of ECL-cell activity. In both stages, we reported significant and sustained decreases in the mRNA abundances of the ECL-cell products CgA and HDC in gastric corpus mucosa. In the first stage, we also observed a decrease in MMP-7 mRNA abundance, but this effect was not seen in stage 2.

Finally, plasma concentrations of netazepide in CAG patients were similar to those in treated healthy subjects(288,323). Such concentrations cause substantial antagonism of gastrin/CCK2R-mediated responses in healthy volunteers. Some oral drugs require low gastric pH to facilitate absorption. Netazepide appears to be well absorbed in the hypochlorhydric stomachs of patients with CAG.

Although serum gastrin concentrations by ELISA were lower than by RIA, both results followed the same pattern. RIA and ELISA are known to give different results for

serum gastrin(324,325). RIA probably measures all active forms of gastrin, whereas that is less certain for ELISA. Netazepide did not affect serum gastrin concentrations in CAG patients, whereas it does in rodents and healthy subjects due to suppression of gastric acid secretion. The absence of a significant effect of netazepide on serum gastrin concentration in CAG patients confirms the diagnosis of achlorhydria. It also emphasises that the effect of netazepide on type 1 gastric NETs is exerted indirectly via suppression of serum gastrin.

In stage 1, twelve-weeks of netazepide did not induce complete tumour regression in any of the 8 subjects, though all but one had smaller and fewer tumours than before treatment. In a series of similar patients treated by antrectomy (to abrogate gastrin production), tumour regression was seen to occur as late as twelve months following surgery(269). The extended dosing employed in stage 2 matched this and in one subject (patient 1) we did report complete and sustained tumour regression after 24 weeks of treatment. A second patient also appeared to have demonstrated complete tumour regression at 24 weeks but we subsequently reported a single, diminutive tumour (1mm in diameter) at the 52-week examination. In retrospect, this tumour was probably present but not identified at the preceding endoscopy. In stage 2 of the study, all but one patient showed a decrease in the number and size of gastric tumours. It must also be remembered that the macroscopic gastric tumours are not necessarily related to neuroendocrine tumour disease and that type 1 gastric NETs might coexist in the same individual with polyps/tumours of other aetiologies such as fundic cystic glandular polyps.

There were limitations in the study design; it was open-label, lacked controls and involved only a small number of patients, and there was a possibility of observer bias by the endoscopist. We decided upon an open, uncontrolled study design for several reasons. First, we took measures to minimise observer bias by the endoscopist. Second, plasma CgA and real-time PCR mRNA abundances are valid outcome measures. Third, type 1 gastric NETs are rare, (258) and we wanted to offer all patients the possibility of active treatment. Fourth, it was the first netazepide study in patients, and a 'proof-of-principle' study. Overall, the findings –

clinical and laboratory – have face validity and provide the first evidence of efficacy by a gastrin/CCK2 receptor antagonist in the treatment of type 1 gastric NETs. That conclusion is strengthened by a parallel study in patients with multiple type 1 gastric NETs, using a similar protocol, which also showed that netazepide reduced tumour number and size and normalises serum CgA(326).

7 Discussion

7.1 Biomarkers of gastric mucosal preneoplasia following infection with Helicobacter pylori

The aims of this study were to investigate the roles played by various genes and proteins during human gastric carcinogenesis following infection with *H. pylori*. In so doing we hoped to identify novel mucosal biomarkers of preneoplastic mucosal lesions in the stomach, which in future might offer non-invasive means of identifying individuals who have an increased risk of developing gastric cancer.

To do this, we measured the mRNA abundance of several putative biomarkers in gastric mucosal biopsies obtained from 1400 study subjects, for whom we had standardised histopathology reports and data from serological analyses including gastrin and pepsinogen concentrations as well as *H. pylori* antibody and CagA status.

Having identified matrix metalloproteinase-7 as the most promising of these putative biomarkers, we sought to determine the effects on its gastric mucosal expression of genetic polymorphisms, and the effect of these polymorphisms on the likelihood of developing gastric preneoplasia.

7.1.1 Gastric preneoplasia

Our study cohort of 1400 subjects included 573 (43.3%) with *H. pylori* seropositivity, 51.3% of whom also had evidence of current infection with this bacterium. Of the 1381 study subjects for whom we obtained gastric histology data, 338 were reported to have histological evidence of gastric preneoplasia. Interestingly, only 14 of these were reported as having preneoplastic lesions macroscopically at upper GI endoscopy, underlining the poor performance of routine endoscopy in detecting gastric preneoplasia as reported in the literature(197).

