Biomarkers of the effect of gastrin on Barrett's oesophagus

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

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Disclaimer

This thesis is the result of my own work. The material contained herein has not been presented, nor is currently being presented, either wholly or in part for consideration of any other degree or qualification. The research was performed in the Department of Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool. All other parties involved in the research presented here, and the nature of their contribution, are listed in the 'Acknowledgements' section of this thesis.

Publications

The following publications contain data either presented in this thesis or obtained using techniques optimised during the course of this research:

Lloyd K, Parsons BN, Burkitt MD, Moore AR, Boyce M, Duckworth CA, Rainbow L, Fang Y, Oxvig C, Dodd S, Varro A, Hall N, Pritchard DM - Netazepide inhibits pregnancy-associated plasma protein-A2 expression in type-1 gastric neuroendocrine tumors. Gastroenterology. In press.

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Varga A, Kumar JD, Simpson AWM, Dodd S, Hegyi P, Dockray G, Varro A - Cell cycle dependent expression of the CCK2 receptor by gastrointestinal myofibroblasts: putative role in determining cell migration. Physiol Rep 2017;5. e13394.

Shawe-Taylor M, Kumar JD, Holden W, Dodd S, Varga A, Giger O, Varro A, Dockray G - Glucagon-like petide-2 acts on colon cancer myofibroblasts to stimulate proliferation, migration and invasion of both myofibroblasts and cancer cells via the IGF pathway. Peptides 2017;91:49-57.

Calvete O, Varro A, Pritchard DM, Barroso A, Oteo M, Morcillo MA, Vargiu P, Dodd S, Garcia M, Reyes J, Ortega S, Benitez J - A knockin mouse model for human ATP4aR703C mutation identified in familial gastric neuroendocrine tumors recapitulates the premalignant condition of the human disease and suggests new therapeutic strategies. Dis Model Mech 2016;9:975-84.

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Abstract

The incidence of adenocarcinoma of the oesophagus (ACO) is rapidly rising, with the UK white male population currently experiencing the highest incidence worldwide. ACO arises on a background of Barrett's oesophagus (BO), a condition characterised by metastatic remodelling of the lower oesophagus in response to reflux of gastric contents. Previous work within our laboratory has shown that Barrett's epithelium expresses the gastrin activated CCK2 receptor, that gastrin stimulates proliferation of BO cells, and that Barrett's patients with the highest circulating gastrin concentrations are more likely to exhibit dysplasia.

Patients diagnosed with BO are often prescribed proton pump inhibitors (PPIs) to reduce damage when acid reflux occurs. A common consequence of PPI usage is a significant increase in circulating gastrin concentration. The idea underlying the present study is that gastrin drives the progression of BO to ACO. If so, there is a need for biomarkers of the effect of gastrin on Barrett's epithelium that might be useful in tracking the progression of BO to ACO and in monitoring the effectiveness of novel anti-gastrin therapies.

Circulating gastrin concentration was analysed by radioimmunoassay and related to putative biomarker mRNA and miRNA expression quantified by qPCR in gastric and oesophageal biopsies from BO patients. Using gastric adenocarcinoma cell lines expressing the CCK2 receptor and treated with gastrin, putative protein biomarkers were analysed using western blot.

Treatment with PPIs was associated with significant increases in circulating gastrin in control and Barrett's patients. Abundance of CgA and HDC mRNA was also significantly elevated in gastric biopsies of both groups treated with PPIs. In gastric biopsies from patients with preneoplastic conditions CgA was significantly decreased in the highest risk group.

Interestingly, BO biopsies exhibited a significantly lower abundance of CgA, MMP-1, MMP-7, COX-2 and SHH transcripts in patients with high circulating gastrin (>100 pM). In serum, miR-21, -221 and 222 abundances were significantly elevated when circulating gastrin was 30-100 pM but not >100 pM; tissue miR-221 and 222 was lower with gastrin >100 pM.

The expression of putative biomarkers in vitro varied between cell lines, although TIMP-1 and MMP-1 exhibited consistent responses to gastrin.

Unexpectedly, in stomach and BO biopsies, CCK2 receptor mRNA abundance was significantly depressed in patients with elevated circulating gastrin concentration. Whilst changes in CCK2 receptor expression relative to gastrin have been suggested previously, the regulatory mechanisms are not completely understood. This result was surprising and may account for the pattern of association of putative gastrin-regulated biomarkers and circulating gastrin. The result also has wider-reaching implications for the interpretation of previous studies and for the design of future studies of the effect of gastrin on the progression of preneoplastic conditions in the gastrointestinal tract.

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List of Abbreviations

Abbreviation	Definition
ACO	Adenocarcinoma of the oesophagus
BE	Barrett's epithelium
BO	Barrett's oesophagus
CCK	Cholecystokinin
CCK2R	Cholecystokinin 2 receptor
CCK2RA	CCK2 receptor antagonists
CgA	Chromogranin A
CNS	Central nervous system
COX2	Cyclo-oxygenase-2
CREB	cyclic AMP-response element-binding protein
ctDNA	Circulating tumour DNA
CTFP	C-terminal flanking peptide
DAG	1,2-diacylglycerol
DNA	Deoxyribonucleic acid
ECL	enterochromaffin-like
EGF	Epidermal growth factor
ENS	Enteric nervous system
ER	Endoplasmic reticulum
ER	Endoplasmic reticulum
G17	Gastrin-17
G34	Gastrin-34
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gastric cancer
GI	Gastro-intestinal
GIT	Gastro-intestinal tract
GORD	Gastro-oesophageal reflux disease
GPCR	G-protein coupled receptor
HDC	Histidine decarboxylase
IHC	Immunohistochemical
IM	Intestinal metaplasia
IP_3	Inositol 1,4,5-trisphosphate
MAPK	MAP kinase
miR	MicroRNA
miRNA	Micro RNA
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MTC	Medullary thyroid cancer
NET	Neuroendocrine tumour
PA	Pernicious anaemia
PAI	Plasminogen activator inhibitors
PAM	Peptidyl-glycine α-amidating monooxygenase
PC	Prohormone convertases
PKC	Protein kinase C

PLC	Phospholipase C
PMA	Phorbol myristate acetate
PPI	Proton pump inhibitor
PPI	Proton pump inhibitor
qPCR	Quantitative polymerase chain reaction
Reg	Regenerating protein
RNA	Ribonucleic acid
SCC	Squamous cell carcinoma
SHH	Sonic hedgehog
SST	Somatostatin
TFF	Trefoil factor
TFF	Trefoil factor
TIMP	Tissue inhibitors of metalloproteinase
VMAT	Vesicular monoamine transporter
ZES	Zollinger-Ellison syndrome

Chapter 1

Introduction

1.1 Perspective

Cancer is a genetic disease characterised by inherited or acquired mutations that disrupt critical gene functions implicated in the regulation of cellular proliferation, migration, differentiation and programmed death. The result of this disruption is the conversion of healthy cells to neoplastic ones which have become unresponsive to the physiological cues that regulate cell proliferation, differentiation, migration, invasion and programmed death, characteristics collectively attributed as the "hallmarks" of cancer.[1] Neoplastic cells can then recruit surrounding normal cells to form a tumorous mass (neoplasm) complete with a protumourigenic microenvironment. Neoplastic cells can also metastasise from their site of origin to form secondary neoplasms in other organs that exhibit a favourable microenvironment,[2] compounding difficulties in treatment and increasing the risk of mortality either via tumour spread to vital organs or through multiple organ failure.