The central hypothesis – that *H. pylori* mediated inflammation is largely responsible for initiation of the cascade of preneoplastic changes – was substantiated by this study. *H. pylori* infection was significantly more prevalent in the individuals who had preneoplasia than in those without (OR 3.8) and the same was true for more virulent strains of this organism as evidenced by CagA seropositivity (OR 2.1).

There are several pieces of evidence to suggest that the antral hormone gastrin plays an important role in gastric carcinogenesis. CCK2R expression increases with the progression of CAG(327) and is found in a large proportion of gastric adenocarcinomas(328). Gastrin induces apoptosis in cancer cell lines(329) and potentiates the expression of mucosal proteases implicated in carcinogenesis including the MMPs, TIMPs and members of the uPA system(33,56). Of course, preneoplasia of the gastric corpus also entails the loss of native functional glandular units and the resulting hypochlorhydria leads to the unregulated stimulation of gastrin secretion.

Our study findings were in line with this hypothesis in that we found fasting serum gastrin concentrations to be significantly higher in individuals with gastric mucosal preneoplasia than in those without. Unsurprisingly, we demonstrated that this effect was greater for those subjects whose gastric preneoplasia was confined to the corpus than those with pangastric disease, which in turn was greater than those with antral-only disease.

The best-studied non-invasive biomarker of gastric preneoplasia and cancer to date is serum pepsinogen concentration. Analyses of circulating levels of PG1 or PG1/2 ratio are used frequently in some high-risk populations but comparatively few studies have examined their use in lower-risk areas(197,330,331). Several authors have reported improved diagnostic performance when serum pepsinogen analyses are combined either with *H. pylori* serology, serum gastrin concentration or both. A commercially available kit – GastroPanel – is available based on ELISAs for serum PG1 and PG2, *H. pylori* IgG antibody and serum gastrin-17. Its use in clinical practice

is yet to be adequately validated and indeed, a recent study in Spain suggested that its sensitivity for gastric disease was inadequate for clinical use(241).

In the present study, we found that serum PG2 concentration and PG1/2 ratio were significantly different in individuals with gastric preneoplasia compared to those with normal stomach histology. Again, this difference was unsurprisingly greater when comparing corpus-only preneoplasia to normal histology groups. As a diagnostic test for gastric preneoplasia, PG1/2 was better than PG2 alone, with areas under the ROC curve of 0.8 and 0.75 respectively. Combining serum PG1/2 ratio with *H. pylori* serology and serum gastric concentration gave a diagnostic test which had good sensitivity (90%) and negative predictive value (96%) for gastric preneoplasia in symptomatic individuals. Whilst this would appear to endorse the approach suggested by the manufacturers of GastroPanel, it is important to highlight that whilst serum pepsinogen and *H. pylori* serology assays were performed by ELISA in this study, we used radioimmunoassay to determine serum gastrin concentrations. ELISA is a comparatively poor technique for this purpose but is readily available and inexpensive(324,325). Whilst RIA is superior, it is no longer widely available. It may be that the future clinical use of biomarker screening panels for the noninvasive detection and risk stratification of gastric preneoplasia will be dependent on the development of more reliable gastrin assays.

7.1.2 MMP-7 expression in gastric preneoplasia

Helicobacter pylori, its constituents and particularly the more virulent strains of the bacterium stimulate the expression of MMP-7, which is also overexpressed in gastric cancer. Its expression in cancer tissue (and circulating levels) seem to correlate with stage of disease, degree of invasiveness and metastatic behaviour(53,332).

We found that gastric mucosal expression of MMP-7 was significantly increased in preneoplastic gastric disease (CAG and IM) and to a lesser degree, in gastric inflammation associated with *H. pylori* infection. The effect was greatest for corpus-

only preneoplasia, which is probably the phenotype that is associated with the highest risk for cancer development. We observed an increase in MMP-7 expression with increasing 'severity' of preneoplasia i.e. expression was greater for subjects with IM and atrophy than in subjects with gastric atrophy alone though this difference was not statistically significant. Interestingly, where IM was reported in the absence of atrophy, the increase in MMP-7 expression over controls was less marked (and did not reach statistical significance). This suggests that the IM in this group might include different metaplastic variants which are not associated with atrophy as a precursor and that these patients may have an attenuated risk for cancer development.

We also demonstrated that mucosal MMP-7 expression was significantly greater in preneoplasia and *H. pylori* associated inflammation than in the 'benign' gastric mucosal conditions of reactive/chemical gastritis (gastritis C) and foveolar hyperplasia. This is an important feature for a putative biomarker. Interestingly, we found that the magnitude of increase in MMP-7 expression for those subjects who were infected with the CagA serovar was not significantly greater than those who were infected with CagA negative strains of *H. pylori*.

MMP-7 expression has been shown to be driven in part by gastrin(126) and this phenomenon may play a part in regulating epithelial cell migration and invasion(333,334). In our study, gastric MMP-7 expression was 2.5 times greater in hypergastrinaemic subjects than in controls. Moreover, in subjects with 'severe' hypergastrinaemia (>100pM), the increase was almost 4-fold. When we controlled for the effect of preneoplasia by comparing MMP-7 mRNA abundance in subjects with normal gastric histology, we found that the severe hypergastrinaemia group exhibited a 7.4-fold change in MMP-7 expression. This suggests that serum gastrin concentration exerts an independent effect on gastric mucosal MMP-7 expression.

We concluded our investigation of MMP-7 as a potential biomarker of gastric preneoplasia by studying the circulating concentrations of the protein. The assay selected was an 'off-the-shelf', commercially available ELISA.

Serum concentration of MMP-7 was significantly greater in the preneoplasia group than in subjects with normal gastric histology, but the magnitude of this change was much smaller than that observed for mucosal MMP-7 expression. When we examined the differences between normal controls and preneoplasia in different topographical sites, only corpus-limited preneoplasia was associated with a significant increase in serum MMP-7 concentration. In the present study, serum MMP-7 concentration was also significantly higher in individuals with gastric preneoplasia when compared with those exhibiting 'benign' gastric disease.

There are two major applications of noninvasive biomarkers of gastric preneoplasia and the clinical relevance of such 'tests' is dependent on the burden of disease in the population in question. In high-risk populations, population-based screening is already employed for the detection of early gastric cancer. In these populations, this is predominantly conducted endoscopically or radiologically, but these systems are expensive and inconvenient, and their sensitivity questionable(197). In the case of gastric preneoplasia, serological testing may indeed prove more sensitive than the incumbent methods, which would make for a simpler, cheaper means of screening large numbers of people.

Our study was not conducted with cancer diagnosis in mind. We identified only 12 cases of gastric adenocarcinoma, which was insufficient to evaluate the performance of MMP-7 as a biomarker. This has been studied previously for other epithelial cancers (colorectal and pancreatic) and in small series for gastric cancer. Blanco-Calvo et al. examined the diagnostic performance of serum concentrations of MMP-7 and GDF15 in 52 cases of gastric cancer (and 23 controls). The area under the ROC curve for MMP-7 as a diagnostic test for gastric cancer was 0.86 with a reported sensitivity and specificity of 79% and 87% respectively. Yeh et al. published their findings from a series of 201 patients including 55 gastric cancers. They reported serum concentrations of MMP-7 (along with MMPs -3 and -9) and found an optimal sensitivity and specificity of the former for the diagnosis of gastric cancer of 66% and 67% respectively. In that study, the authors concluded that increasing serum

concentrations of both MMP-3 and -7 were associated with diminishing survival rates from gastric cancer. Before non-invasive methods for the diagnosis and risk-stratification of gastric preneoplasia are accepted in these populations, they would need to be shown to be at least as effective for the diagnosis of early cancer as the current modalities.

Less attention has been paid to the diagnosis of preneoplastic lesions, as there has previously been limited evidence to guide subsequent surveillance and management. A landmark in this field has been the development of the OLGA/OLGIM scoring systems(227,229) – themselves developments of the Sydney scoring system(199). Using a standardised sampling protocol, gastric mucosal biopsies can be used, not only to diagnose the presence if preneoplastic disease but also to estimate the risk of future cancer development. In low-risk gastric cancer populations such as those found in Western Europe and the United States, these systems could be used to accurately identify those patients who have a substantially increased risk of gastric cancer development and in whom it might be appropriate to offer a surveillance regimen. It is impractical however to implement endoscopic, population-based screening in these populations. In these settings, symptomatic individuals or those at higher risk (such as those with family histories etc.) could be screened using a non-invasive biomarker panel. If the results suggested a diagnosis of gastric preneoplasia, then upper GI endoscopy with Sydney-protocol biopsies could be undertaken and the patient's risk of cancer development determined using the OLGA/OLGIM systems. Based on the risk-stratification determined from gastric 'mapping' biopsies, individuals could then be enrolled into an endoscopic surveillance system if appropriate. The available data suggest that the cancer risk in patients with gastric preneoplasia is similar to that reported in Barrett's oesophagus and the benefit of making earlier diagnoses in these cancers is analogous as both tumours usually present late and are associated with very poor survival. It should be noted however, that in these low-risk populations, individuals for whom upper GI endoscopy is currently indicated (older patients, 'alarm' symptoms, iron-deficiency etc.) would not be safely spared such examination by negative serological biomarker testing alone.