This process of development is similar for all solid tumours including those found in gastro-intestinal (GI) cancers. Neoplasms can, in principle, occur in any region along the length of the GI tract. However they occur predominantly at the upper (oesophagus, stomach) or lower (colon) ends, indicating that the anti-oncogenic mechanisms of the small intestine are normally extraordinarily efficient compared with other regions of the gut. Gastrointestinal malignancies are a leading cause of morbidity and mortality worldwide with three million new cases identified each year resulting in two million deaths annually (data collected in 2000).[3] Gastric cancer (GC) is currently the fourth most common cancer worldwide and second most common cause of cancer-related death, with 990,000 people diagnosed each year of whom 738,000 die of the disease.[4] Oesophageal cancer is currently the eighth most common cancer worldwide and the sixth most common cause of cancer-related deaths, with an overall ratio of mortality to incidence of 0.88 (data collected in 2012).[5]

The progression of healthy cells towards neoplastic ones usually occurs over a relatively long time period and involves many stages of transition, during which cells slowly acquire pro-oncogenic genetic mutations.[6] As cells undergo this progression they become further removed, morphologically, functionally and genetically, from the normal cells of their tissues of origin. The conditions that lie intermediate between

normal tissue and cancer are known as preneoplastic or precancerous conditions;[7] they represent a stage at which there is a significantly increased risk of cancer, although many patients may die of unrelated conditions before development of cancer. Preneoplastic conditions are often associated with chronic inflammation, an example of this being Barrett's oesophagus (BO) arising on a background of oesophagitis due to gastric contents reflux being associated with increased risk of progression to adenocarcinoma of the oesophagus (ACO).[8] Identifying patients with preneoplastic changes, in particular those most at risk of progression to cancer, is essential for early cancer detection to improve treatment outcome. Diagnosis based on histopathology tends to be invasive e.g. requiring endoscopy and biopsies, whereas assays based on the quantification of validated biomarkers, for example in blood, are clearly an attractive alternative for monitoring both cancer risk and treatment in high risk patients.

1.2 Organisation and control of the gastro-intestinal tract

The primary function of the gastro-intestinal tract (GIT) is the transport of water, electrolytes and nutrients into the body and the provision of an environment that promotes the conversion of food into its constituent nutrients that are appropriate for transport. At the most basic level, the morphology of the GIT consists of four distinct layers; the mucosa forming the innermost, lumen-facing, layer consisting of epithelial cells responsible for secretion (fluid, electrolytes, enzymes and mucous) and absorption, the submucosa comprising connective tissue that supports the mucosa, the muscularis externa surrounding the submucosa which provides muscular control of the GIT and so determines the rate of progression of luminal contents along the tract, and the outermost serosa, a mixture of epithelial and connective tissues encapsulating the tract.[9] The common GI cancers all arise from epithelial cells of the mucosa (some rare exceptions being MALT lymphoma and GI stromal tumours).

1.2.1 Control mechanisms of the GI tract

The GIT is unique in that it is controlled by neurons of both the central nervous system (CNS) and its own intrinsic nervous system, the enteric nervous system (ENS), located in the wall of the tract. Neuronal control mechanisms are integrated with a complex network of paracrine (local) signalling molecules e.g. cytokines, chemokines and growth factors, as well as a system of hormonal (blood-borne) control factors.

Collectively the constituents of this network make an important contribution to the cancer microenvironment and may influence tumour progression, or not, depending on the circumstances.

The peptide hormones of the gut normally regulate the secretion of digestive enzymes, the transit of nutrients through the tract and food intake. They modulate the functions of many different cell types located both within and outside the GIT including the brain and secondary digestive organs such as the pancreas. They are produced by specialised endocrine cells dispersed within the GI epithelium, from the stomach to the rectum; important examples include the cells producing gastrin, cholecystokinin (CCK), somatostatin, ghrelin, secretin and glucagon-like peptides.[10]

In addition to nutrients, the GIT inevitably has direct contact with ingested microorganisms and toxic substances that may lead to inflammation and increased risk of cancer.[11] Protective mechanisms are therefore well developed to ensure separation of the luminal contents and potential pathogens contained therein from the internal environment of the host. The first layer of defence within the GIT is tight junctions between epithelial cells, which are selective and capable of excluding noxious luminal contents such as gastric acid, colonic bacteria and bacterial antigens.[12] The GIT also exhibits well developed adaptive immune defence mechanisms that limit the attachment, colonisation and potential damage caused by pathogens.[13]

A particularly well characterised example of the interactions between the microbiota and inflammatory/immune mechanisms relevant to cancer development is the relationship between *Helicobacter pylori* infection in the stomach and GC. Chronic inflammation caused by an immune response to dysbiosis in patients with inflammatory bowel disease has also been linked to the increased risk of development of colorectal cancer.[14,15]

An area of considerable research interest in recent years has been the elucidation of the human microbiome i.e the “human microbiome project”, particularly with respect to changes in the intestinal microbiome in health and disease.[16] The gut microbiome, consists of bacteria, archaea, viruses and eukaryotic microbes; the precise balance between the different constituents of the microbiota is fundamental to maintaining

healthy human physiology and plays a pivotal role in metabolic function, protection against pathogens and educating the immune system.[17] Dysbiosis of the GIT is also suspected to play a role in diseases not typically associated with the GIT including cardiovascular disease, Parkinson's disease[18] and systemic metabolic diseases such as diabetes and obesity.[19]

1.2.2 Hormones of the GI tract.

The first GI hormone to be discovered was secretin in 1902.[20] The landmark discovery by Bayliss and Starling was then followed by the discovery of gastrin by Edkins in 1906.[21] The two hormones account for the regulation of pancreatic and gastric secretion, respectively. In 1928 Ivy and Oldberg discovered a hormone capable of contracting the gallbladder to secrete bile into the intestine – cholecystokinin.[22] In the 1960s and 70s advances in peptide biochemistry spurred the purification to homogeneity, elucidation of structure and subsequent synthesis of these hormones and also led to the discovery of more regulatory peptides including vasoactive intestinal peptide[23], gastric inhibitory peptide,[24] motilin[25] and gastrin-releasing peptide.[26] via the work of several international teams of researchers including Gregory, Dockray and Kenner in the UK, Rehfeld in Denmark, Wunsch in Germany, Jorpes and Mutt in Sweden and Yahaihara in Japan.[27] Relatively recently it has become clear that these hormones may also influence cell proliferation and contribute to cancer progression.

1.3 Gastrin

Edkins' original findings became controversial when it was later discovered that histamine also stimulated acid secretion, since many then believed that histamine was the main active factor in tissue extracts of the type prepared by Edkins. The matter was resolved in 1942 when S.A. Komarov developed a histamine-free method of producing mucosal extracts and reproduced Edkins' findings.[28] In 1964, gastrin became the first GI hormone for which the molecular structure was elucidated following the work of Gregory and Tracy who isolated heptadecapeptide gastrin (G17) from pig antrum.[29] The elucidation of the molecular structure and the ability to produce pure gastrin, then facilitated the development of a gastrin radioimmunoassay in 1968[30] that could accurately quantify circulating serum gastrin concentrations from patients and animals and thereby gave birth to a new generation of gastrin research.[31]

1.3.1 Gastrin biosynthesis

Under normal conditions the *gastrin* gene is expressed in specialised entero-endocrine cells of the antrum known as G-cells. It is encoded by a 4.3 kb gene located on chromosome 17(q21) [32] and consists of three exons that encode for a prepropeptide of 101 amino acid residues (in humans) with a calculated molecular mass of 11.4 kDa.[33, 34] Preprogastrin consists of an N-terminal 21 amino acid signal sequence followed by a spacer peptide, a bioactive domain and a C-terminal hexapeptide flanking peptide (CTFP). The precursor undergoes extensive post-translation processing within G-cells before secretion. During translation, the signal sequence determines the translocation of the elongating polypeptide to the endoplasmic reticulum (ER), where it is cleaved to produce progastrin. As progastrin travels through the Golgi complex to the *trans*-Golgi network it is *O*-sulphated on tyrosine-87[35, 36] and/or phosphorylated on serine-96.[37] The extent of *O*-sulphation depends on the species, cellular origins and developmental stage with approximately 50% of adult human G-cell derived gastrin sulphated. The effects of progastrin phosphorylation are currently not completely understood as it is not essential for progastrin processing, however it is believed that it may modulate cleavage of the CTFP which subsequently affects the conversion of glycine-extended gastrin intermediates to C-terminal amidated peptides.[38] Following sulphation and/or phosphorylation progastrin exits the *trans*-Golgi network into immature secretary granules where it undergoes major proteolytic processing into biologically active peptides as the secretary granules mature.