7.1.3 Genetic polymorphisms in *MMP-7*

Single nucleotide polymorphisms in *MMP-7* have been associated with an increased risk of developing digestive (including oesophageal adenocarcinoma, oesophageal squamous-cell carcinoma, colorectal carcinoma and gastric carcinoma), gynaecological and breast cancers(313,335). The best studied of these is rs11568818 (usually referred to in the literature as '*MMP-7* -181A>G'). Wu et al. conducted a meta-analysis of 27 case-control studies(314) and concluded that the G allele was associated with a significantly increased risk of cancer in Asian populations but not in Europeans. Conversely, for gastric cancer, Fang et al. reported an association study and meta-analysis which suggested an increased risk of gastric cancer for the A allele of this SNP(336). European studies are however comparatively scarce. Kubben et al. found that the G allele at rs11568818 was found more frequently in gastric cancer cases than in controls and that this was associated with poorer survival(337). The role of MMP-7 SNPs in the development of preneoplastic lesions of the stomach has not been studied extensively. Hellmig et al. reported that carriage of the G allele of rs11568818 was associated with gastric ulcer development(338), while Achyut et al. found that the same allele was associated with an increased risk of lymphoid follicle formation in dyspeptic patients with *H. pylori* infection(339).

The present body of literature therefore asserts that although there appears to be an association between SNPs of *MMP-7* and gastric cancer, the supporting evidence is sometimes conflicting and this association has not been extensively examined in European populations. Additionally, few studies have examined the influence of MMP-7 polymorphisms upon the development of gastric preneoplasia.

In the present study, we demonstrated that the nine SNPs studied were distributed in Hardy-Weinberg equilibrium and that for SNP (rs17352054), the minor allele homozygote was significantly more frequent in cases of gastric preneoplasia than in normal controls. We demonstrated that carriage of the minor allele for SNP rs11225297 was assoicated with gastric preneoplasia amongst individuals with *H. pylori* seropositivity. We also studied the influence of these polymorphisms on

mucosal expression of MMP-7. Six SNPs were associated with a significant increase in mucosal MMP-7 mRNA abundance in their minor allele homozygote form, heterozygote form or in both. We have also shown that in our European cohort, the SNPs commonly associated with gastric cancer in the literature were not associated with the presence of preneoplasia. The identification of rs17352054 and rs11225297 as a polymorphism associated with gastric preneoplasia is novel and merits further study, not least in cases of gastric cancer.

7.1.4 Strengths and limitations of the study

The present study design proffered several advantages. First, the large number of study subjects provided us not only with a substantial number of individuals in whom we identified *H. pylori* infection and gastric preneoplasia but also a sufficiently large group of individuals with normal gastric mucosal histology that we were able to generate statistically powerful control groups. This allowed us to control for variables such as PPI use, which might not have been possible in a smaller study population. Secondly, we benefitted from the input of a single, expert GI pathologist, whose reporting of all pathology specimens obviated the risk of interobserver variance, thus yielding highly consistent pathology reports.

There were however some limitations to the present study. In a disease process such as gastric preneoplasia, there is considerable field variation in histological changes across the mucosal surface. We were limited by ethical and safety considerations in the number of biopsies we were able to take. Although we were able to obtain sufficient biopsies to satisfy the minimum criteria for Sydney scoring, additional mucosal biopsies might have increased our pickup rate of gastric preneoplasia and better defined the topographical phenotypes being studied. We have asserted that the biomarkers of gastric preneoplasia described in this thesis can be used to estimate the ‘stage’ or ‘severity’ of disease. It could be argued that the finding of intestinal metaplasia in histology series from a patient with gastric atrophy is representative of more severe atrophy than of a more advanced lesion *per se*. The

conventional view however is that IM itself confers an increased risk of malignant remodelling (as per Correa's cascade of gastric oncogenesis).

Secondly, we did not set out to include gastric cancer in this study. Those small number of cases included were captured 'fortuitously' and are too small to inform meaningful conclusions about biomarker performance. Before moving forward with the development of noninvasive biomarkers for gastric preneoplasia, the performance of these biomarkers should be validated for the diagnosis of gastric cancer in the same study setting.

It is also worthy of note that we omitted testing for serological biomarkers of pernicious anaemia/autoimmune atrophic gastritis in this study. We recognise that this disease would account for a small proportion of the cases we included in our 'preneoplasia' group. In European populations, the prevalence of PA is estimated to be less than 5% and the sensitivity of antibody serology for the syndrome to be less than 80%(340,341). Assuming that these estimates are broadly applicable to our study population, the number of cases of pernicious anaemia/autoimmune atrophic gastritis included in our 'preneoplasia' group is likely to be small and unlikely to substantially affect our results and conclusions. On this basis and given resource constraints, we elected not to perform anti-gastric parietal cell and anti-intrinsic factor antibody serology in this study.

We have also not yet described the effect on MMP-7 protein abundance in this study, although work is currently underway to determine this using immunohistochemistry on slides that have been cut from the original histopathology blocks.

7.1.5 Conclusions

This study has provided evidence to support the hypothesis that the gastric epithelial protease MMP-7 is overexpressed in the presence of gastric preneoplasia. Based on previous experimental work, it seems likely that this response to *H. pylori* induced chronic inflammation is one of the factors responsible for driving mucosal remodelling towards cancer development. We have shown that MMP-7 has the potential for clinical use in combination with other established methods as a noninvasive biomarker of gastric mucosal preneoplasia. Development of MMP-7 as a biomarker will however require a number of additional steps. Its use as a biomarker will depend on its ability to reliably detect (or reliably exclude) gastric cancer. An accurate assay for circulating MMP-7 in this context will also be required and our group is presently working on such an assay. Finally, biomarker performance will require validation in populations of different ethnicity.

We have highlighted the role of genetic polymorphisms in MMP-7 in affecting gastric mucosal expression and in the case of SNPs rs17352054 and rs11225297, identified SNP genotypes which might predispose individuals from this European population to gastric preneoplasia development.

These data might contribute towards a means of determining an individual's risk of developing gastric cancer either in place of or in conjunction with histopathological sampling and scoring systems such as OLGA/OLGIM. This is likely to be most relevant in high-risk populations.

7.2 The use of netazepide for the treatment of type 1 gastric neuroendocrine tumours

The development of CCK2R ligands for therapeutic purposes outside the CNS has been grounded in the observations that amidated gastrins are major hormonal regulators of gastric acid secretion and that they exert trophic effects on a range of

tissues including several neoplasms. There has been recent work describing immunotherapy for gastrin using G17DT in pancreatic cancer(342). Our own work on netazepide has been published, as has that of a group who performed a parallel study(326,343).

In the present study, we demonstrated the efficacy of the novel gastrin/CCK2R antagonist netazepide for the treatment of type 1 gastric NETs. The drug was safe and well tolerated and the 8 patients in the study all exhibited a decrease in the size and/or number of gastric tumours, along with a decrease in both mucosal and circulating biomarkers of ECL-cell population and activity. Randomised, placebo-controlled trials are now required to validate this treatment. Type 2 gastric NETs are also dependent on gastrin for growth and proliferation. A trial of the use of netazepide for this indication is ongoing.

There are several other potential clinical uses for gastrin/CCK2R antagonists. Histamine receptor antagonists (H2RAs) and proton-pump inhibitors (PPIs) are effective treatments for acid-secretory disorders and exhibit low toxicity. Nevertheless there continues to be interest in the development of CCK2R antagonists as anti-secretory agents for two reasons. First, suppression of acid secretion reduces the feedback-inhibition of gastrin release, resulting in hypergastrinaemia which can lead to a rebound in acid secretion on cessation of treatment; the “acid-rebound” effect has been shown to persist for up to 8 weeks after discontinuing PPI use, and can lead to a state of physiological dependence by patients using anti-secretory drugs. Second, in patients on long-term therapy acid suppressing therapies (e.g. for the treatment of gastro-oesophageal reflux disease, GORD), there has been speculation that the resulting hypergastrinaemia might increase the risk of developing gastrointestinal malignancies. Netazepide has previously been shown to be well tolerated and orally active in inhibiting acid secretion(288,323). A dose of 25 mg was superior to 150 mg ranitidine in raising 24-hour gastric pH, and the inhibition lasted longer. The data contribute to the idea that gastrin is a physiological regulator of acid secretion in man, and this paves the way for future trials involving this compound. With respect to gastrin-related risks from

PPIs, recent work in Mongolian gerbils has shown that PPI treatment increases the risk of developing *H. pylori* -induced gastric adenocarcinoma(344). However, while some patients taking PPIs may be at risk of developing ECL cell hyperplasia, there does not at present appear to be evidence of an increased incidence of gastric neuroendocrine tumours in humans(345). A recent Cochrane review of 7 randomised controlled trials examining the effect of long-term PPI use on the development of gastric preneoplasia concluded that there was no clear evidence causation or accelerated development of gastric preneoplasia(346). Moreover, no increase in gastric adenocarcinoma was found amongst PPI/H2RA users in a prospective cohort study(347), and in a recent case–control study, there was no association between PPI use and pancreatic cancer(348). These studies did not, however, stratify subjects by serum gastrin concentration, and so they leave open the question of a role for hypergastrinaemia in a subset of patients in whom CCK2R antagonists might be beneficial.