Processing of progastrin requires convertase-specific cleavage by prohormone convertases (PC); in immature granules with a neutral pH, progastrin is cleaved by PC1/3 to release the CTFP and spacer peptide to generate G34-Gly-Arg-Arg before the two basic residues are removed by carboxy-peptidase E resulting in glycine-extended G34 (G34-Gly). As secretory granules mature and the internal pH becomes acidic, PC2 is activated and cleaves a proportion of G34-Gly into G17-Gly.[39] Both glycine-extended gastrin forms are substrates for peptidyl-glycine α-amidating monooxygenase (PAM) for conversion to the bioactive amidated gastrins, G34 and G17 (fig. 1.3.1.1).[39] In humans > 98% of progastrin is converted to α-amidated bioactive gastrins, the majority being G17 (85%) and G34 (10%) forms, however G34 is considered the major biologically active circulating form of gastrin due to its much

longer half-life and preferential release.[40, 41] A detailed schematic showing how different lengths of gastrin hormone are derived from the common precursor preprograstrin is displayed in figure 1.3.1.1.

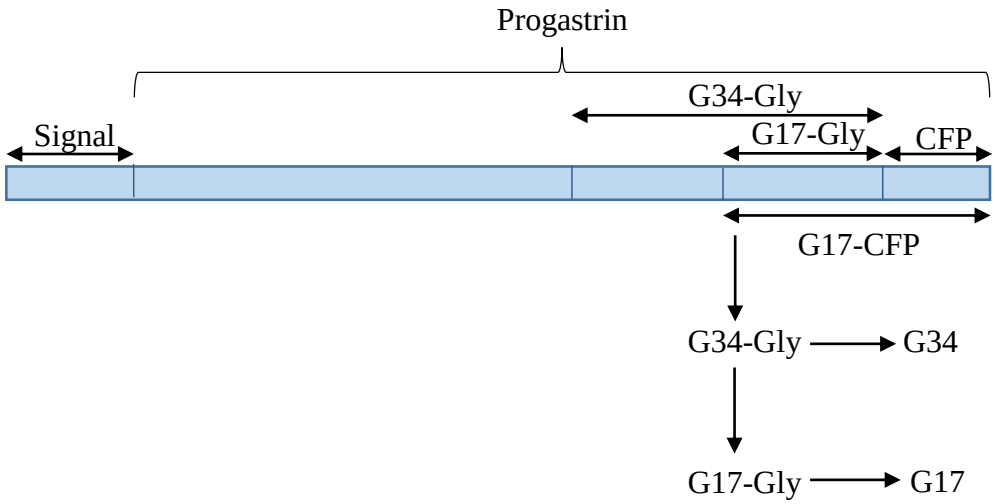

Figure 1.3.1.1 – **A schematic representation of preprogastrin and the pathways of progastrin proteolytic processing to produce amidated gastrins G34 and G17**.

1.3.2 Stimulation of gastrin synthesis and release

The secretion of gastrin from G-cells is controlled by gastric luminal contents including gastric pH, nervous reflexes and paracrine signalling from adjacent cells. Expression of the *gastrin* gene within G-cells is also regulated by luminal contents[42] and pH of the stomach,[43] secondary to paracrine signalling exerted by the GI inhibitory hormone somatostatin. G-cells exhibit an open morphology whereby different domains in the plasma membrane are in contact with either the basement membrane or gut lumen; the latter allows the cells to sense luminal content of which protein and amino acids, notably phenylalanine and tryptophan, are the most potent stimulators.[44, 45] Pepsin, released as pepsinogen from gastric chief cells and activated by gastric acid, digests dietary protein into the amino acids including those previously mentioned which stimulate gastrin release from G-cells.[10] The release of gastrin is also stimulated if gastric pH rises above 3 via both direct action on the G-cell[46] and indirectly through reduction in the release of inhibitory somatostatin from adjacent D-cells[47] (fig. 1.4.1). However in normal subjects an acute rise in pH is not sufficient to stimulate gastrin release. Modulation of both the parasympathetic and sympathetic nervous pathways

has also been shown to regulate gastric acid secretion via the release of gastrin-releasing peptide.[48, 49]

The plasma concentration of amidated gastrins in fasted humans ranges up to 30 pmol/L, however after a mixed meal consisting of carbohydrates, protein and fats the concentration of plasma gastrin increases 2-3 fold with a postprandial peak after 30-60 minutes.[50]

1.3.3 Inhibition of gastrin synthesis and release

Somatostatin plays a dual role in inhibiting gastrin release, both by inhibiting secretion of gastrin from G-cells via activation of somatostatin receptor 2 and by inhibiting *gastrin* gene expression. Whilst the paracrine signalling pathways regulating transcription are not completely understood, a number of pathways have been reported to be involved - the most well elucidated being the epidermal growth factor (EGF) pathway. EGF stimulates gastrin gene expression via a GC-rich sequence in the *gastrin* gene promoter, EGF stimulates the mitogen-activated protein kinase pathway, which in turn phosphorylates transcription factor specificity protein 1 and allows binding to the promotor region and gene transcription.[51] Somatostatin has been shown to inhibit basal *gastrin* gene expression and EGF-stimulated gene transcription via suppression of protein kinase A activity and interaction with sequences adjacent to the gastrin promotor sequence induced by EGF.[52, 53] Transforming growth factor-β, Wnt and nuclear factor-κ-B signalling pathways have also been reported as being involved in transcription of the *gastrin* gene.[54] In terms of *gastrin* gene transcription inhibition, recent experimental data has identified a role of the inflammatory cytokine interleukin-1β as an inhibitor of gene transcription via a NF-κB/histone deacetylase repressor complex.[55]

1.4 Overview of the actions of gastrin

The main biological actions of amidated gastrins are exerted via activation of the G-protein coupled cholecystokinin 2 receptor (CCK2R). The latter is typically expressed in the periphery by parietal cells and enterochromaffin-like (ECL) cells and there is species specific expression in pancreatic acinar cells. The primary physiological function of gastrin is the stimulation of gastric acid secretion from parietal cells. This is achieved via the activation of CCK2Rs by gastrin on neighbouring ECL cells,

resulting in histamine release which acts as a paracrine mediator stimulating acid release from parietal cells (fig 1.4.1). In addition, gastrin also regulates ECL and parietal cell numbers, ECL cell proliferation, gene expression and parietal cell maturity[56] and migration along gastric glands[57] playing a pivotal role in the organisation and function of the gastric epithelium.[58]

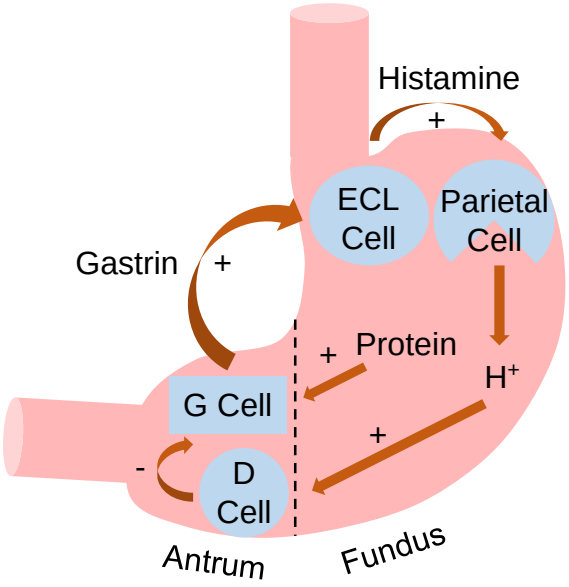

Figure 1.4.1 – **Overview of gastrin release and stimulation in the stomach**.

1.4.1 Proliferative effects of gastrins

Changes in cell proliferation and cell loss induced by gastrin through the CCK2R were first described by Johnson in 1974[59] and his continued research then went on to identify the proliferative role of gastrin in a rat duodenal ulcer healing model.[60] Following on from this, the utilisation of genetically manipulated gastrin-deficient mouse models identified that gastrin production was crucial for regulating the maturation of gastric parietal cells and that gastrin is also a trophic factor for development of the colonic mucosa.[56] Similar mouse studies utilising transgenic models overexpressing Gly-extended gastrin and progastrin reported increases in colonic mucosal thickness and colonic proliferation when compared to wild type and gastrin-deficient mouse models[61] and this effect was reversed through deletion or antagonism of the CCK2R.[62] Moreover, the main physiological target of gastrin, enterochromaffin-like (ECL) cells, have been shown to proliferate in response to gastrin in both culture[63] and *in vivo*[64] and hypergastrinaemia is associated with increased ECL cell numbers[65] and spontaneous ECL cell carcinoid tumours.[66]

Collectively this work provides an experimental foundation for understanding the possible role of gastrin in cancer.