Previous clinical trials of CCK2R antagonists in gastrointestinal cancer have largely focused on the treatment of pancreatic cancer. A benzodiazepine-derived CCK2R antagonist, Z-360, which has good oral activity and high receptor affinity, was shown in a small phase Ib/IIa trial (33 patients) to result in no improvement in tumour control in advanced pancreatic cancer, but there was an encouraging trend towards improved survival when Z-360 plus chemotherapy was given compared with chemotherapy alone(349). It should be noted, however, that in a mouse model of cancer pain, Z-360 inhibited *ephrin B1* gene expression and phosphorylation of the NR2B subunit of N-methyl-D-aspartate receptor, and that the CCK1R antagonist, devazepide, had similar effects(349). Moreover, in another model of induced pain, the same group found that Z-360 had analgesic actions that were replicated by devazepide but not by netazepide(350). It seems therefore that in some circumstances Z-360 may act through CCK1R.

7.2.1 Limitations of the study

There were a number of limitations of this study. The study design was one of an open-label, uncontrolled pilot study and only eight patients were included. Though the observed effects on endoscopic characteristics and biomarkers were statistically significant, the nature of the study allows for a number of sources of bias. The inclusion of endoscopic characteristics as a means to monitor disease response was important but particularly prone to inconsistency – though the procedures were conducted by a single endoscopist, he was an investigator in the trial and thus open to observer bias. Not only this, but the endoscopic assessment of tumour size is notoriously inconsistent(321,351) and involved determining very small changes in already very small tumours. Finally on this point, the use of largest tumour size as a measure of disease response might have been affected by the requirement to biopsy the very tumours under follow-up at each study endoscopy.

7.2.2 Conclusions

We have shown that netazepide is an efficacious, safe and well-tolerated treatment for type 1 gastric NETs. Larger, randomised, placebo-controlled trials are now required to validate our findings. We have also shown that serum CgA concentration and mucosal abundance of CgA, HDC and MMP-7 mRNA are useful biomarkers of disease activity and treatment response.

It is also clear that there are other potential clinical uses of gastrin/CCK2R antagonists and this study provides proof-of-principle that netazepide inhibits gastrin stimulation of the receptor in patients in order to deliver a clinical response. Trials of its use and of other small-molecule antagonists of the CCK2 receptor are therefore warranted.

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Publications and Communications

Research publications

Kenny S, Steele I, Lyons S, Moore AR, Murugesan SV, Tiszlavicz L, et al..
The role of plasminogen activator inhibitor-1 in gastric mucosal protection.
Am J Physiol - Gastrointest Liver Physiol. 2013 May 1;304(9):G814–G822.

Moore AR, Boyce M, Steele IA, Campbell F, Varro A, Pritchard DM.
Netazepide, a Gastrin Receptor Antagonist, Normalises Tumour Biomarkers and Causes Regression of Type 1 Gastric Neuroendocrine Tumours in a Nonrandomised Trial of Patients with Chronic Atrophic Gastritis.
PLoS ONE. 2013 Oct 1;8(10)

Review articles

Dockray G, Moore A, Varro A, Pritchard D.
Gastrin Receptor Pharmacology.
Curr Gastroenterol Rep. 2012;14(6):453–9.

Moore AR, Varro A, Pritchard DM.
Zollinger-Ellison Syndrome.
Gastrointest Nursing 2012 Jun 21;10(5):44–9.

Oral abstract presentations

Moore AR, Steele IA, Boyce M, Varro A, Pritchard DM.
The gastrin/CK2 receptor antagonist netazepide (YF476) is well tolerated and causes regression of type- 1 gastric neuroendocrine tumours.
UKI NETS 10th National Conference, 2012. London, UK

Moore AR, Ball L, Boyce M, Varro A, Pritchard DM.
The novel gastrinCCK2 receptor antagonist YF476 induces clinical responses and is well tolerated in patients with type I gastric neuroendocrine tumours.
DDF, 2012. Liverpool, UK.

Moore AR, Steele IA, Murugesan S, Tiszlavicz L, Dockray G, Varro A, Pritchard DM.
A combination of fasting serum gastrin concentration, pepsinogen 1/2 ratio and Helicobacter pylori IgG antibody serotype accurately predicts gastric mucosal preneoplasia in a large European cohort.
DDW, 2015. Washington, USA – *In press*

Poster presentations

Parsons BN, Moore AR, Boyce MJ, Rainbow L, Fang Y, Varro A, Hall N, Pritchard DM
Identification of potential biomarkers of Netazepide (YF476) treatment of type I gastric neuroendocrine tumours.
UKI NETS 12th National Conference, 2012. London, UK

Moore AR, Boyce MJ, Steele I, Campbell F, Varro A, Pritchard DM.
Netazepide, a Gastrin/CCK-2 Receptor Antagonist, Causes Regression of Type 1 Gastric Neuroendocrine Tumors and Reduces Expression of Chromogranin A and Histidine Decarboxylase Genes in Chronic Atrophic Gastritis.
Gastroenterology. 2013 May 1;144(5):S–360.

Murugesan SV, Steele I, Tiszlavicz L, Farragher T, Moore AR, Pritchard DM, et al..
Determinants of Human Fasting Serum Gastrin Concentration- Interaction Between *H. pylori* Infection, Gastric Preneoplastic Pathology and Proton Pump Inhibitor Use.
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Murugesan SV, Steele I, Tiszlavicz L, Farragher T, Moore AR, Varro A, et al..

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The Effects of Host, Environmental and Bacterial Factors on Development of Gastric Preneoplastic Pathology.
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Moore AR, Parsons BN, Boyce MJ, Rainbow L, Fang Y, Varro A, Hall N, Pritchard DM
Identification of potential biomarkers of Netazepide (YF476) treatment of type I gastric neuroendocrine tumours.
DDW, 2015. Washington, USA – *In press*

Appendices

A) Novel biomarkers of gastric preneoplasia - histopathology reporting form

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H-6720 **Szeged**, Állomás u. 2. (6701 Szeged Pf. 427.)
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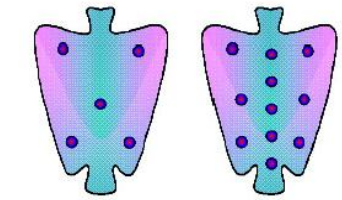
Histology study code: ……

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:……..

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

B) Novel biomarkers of preneoplasia – patient information leaflet

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

PATIENT INFORMATION

Factors Which Affect the Outcome Of Helicobacter Pylori Infection in the Stomach

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your family doctor (GP) if you wish. If there is anything that is not clear, or if you would like more information, please ask.

This is a voluntary project and, if you would prefer not to take part, your decision will be accepted without question and **will not** affect the standard of care you receive.

What is the purpose of the study?

Cancer of the stomach is the second most common cause of cancers worldwide. There are several important factors that have been identified that are associated with the development of stomach cancer. Infection of the stomach with bacteria – Helicobacter pylori - in addition to causing inflammation and ulcer of the stomach, also increases the risk of stomach cancer. However, only a small proportion of people who have this infection go on to develop stomach cancer. Therefore additional factors must be involved. Recent studies by our research group have identified several proteins in the stomach that have been shown to play a part in this process.

In this new research, we wish to study these proteins and genes that could possibly make people susceptible to the development of stomach cancer. The results could in the long term help in developing new ways to halt the progression of cancer in the stomach.

We aim to recruit about 1000 patients who have had a gastroscopy (a camera into the stomach /duodenum) for investigation of stomach problems. Patients will be identified and recruited for the study from the endoscopy unit at the Royal Liverpool University Hospital.

Why have I been chosen?

You have been chosen for the study because you are attending for a gastroscopy to investigate stomach problems.

PIF 1259/V2

Do I have to take part?

No, it is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw from the study at any time without giving a reason. This will not affect the standard of care you receive in any way.

What will happen to me if I take part?