The proliferative effects of gastrin have also been identified in pancreatic and CHO cell lines through a PKC/MAPK pathway[67] and in pituitary GH3 cells however this proliferation mechanism was MAPK independent.[68] In some cell lines, however, gastrin acting at the CCK2R has been identified as inhibiting cellular proliferation including certain pancreatic cancer cell lines,[69, 70] CHO cells[70] and AGS cells transfected with the human CCK2R.[71] Conversely, gastrin has also been shown to induce proliferation of transfected AGS cells in a co-culture cell model[72] indicating that gastrin can play a dual role – directly inhibiting or indirectly stimulating proliferation through a paracrine mechanism.

1.4.2 Gastrin in disease

A number of conditions and medications are associated with hypergastrinaemia. These fall into two major categories: those accompanied by gastric acid hypersecretion and those associated with hypochlorhydria or achlorhydria. Well studied examples of conditions resulting in hypergastrinaemia accompanied by gastric acid hypersecretion include duodenal ulcer disease, Zollinger-Ellison syndrome i.e. gastrin-secreting tumours, antral G-cell hyperplasia and gastric outlet obstruction. All of these share the clinical features of peptic ulcer disease and/or gastroesophageal reflux disease and complications thereof.[73] Conditions resulting in hypergastrinaemia accompanied by reduced or absent gastric acid hypersecretion (and attributable to loss of suppression of gastrin release by gastric acid) include patients treated with protein pump inhibitors (PPIs),[74] corporal atrophic gastritis[75] and some sub-groups of patients with *H. pylori* infection.[76]

1.4.3 Causes of hypergastrinaemia

1.4.3.1 Zollinger-Ellison Syndrome

Zollinger-Ellison syndrome (ZES) is a characterised by significantly increased serum gastrin concentration due to gastrin production from neuroendocrine tumours – gastrinomas – which either develop sporadically or as one of the tumours in patients suffering from multiple endocrine neoplasia (MEN)-1 syndrome. Symptoms of ZES

reflect the hypergastrinaemia caused by the presence of gastrinoma and commonly include diarrhoea, multiple relapsing ulcers in atypical locations throughout the GI tract, and gastric mucosal hypertrophy.[77] Diagnosis of gastrinoma is typically performed based on the response of serum gastrin concentration to an IV injection of secretin, with an increase of ≥50 pM considered positive.[78]

1.4.3.2 Autoimmune gastritis

Chronic atrophic corporal gastritis type A is an autoimmune inflammatory disease characterised by the destruction of the oxyntic mucosa caused by the presence of autoantibodies to the H+/K+-ATPase proton pump expressed by gastric parietal cells responsible for gastric acid production.[79] Loss of parietal cells leads to achlorhydria and so removes the negative feedback mechanism provided by somatostatin on G-cells resulting in hypergastrinaemia; the secondary loss of intrinsic factor which is produced by parietal cells results in pernicious anaemia (PA).[80] Hypergastrinaemia secondary to hypochlorhydria in patients with PA, is a well-known risk factor for ECL cell hyperplasia and gastric carcinoid neuroendocrine tumours. [81-83] Loss of intrinsic factor prevents absorption of vitamin B_{12} from the diet resulting in PA and presenting as fatigue, typically most affecting the elderly.[84]

1.4.3.3 Proton pump inhibitors

The widespread use of PPIs, such as omeprazole, lansoprazole, rabeprazole and others, has been common for nearly 30 years. It has recently become a topic of increased clinical interest due to reports detailing their usage as a potential risk factor for GC[85, 86] amongst other adverse effects including increased risk of pneumonia, *C. difficile* infection, bone fractures, hypomagnesaemia and acute interstitial nephritis.[87] Treatment with PPIs blocks the H^+,K^+-ATPase proton pump responsible for H^+ ion secretion into the lumen of the stomach from parietal cells[88] increasing gastric pH and stimulating gastrin release from G-cells, with prolonged usage resulting in hypergastrinaemia. They have also been linked to morphological changes in the stomach including parietal cell hyperplasia,[89] ECL cell hyperplasia,[90] fundic gland polys[91] and, in rare cases, gastric carcinoids.[92] Recent research has identified that PPIs can also influence the gastric microbiome and increase the risk of certain bacterial infections including *C. difficile, Salmonella* and *Campylobacter* populations.[93]

ECL-cell proliferation as a result of PPI-induced hypergastrinaemia can lead to increased acid secretion once PPI treatment is discontinued.[94] A number of studies have documented the phenomenon of rebound acid hypersecretion following cessation of a minimum of 8 weeks of PPI treatment.[95, 96] The increased secretory capacity of the gastric mucosa reflects the degree of ECL cell hyperplasia and increased gastrin stimulated acid secretion can be detected 2-3 months post PPI withdrawal.[97] In the most extreme cases rebound hypersecretion has been reported 11 months after cessation of only a 2 month PPI treatment period.[98] The consequences of rebound hypersecretion mimic those for which PPIs are originally indicated, typically dyspepsia, heartburn or gastro-oesophageal reflux disease[99, 100] even in patients that were asymptomatic before PPI administration.[101]

1.4.3.4 H. pylori infection

Infection with *H. pylori* is one of the most common causes of moderate hypergastrinaemia. The bacteria cause damage to gastric glands via infiltration and release of virulence factors[102, 103] which stimulates cytokine release and recruitment of inflammatory cells (lymphocytes, neutrophils etc.) and activation of inflammatory regulators such as cyclooxygenase-2 (COX-2).[104, 105] The resulting chronic inflammation of the gastric mucosa leads to gastric atrophy[106] characterised by the loss of parietal cells and gastric glands resulting in hypochlorhydria.[107] Depending on the gastric location and severity of the *H. pylori* infection, an increase in gastrin concentration can be caused either via removal of G-cell inhibition by suppression of D-cells (antral infection) or via parietal cell loss resulting in decreased acid secretion (corpus infection).[108] There is also evidence to suggest *H. pylori* infection can inhibit acid secretion from parietal cells by inhibiting H^+/K^+-ATPase α subunit gene expression.[109] The chronic inflammation induced by *H. pylori* infection has also been linked to being the greatest risk factor for the development of sporadic GC.[110]

However, despite *H. pylori* infecting >50% of the world's population, only approximately 1% of those infected will develop GC; indicating currently unknown additional factors that play a role in GC development. Recent research has focused on the possible role of the gastric microbiome in facilitating *H. pylori* driven progression to GC. A study performed in China found that GC mucosa was associated with marked differences in the microbiome compared to samples obtained from patients with

superficial gastritis, atrophic gastritis and intestinal metaplasia (IM).[111] Specifically, gastric mucosa exhibited enriched bacterial populations typically associated with the oral cavity, although it is unclear whether the changes in the microbiome predate GC development and contribute to it or result as a consequence of the creation of the tumour microenvironment favouring the opportunistic growth of the microorganisms.[112] Significant differences in the gastric microbiome between patients with autoimmune associated atrophic gastritis and *H. pylori* associated atrophic gastritis (both associated with the development of different types of GC) which was independent of *H. pylori* induced hypochlorydia have also been identified.[113]

1.4.4 Role of gastrin in cancer

1.4.4.1 Gastric cancer

Gastric adenocarcinoma has been associated with a significant elevation in serum gastrin concentration for approximately 40 years.[114] Within animal models exhibiting preneoplastic phenotypes there appears to be a synergistic relationship with hypergastrinaemia that accelerates the progression to cancer[115] which implicates gastrin as a potential driving factor of progression to cancer in humans. Very recently a Finnish group identified that patients with high gastrin concentrations measured in the 1980s had an increased risk of gastric cancer development decades later[116] and multiple groups have identified increased risk of gastric cancer in patients treated long-term with PPIs[85, 117] potentially via PPI induced hypergastrinaemia.

1.4.4.2 Colorectal cancer

Typically the colorectal mucosa does not appear to regularly express CCK2Rs[118] however some early studies did suggest that certain colorectal carcinomas and cell lines synthesised gastrin and exhibited expression of the CCK2R.[119, 120] Moreover, the growth of some colorectal carcinoma cell lines was stimulated by exogenous gastrin stimulation, which was then inhibited in the presence of a gastrin receptor antagonist.[121] Further research then identified increased serum gastrin precursor concentration in colorectal carcinoma patients[122] which was reduced post-resection, but amidated gastrin concentration remained similar.[123] These results lead to the current hypothesis, that colorectal cancers produce progastrin but are unable to process

progastrin to amidated gastrins[122] and it is these non-amidated forms of gastrin that promote the acceleration of colon carcinogenesis (see section 1.6.1 below).

1.4.4.3 Thyroid cancer

Medullary thyroid cancer (MTC) results from the malignant de-differentiation of calcitonin-producing C cells. It was discovered that high-affinity CCK2 receptors were present in MTC however they were absent in non-medullary thyroid carcinomas or in normal thyroid glands.[124] Prior to this, diagnosis of MTC was performed using a pentagastrin provocation test where IV administration stimulated a significant increase in plasma calcitonin concentration, secreted from the thyroid [125] this test remains the gold standard for MTC diagnosis.[126]

Gastrin has also been suspected to stimulate the growth of other cancers, in epidemiological studies of patients with ZES and atrophic gastritis there is a significant increase in cancer risk in the stomach, oesophagus and pancreas.[127-129]

1.5 Novel functions of gastrin

In addition to its well documented gastric acid regulatory and proliferative effects, recent research has identified a plethora of previously unrecognised potential functions of gastrin including roles in stimulating cell migration, invasion, apoptosis and tubulogenesis.[130, 131] The role of gastrin in cell migration and invasion can be linked to its regulation of a number of proteins that exert effects on tissue remodelling including matrix metalloproteinases (MMPs),[132, 133] plasminogen activator inhibitors (PAIs)[134, 135] and tissue inhibitors of metalloproteinases (TIMPs).[136] In the context of cell survival, gastrin has been implicated in the upregulation of pro-apoptotic PAI-2[137] and conversely the upregulation of pro-survival factors clusterin[138] and the putative protective trefoil factors (TFFs).[139] The expression of some of these novel gastrin-regulated proteins have been considered potential biomarkers of gastrin responsiveness (see section 1.8.1).

1.6 "Non-Classical" gastrins

Originally only the common C-terminally amidated gastrins, G17 and G34, were considered to be biologically active. Subsequent research however has identified

biological actions attributed to other progastrin derivatives, collectively called the non-classical gastrins.[61, 140, 141] As explained previously, the extensive processing of preprogastrin translated from the *gastrin* gene generates a number of intermediate peptides and fragments including a CTFP, progastrin, G34-Gly, G17-Gly, G17-CFP and G34-CFP; these may actually be the predominant forms found in cells lacking the regulated secretory pathway.[120] A recent review summised that although progastrin and glycine-extended gastrins are the predominant forms found in many cancers and cell lines including colon,[142] oesophagus[143] lung[144] and ovarian[145] that express the *gastrin* gene, they are unable to process the translation products fully to the amidated gastrins, except in negligible amounts. [146]

1.6.1 Progastrin

Research concerning the biological effects of progastrin and its derivatives has mainly focused on the colon. Transgenic mice over-expressing progastrin exhibit increased colonic proliferation and susceptibility to cancer formation,[147, 148] whilst progastrin expression by human primary colorectal cancer cells was found to be vital for tumour formation.[149] Overexpression of progastrin has also been shown to impart tumourogenic and metastatic potential to human embryonic epithelial cells.[150] Progastrin has also been identified as playing a possible role in gastric antral stem cell expansion and carcinogenesis.[151]

How progastrin exerts these effects is currently not completely understood. Several putative receptors for progastrin have been proposed[152] including the classical gastrin activated CCK2 receptor.[153] Recently G-protein coupled receptor 56 activation has been implicated[154] and a novel mechanism of clathrin-dependent endocytosis via cell-surface annexin AnxA2 has also been proposed.[155] Despite these advances, more research is required to fully elucidate the role of progastrin in carcinogenesis and the mechanisms involved.

1.6.2 Gly-gastrins

Discovery of the proliferating effect of Gly-gastrins was initially described in a pancreatic cell line[141] and since then has also been identified in colonic mucosa[156] and oesophageal cancer cell lines.[157] G17-Gly has also been identified exhibiting anti-apoptotic activity in both Barrett's oesophagus and oesophageal adenocarcinoma cell

lines.[158] Evidence has emerged suggesting that G17-Gly acts synergistically with amidated gastrins in the stomach[159] and, considering amidated gastrin has been linked to proliferation in Barrett's metaplasia,[160] this may also the case for the oesophagus although so far it has not been investigated.

As with progastrin, a specific receptor for Gly-extended gastrins has not been identified, with arguments for and against the involvement of the CCK2R.[152] Recent work suggests a role for cell-surface bound F1-ATPase, at least within colonic epithelial cell proliferation, as a Gly-gastrin receptor.[161] As with progastrin, more research is required to explore the role of Gly gastrins in carcinogenesis and the mechanisms involved.

1.7 The CCK2 receptor

The first of the CCK family of peptides to be isolated was CCK33, in 1968 by Mutt and Jorpes.[162] Another form, CCK8, was one of the first gastrointestinal peptides to be discovered in the brain.[163] Cholecystokinin receptors were pharmacologically classified into two subtypes – CCKA and B[164] with the latter also being referred to as gastrin-CCK-B[165] (now referred to as CCK1 and 2 respectively). The CCK1 receptor was initially cloned and sequenced from rat pancreatic mRNA, later both receptors where cloned from rat brain and rat pancreatic cell lines in 1992 by Wank *et. al.*[166, 167] This work was also important in identifying that the cDNA sequences of CCK1 and CCK2 receptors in the brain were the same as those of the corresponding receptor in the GI system. Cholecystokinin contains an identical C-terminal pentapeptide sequence to that of gastrin, which explains why both gastrin and CCK are able to bind to CCK receptors. However differences in receptor affinity for gastrin are striking. The CCK1 receptor binds gastrin with a relatively low affinity, 500-1000 times lower than sulphated CCK, whereas the CCK2 receptor binds gastrin and CCK with similar affinities.[168] In the central nervous system CCK is the predominant ligand for the CCK2R because it is expressed in high concentrations by neurons, while gastrin is the predominant ligand in the periphery as, under physiological conditions, its plasma concentration is 5-10 times higher than that of CCK.[169]

In the GI tract, CCK1 receptors are expressed on gastric D-cells, chief cells, smooth muscle cells, enteric neurons, smooth muscle cells, vagal afferent neurons and

pancreatic acinar cells. In the CNS, CCK1 receptors are expressed in the anterior pituitary and areas of the midbrain.[170] Whereas CCK2R are normally expressed by parietal cells, ECL cells, some smooth muscle cells, neurons in many parts of the CNS as well as in the peripheral nervous system and pancreatic acinar cells.[10]

1.7.1 Activation of CCK2R by gastrin

Gastrin regulates acid secretion from parietal cells in large part by stimulating release of histamine from ECL cells via CCK2R activation, which in turn activates histamine subtype 2 receptors on parietal cells to induce acid secretion. In most cell types studied, including ECL cells, CCK2R is coupled to members of the $G\alpha_{q/11}$ subfamily of heterotrimeric GTP-binding proteins which mediate receptor activation of phospholipase C (PLC)-β. In turn PLC-β produces secondary messengers inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG); IP_3 binds to and activates Ca^{2+} channels in the ER membrane resulting in rapid Ca^{2+} efflux into the cell cytoplasm. Elevated cytoplasm Ca^{2+} concentration and DAG go on to activate numerous Ca^{2+} and lipid-regulated proteins including members of the protein kinase C (PKC) family.

Recently the CCK2R has been indicated in playing a role in various cancers. It has been known for some time that there are multiple splice variants of the CCK2R. Of particular interest is variant CCK2R i4sv which was identified in 100% of insulinomas and gastrointestinal stromal tumours and 67% of small cell lung cancers across a panel of 81 different tumours.[171] It is thought to regulate cancer cell proliferation, however this mechanism may be independent of gastrin stimulation.[172] Studies of archived pancreatic cancer material have also shown expression of CCK2R mRNA and variants thereof.[173]

1.7.2 Expression of the CCK2R

Although the mechanisms controlling CCK2R expression are not completely understood, it is known that binding sites for transcription factors SP1, C/EBP and GATA are essential for functional receptor production. Some evidence suggests that gastrin itself is capable of upregulating the expression of the CCK2R. Gastrin treatment of RGM1 cells significantly increased endogenous CCK2R expression

whilst in the stomach of hypergastrinaemic animals increased CCK2R abundance has also been observed.[174] In cases of gastric mucosal damage CCK2R expression increases progressively at the repair margin and *de novo* expression of the CCK2R has been observed in the submucosa beneath the repairing mucosa. Many of the cells that expressed the CCK2R in response to injury were identified as myofibroblasts. These data suggest that increased CCK2R expression might influence the outcome of epithelial inflammation or injury and that the response may be mediated in part by myofibroblasts.[174] There is also emerging evidence of regulation of CCK2R expression by miR148b in gastric and colorectal cancers.[175, 176]

1.7.3 Antagonists of the CCK2R

The initial discovery of CCK2 receptor antagonists (CCK2RA) began with asperlicin, a benzodiazepine-related natural product with weak affinity for CCK1 receptors. This discovery led to the development of a number of benzodiazepine-derived CCK2RA such as L-364,718 (devazepide) and L-365,260. Other classes of antagonists include the dipeptoid CI-988,[177] benzodiazepine derivatives YM022,[178] Z-360[179] and YF476 (netazepide).[180, 181] Two new classes of CCK2RA have also recently been invented: JB95008 which is a substituted imidazole[182] and JNJ-26070109[183] which is a benzamide derivative. Whilst no CCK2RA are currently on the market, the most promising candidates netazapide and Z-360 are in active clinical development indicated for the treatment of type 1 gastric NETs[184, 185] and pancreatic cancer[179, 186] respectively. A new benzodiazepine derivative CCK2RA – ceclazepide – that offers greater selectivity, solubility and bioavailability compared to netazepide is also in pre-clinical development.[187]

In their use as a research tool, animal models treated with JNJ-26070109 and netazepide[188] both exhibited dose-dependent inhibition of pentagastrin-stimulated acid secretion in rats and dogs. Netazepide also prevented the increases in ECL-cell activity and density, oxyntic mucosal thickness, mucosal histidine decarboxylase (HDC) activity and serum pancreastatin caused by typical PPI induced hypergastrinaemia in rats.[189] Netazepide has also been identified as preventing ECL-derived gastric carcinoids accelerated by PPI induced hypergastrinaemia and causing the regression of existing carcinoids.[190] In human trials, L-365,260[191] and netazepide[192] have both exhibited dose-dependent inhibition of pentagastrin-stimulated acid secretion, with the

latter suppressing acid production as effectively as the PPI rabeprazole. Furthermore, netazepide treatment of patients with chronic atrophic gastritis, achlorhydria, hypergastrinaemia and multiple gastric neuroendocrine tumours (NETs) caused a reduction in tumour number and size and normalised expression of the NET biomarker chromogranin A (CgA).[184]

The latest generation of CCK2RA represent a leap forward in their suitability for use in indications such as hypergastrinaemia, acid-related conditions, *H. pylori* infection and conditions of CCK2R overexpression without the potential side effects associated with PPIs.

1.8 The ECL cell: significance for biomarkers of gastrin responsiveness

ECL cells reside in the oxyntic glands, located in the corpus of the stomach alongside parietal and chief cells. They are the predominant expressors of the CCK2 receptor in the periphery, activation of which via gastrin stimulates both the release of histamine (see above) from the cell and increases its synthesis within the cell.[58] Histamine synthesis is dependent on the activation of HDC and thus, as the expression of HDC is induced by gastrin, HDC transcript abundance can be (and has previously been) used as a biomarker of gastrin stimulation of ECL cells.[193-195] Histamine is generated in the ECL cell cytosol and transported into secretory vesicles by vesicular monoamine transporter 2 (VMAT)-2, the expression of which is also regulated by gastrin so provides a further potential biomarker of gastrin responsiveness of ECL cells.[196, 197]

The majority protein component of ECL cell secretory vesicles is contributed by members of the chromogranin family. There are nine members, with the most well investigated being chromogranins A, B and C.[198] Members of this family occur in neuroendocrine cells throughout the body and play a chaperone role in the packaging of secretary products into the regulated secretory pathway and are secreted alongside them.[199] They can also act as precursors of biologically active peptides; for example, processing of CgA produces pancreastatin and pancreastatin-related products of which ECL cells contain and secrete the majority in the body.[194] Circulating CgA is therefore often used as a biomarker of ECL cell function. Gastrin regulates CgA gene expression in ECL cells[200, 201] and measurement of the concentration of circulating CgA has also been used as a biomarker not just of ECL mass and activity, but also as an indicator

of responsiveness to gastrin;[185] CgA has been well characterised as such, particularly for diagnosis of ECL cell hyperplasia and NETs.[202-204]

Gastrin plays an important role in regulating the ECL cell population.[63] Sustained hypergastrinaemia however induces ECL cell hypertrophy after approximately one week, hyperplasia after approximately 10 weeks (with ECL cell number plateauing at 20 weeks), signs of linear hyperplasia (focal multiple micronodules) at approximately one year[205] and a transition to dysplasia (defined as ECL tumours) after two years.[194] The development of hypertrophy and subsequent hyperplasia and dysplasia can be blocked via the use of CCK2RAs, demonstrating that progression is gastrin driven.[189, 190, 206]

1.9 Gastrin-regulated gene expression

A number of downstream targets of gastrin (that represent potential biomarkers of cellular response to gastrin) have been studied in addition to the well characterised examples of CgA and HDC mentioned previously.[185, 195, 207, 208] These include MMPs -1, -7 and -9, TFFs -1 and -2, the regenerating family of proteins (Reg), COX-2 and microRNAs -21, -221 and -222.

1.9.1 Putative biomarkers of gastrin responsiveness

1.9.1.1 Matrix metalloproteinases

The MMPs are a family of zinc-dependent endopeptidases that play crucial roles during tissue remodelling and organ development via rearrangement of the extracellular matrix.[209] If the function or expression of MMPs goes awry however, they can contribute to virtually all steps in tumour progression[210] through extracellular matrix degradation paving the way for tumour cell invasion and metastasis. Gastrin has been shown to stimulate the expression of multiple members of the MMP family including MMP-1[211] in parietal and chief cells and MMP-7[132] and MMP-9[212] in ECL cells.

1.9.1.2 Trefoil factor family

The TFF family of peptides are a polypeptide family secreted onto epithelial surfaces in conjunction with mucus by mucus-secreting cells. They are believed to play roles

in the maintenance and repair of the gastric mucosa and as a tumour suppressor.[213] Specifically TFF1 and 2 are implicated in repairing the GI mucosa through cell migration post-injury.[214, 215] TFF1 protects against inflammation of the gastric mucosa[216] and both have been indicated as tumour suppressors in GC[217, 218]. A number of studies have suggested that hypergastrinaemia can regulate the expression of both TFF1[139] and TFF2[215] mRNA abundance via both direct CCK2 receptor activation and indirect paracrine signalling pathways. Reduced expression of TFF1 and 2 has also been observed in gastric preneoplastic conditions and cancer.[218]

1.9.1.3 Regenerating protein family

The Reg family of proteins (also known as pancreatic stone protein, pancreatic thread protein, lithostathine and islet of Langerhans regenerating protein) are a large family of proteins associated with binding of extracellular calcium, cellular proliferation and differentiation; they show a characteristic distribution in various organs with strong expression along the entire GIT and accessory organs such as the pancreas.[219] Members of the group also exhibit dramatic (>100 fold) increases in expression in response to cell stimulation in a wide variety of circumstances. Gastrin has been identified in regulating the expression of the *Reg1* mRNA in both chief and ECL cells[220] suspected to be responsible for gastrin-induced cell growth.[221]

1.9.1.4 Cyclooxygenase-2

The enzyme COX-2 catalyses the conversion of arachidonic acid to prostaglandins, responsible for inflammatory reactions and tumour development.[222, 223] Research has identified that *H. pylori* induced hypergastrinaemia is often associated with increased COX-2 expression.[224] Further work has identified CCK2 receptor activation by gastrin stimulates expression of COX-2 mRNA in GC cells irrespective of *H. pylori* infection[225] via activation of the secondary messaging JAK2/STAT3/PI3K/Akt pathway.

1.9.1.5 MicroRNAs

Recent advancements in experimental techniques have allowed the analysis of small noncoding RNA species known as microRNAs (miRNAs) that have been shown to regulate gene expression and play important roles in a wide range of physiological and

pathological processes, including the promotion of GI cancers.[226-229] Gastrin has previously been shown to induce the expression of miRNA-222 in CCK2R transfected AGS cells and in mouse models exhibiting hypergastrinaemia.[230] There may also be other miRNAs that are regulated by gastrin, however there has been very little research comparing the two.

1.9.2 Cellular mechanisms of gastrin-regulated gene expression

Direct gastrin-dependent gene expression within ECL cells is responsible for the increased expression of *HDC*, *VMAT* and *CgA*.[231] Increased *HDC* gene transcription in response to gastrin is mediated by an enhancer-like response element which consists of two overlapping nuclear factors – gastrin response elements 1 and 2.[232] Gastrin stimulation of HDC transcription was shown to be mediated by PKC[233] indirect activation of c-*fos* and c-*jun*[234] and Raf dependent MAP kinase stimulation (MAPK).[235] Gastrin regulated transcription of *CgA* has been identified as being under the control of transcriptional factors Sp1, Egr-1 and cyclic AMP-response element-binding protein (CREB),[236] the activation of which is modulated via a PKC/MEK-1/ERK-1/-2 pathway.[231] Transcription of *VMAT* regulated by gastrin is dependent on the binding of a currently uncharacterised transcription factor to an AP2/Sp1 site within the promotor region and CREB binding to a upstream CRE consensus element.[237] This activation is again suspected to be modulated downstream of PKC[238] identifying a common mediator of gastrin stimulated expression within ECL cells that activates a diverse network of secondary signalling pathways to differentially regulate CgA and HDC production.

1.9.3 Indirect actions of gastrin

In addition to direct effects of gastrin on cells expressing the CCK2R (parietal, ECL) recent work has suggested that gastrin also activates a number of paracrine signalling pathways involved in cell secretion, proliferation and inflammation. These include release of somatostatin,[239] activation of COX-2,[240-242] shedding or induction of members of the EGF family,[72, 243] fibroblast growth factor (FGF)-1,[244] Reg[245, 246] and the chemokine IL-8.[247]

1.10 Gastric cancer and preneoplastic conditions

The development of intestinal type gastric adenocarcinoma – the most prevalent form of GC[248] – represents the culmination of an inflammation-metaplasia-dysplasia-carcinoma sequence originally characterised by Correa.[249-251] Progression typically proceeds from normal mucosa through chronic non-atrophic gastritis (for which *H. pylori* infection is the most frequent cause), atrophic gastritis (defined as loss of parietal cells and oxyntic glands) and IM (defined as presence of goblet cells) to dysplasia and finally carcinoma. In this sequence, chronic atrophic gastritis and IM both confer a high risk for the development of GC as they constitute the background on which dysplasia and carcinoma arise[252] and are considered to be preneoplastic conditions.[253] Therefore conditions known to induce inflammation of the gastric mucosa and gastritis such as *H. pylori* infection and autoimmune atrophic gastritis themselves also represent preneoplastic conditions. Despite increasing the risk of GC however, not all patients with preneoplastic changes progress to GC. A recent large-scale study within a western population identified that patients with atrophic gastritis and IM had a 20 year average risk of progression to GC of 2% and 2.6% respectively.[254] There exists an obvious clinical need to be able to identify those patients most at risk of progression to cancer from those simply harbouring the preneoplastic condition, we believe that finding the right gastrin-regulated biomarkers has the potential to do this.

1.11 Oesophageal adenocarcinoma and Barrett's oesophagus

Gastro-oesophageal reflux disease (GORD) is defined as reflux of the gastric contents that causes troublesome symptoms, mucosal injury in the oesophagus or a combination of both.[255] It is currently the most common gastrointestinal diagnosis in the USA with the western world in general exhibiting the highest prevalence worldwide of between 10-30%[256] with evidence suggesting a continuing upward trend.[257] Evidence shows a strong link between obesity and GORD,[258] thought to be due to increased abdominal pressure facilitating reflux. The chronic inflammation caused by reflux then leads to the development of BO.[259]

1.11.1 Barrett's oesophagus

Barrett's oesophagus is the metaplastic replacement of the stratified squamous epithelium that typically lines the oesophagus with specialised intestinal columnar epithelial cells in response to inflammation initiated by GORD (fig 1.10.2.1).[260] Although IM is the most common form and the epithelial type linked to adenocarcinoma development,[261] BO can also present with cardia and gastric fundic-type columnar epithelium.[262] Barrett's metaplasia appears to be a protective adaptation for the lower oesophagus against the damaging consequences of reflux of gastric acid and bile salts,[263] however in doing so it predisposes towards oesophageal adenocarcinoma.

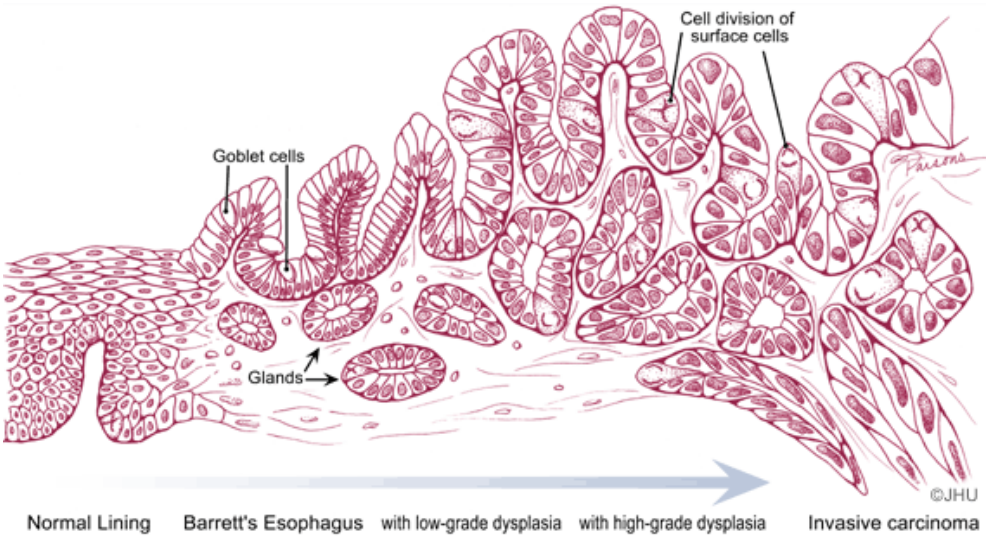

Figure 1.10.2.1 – **A diagrammatic representation of typical cells of the oesophagus transitioning through BO and epithelial dysplasia towards oesophageal carcinoma**. Produced by Jennifer Parsons Brumbaugh © (2000–2004) Johns Hopkins University and taken from http://pathology.jhu.edu/beweb/understanding.cfm.

1.11.2 Cancer of the oesophagus

The majority of oesophageal cancers can be subdivided into two histologically distinct subtypes – ACO and squamous cell carcinoma (SCC) and typically develop in the lower third and upper two thirds of the oesophagus respectively.[264] Of the two subtypes, the epidemiology of ACO has evolved rapidly over recent years and in many higher-income western countries has become the predominant histological type with incidence increasing more rapidly than that of other GI cancers. The UK in particular

has experienced an alarming increase in incidence of ACO with a cumulative risk increase of 10-fold for men and 5-fold for women within just over a generation. In contrast, SCC is more common in eastern Asia and the incidence has remained stable or decreased within the same time frame.[265]

The incidence of BO within the adult western population is approximately 2%,[266] increasing up to 10% in individuals with symptoms of GORD.[267] However, not all patients suffering from BO progress to cancer and risk of developing cancer increases progressively in patients as the severity of epithelial dysplasia increases (see fig 1.10.2.1). The latest estimated progression rate is 0.22-0.83% per annum for patients with non-dysplastic Barrett's,[268, 269] however there is a large degree of variance within the literature depending on sample size and location in which the study was performed. In the UK alone, there were around 8,900 diagnoses of ACO a year (data collected in 2014) with five year survival rates as low as 15% (data collected 2010-2011).[270] A combination of rapidly increasing incidence and a dismal five year survival rate positions ACO as a serious current and future public health risk, particularly within the UK's white male population who currently represent the most at-risk group. However, it is possible to improve the poor prognosis of the disease through earlier detection, with data from the Netherlands showing an improvement in 5-year survival rates from 17% to 74%[271, 272] when patients with BO of at least 2 cm in length underwent endoscopic surveillance every three years and patients with low-grade dysplasia surveyed every year.

Clinical guidelines state the need for periodic endoscopy surveillance of patients to monitor progression of the condition.[273] However this is time-consuming, invasive for the patient and expensive, considering the low percentage of patients that will experience progression towards ACO. There exists then a need to more accurately determine which BO patients will progress using, where possible, less invasive methods. Well characterised biomarkers have the potential to meet this need.

1.12 Causes of Barrett's Oesophagus

The most important pathogenic factor for the development of BO has been shown to be the presence of severe, chronic, reflux disease. Patients with BO typically have decreased cardiac sphincter pressures and oesophageal dysmotility compared with

other forms of GORD reducing their ability to prevent gastric reflux and clearance of gastric contents when reflux does occur.[274] Reflux leads to long-term exposure of the lower oesophageal epithelium to gastric acid and bile salts, with bile salt presence being more common in BO patients than in other forms of GORD.[275] It is believed that the combination of both gastric acid and bile salt reflux, experienced to a greater extent in BO than other forms of GORD, explains why only 5-15% of chronic reflux sufferers present with BO.[276] Exposure to bile salts alone does not seem to contribute to the development of BO,[277] but exposure of oesophageal cells to bile salts and a low pH environment results in oxidative stress and DNA damage[278] activating the NF-κB pathway for programmed cell death.[279]

Recently, other potential noxious components of gastro-oesophageal reflux have emerged and a role for nitric oxide (NO) produced from dietary nitrates has been implicated. Following a meal rich in nitrates, NO generated from those nitrates has been shown to reach genotoxic concentrations at the gastro-oesophageal junction in GORD patients both with and without Barrett's oesophagus.[280] Dietary nitrate supplementation has also been shown to accelerate the development of oesophageal metaplasia in rat models with surgically-induced reflux oesophagitis.[281] Oesophageal tissue samples from rat models with surgically-induced reflux oesophagitis, fed a diet supplemented with nitrates, exhibited reduced expression of transcription factors that promote stratified squamous epithelium development, SOX2 and p63,[282] alongside increased expression of CDX2, a transcription factor that promotes intestinal differentiation. [282, 283] The combination of these events implicates that NO can drive the development of Barrett's by inhibiting the production of normal stratified squamous epithelium and create an environment favouring intestinal epithelium differentiation instead.

1.13 The role of gastrin in Barrett's oesophagus

The current first-line therapeutic treatment for patients with BO is PPIs which, as outlined previously, inhibit gastric acid secretion causing an increase in gastric pH.[284] This in turn causes reduced damaged to the lower oesophagus when reflux of the stomach contents does occur. However, treatment with PPIs at therapeutic doses may induce hypergastrinaemia in some patients. It is therefore significant that studies have suggested increased expression of functional CCK2 receptors in Barrett's epithelium

(BE)[160] compared to normal squamous epithelium indicating a potential role for gastrin to act on BE.

The well-known role of gastrin as a growth factor for gastrointestinal cells expressing the CCK2 receptor has already been mentioned[285] and further research on CCK2 receptor expressing BE and oesophageal adenocarcinoma cell lines have demonstrated gastrin stimulated proliferation[129] and protection from apoptosis.[286] Gastrin is also capable of stimulating expression of COX-2 in CCK2 expressing oesophageal cells which is known to be associated with the development of oesophageal and other GI malignancies.[287, 288] A recent meta-analysis of Barrett's patients including patients with dysplasia (low and high grade) and ACO showed that patients in the highest quartile of serum gastrin had a significantly increased risk of high-grade dysplasia and ACO.[289] Collectively, the data support the hypothesis that gastrin could be a driver of BO progression towards ACO via its role as a known growth factor and inducer of genes associated with inflammation and cellular invasion through degradation of extracellular matrix.

1.14 Biomarkers of Barrett's progression

The prospect of using biomarkers to track Barrett's progression toward adenocarcinoma has been approached before using immunohistochemical (IHC) techniques on biopsies retrieved during routine surveillance however success has been varied. A recent systematic review and meta-analysis comparing 16 different biomarkers across 36 studies identified a significant relationship between aberrant p53 expression and increased risk of development of high-grade dysplasia or ACO from non-dysplastic and low-grade dysplasia patients; aberrant *Aspergillus oryzae* lectin (AOL) staining was also identified as a cross-study potential predictor of neoplastic progression in BO biopsies.[290] Whilst IHC techniques may offer benefits in prediction of progression due to their ease of use and having multiple studies corroborating suspected targets, they remain reliant on the collection of biopsies by endoscopy which is costly and invasive for the patient.

Novel technologies are currently being developed to avoid the use of endoscopy to collect oesophageal cells for analysis and make the process less invasive. Promising results have been obtained using a device called the *Cytosponge* which consists of a

foam sponge inside a gelatin capsule attached to a string which is swallowed by the patient. After approximately five minutes, the gelatin capsule is dissolved allowing the sponge to expand within the oesophagus. Retrieval of the sponge by pulling the string collects epithelial cells from the length of the oesophagus for analysis by a variety of techniques.[291] Another approach has been to use untethered microgrippers that are dispersed in water and swallowed by the patient. Once swallowed, body heat closes the grippers which can then be retrieved from biopsies using a magnet. Currently the technology has only been successfully tested in pigs.[292]

The ideal biomarker-based assay would be minimally invasive (ideally blood-based), compatible with existing techniques that could be performed in the majority of diagnostic laboratories and thoroughly validated using multiple targets. The correlation between serum gastrin and risk of progression towards ACO may represent the first step in a blood-based biomarker for progression and proteins regulated by gastrin an extension of that. Currently the literature has focused solely on biopsy-based biomarkers (see table 1.14.1) so there exists a gap in knowledge of whether blood based biomarkers alongside biopsy based biomarkers could be a useful tool in monitoring BO.

Paper	**Biomarkers examined**
Moore et. al[**185**]	CgA, HDC, MMP-7, PAI-1 and -2
Boyce et. al[**293**]	CgA
Higham et. al[**195**]	CgA, HDC, VMAT2, Somatostatin

Table 1.14.1 – **Published works identifying biomarkers of gastrin stimulation in cells of the GIT**. A table consisting of the papers that have previous identified and used biomarkers of gastrin responsiveness in humans.

1.15 Aims and Objectives

This investigation focuses on the idea, suggested above, that gastrin stimulates the progression of BO progression towards dysplasia and finally cancer. To approach this, it was hypothesised that putative biomarkers of gastrin responsiveness in Barrett's tissue biopsies would provide a novel resource for further study. Since much of the existing relevant literature is based on the stomach, and to provide a basis for comparison, the potential of putative biomarkers of gastrin-responsiveness in gastric preneoplastic conditions was also examined. Since determination of serum gastrin is important in both cases, there was a