All patients who decide to take part in this study will receive the usual clinical care. That is, you will have had a gastroscopy as part of your routine clinical care and will still remain under the care of your clinician. If you decide to take part, the following will also take place

During the camera test, routine biopsy samples will be taken to examine the lining of the stomach under a microscope. Taking a biopsy involves taking a small piece of your stomach lining (smaller than a pinhead) using a specially designed piece of equipment. All of this is done down the middle of the camera, at the time of your procedure. Taking a biopsy sample is completely painless and extremely safe, and is a routine part of any camera procedure. The samples are then processed and examined in a laboratory. As well as routine biopsy samples of your stomach being taken, we would like to take some additional biopsy samples in order to process these in our research

An additional blood sample (approximately 20 ml), will be taken to try to identify genes and their products important in the development of stomach cancer

We will also ask you a few questions about your general health and that of your family in relation to stomach disease, in the form of a questionnaire

A review of your medical casenotes will be carried out by the research doctor.

What are the possible disadvantages and risks of taking part?

There may be some minor but short-lasting discomfort from having a blood test. You won't feel anything while the biopsies are being taken. There is a very small risk of complications (such as bleeding or perforation) from having any biopsy of the stomach, but it is important to note that the biopsy will be taken by an experienced endoscopist and will be done in a unit which has extensive experience of this procedure. Taking part in the study will not affect your current treatment, nor will it affect your ability to obtain insurance for health purposes.

What are the possible benefits of taking part?

The study will not be of direct benefit to you; however, it may benefit patients in the future who develop stomach cancer.

What will happen to my biopsies and blood test?

Blood will be divided into serum and genetic samples which will be stored at the University of Liverpool.
DNA will also be extracted from the genetic sample at the University. It will used to look at genes that may be involved in causing the development of stomach cancer. We will look at many genes in order to identify any predisposing factors.

Our aim is to develop a test that predicts the development of stomach cancer, and this may allow us to prevent its development in the future. Your sample will be stored at the University of Liverpool until it is used up. The serum samples will be used to look for markers that define the development of stomach cancer. Again, the aim here is to develop tests that allow us to predict the development of cancers.

From the biopsies we will look at the expression of various genes which will tell us how the stomach responds to Helicobacter pylori infection. Some of the results obtained from these blood and biopsy tests will be linked with information that we obtain about your current and past medical history from the questionnaire and from your casenotes.

It is important to note that all blood and biopsy samples going outside the hospital, and any notes relating to it, will be identified only by a code number. All clinical details will be kept securely, either at the Royal Liverpool University Hospital or the University of Liverpool. Once the study has been completed, we will anonymise your clinical details and blood and biopsy samples, and therefore it will not be possible to trace the samples back to you. After anonymisation, it will also not be possible for you to withdraw the samples. Once anonymised, the DNA samples may be used for other research, but as this cannot be traced back to you, it will have no direct bearing on your clinical care. Approval will be sought from the Ethics Committee for any future studies.

Your blood and biopsy samples will be considered to be a gift to the University of Liverpool, which will act as custodian of all the samples obtained as part of this project. In some cases, a small amount of your sample will be provided to other researchers either in the UK or other parts of the world. However, it is important to remember that this will only be identified by a code.

In the short-term, it is unlikely that the samples will be of any commercial value to the University or the hospital. However, it is possible that they may be some commercial value in the future, although it is important to note that any commercial value is likely to be due to findings in a group of patients rather than from samples from a single patient. You will not be paid for taking part in the study, nor will you get financial benefit from future discoveries.

Will my taking part in this study be kept confidential?

As stated above, your sample will be anonymised, and therefore the genetic information obtained from it will be kept strictly confidential and not be disclosed to anyone. All information collected about you during the course of the research will also be kept strictly confidential. Any information about you, which leaves the research centres taking part, will have your name and address removed so that you cannot be recognised from it. We will inform your family doctor (GP) that you have participated in the study.

What will happen to the results of the research study?

Results from the project will be published in leading international medical journals.

Who is organising and funding the research?

This study has been designed by The University of Liverpool and the Royal Liverpool and Broadgreen University Hospital NHS Trust. It is funded by the National Institute of Health Research (NIHR).

The principal investigators are Professor A Varro and Professor D M Pritchard.

Who has reviewed the study?

The research has been reviewed by a Research Ethics Committee and has been granted approval.

Thank you for reading this information leaflet

Further information

If you need further information or are worried about any aspect of the study, please do not hesitate to contact Dr Andrew Moore, the Research Fellow running the study.

Tel: 0151 706 2000 Ext 3353

Author: Dr Andrew Moore
Date: March 2011
Review Date: January 2013

This leaflet is available in large print, audio/computer disc, Braille, and other languages on request.

C) Novel biomarkers of preneoplasia – patient data questionnaire

Trust R&D No:3592

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

Patient identification data

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☐
Haematemsis/Melaena:	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☐

Ischaemic 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☐
Ex-smoker: Y☐ N☐ when stopped: Cpd: Years:

1

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: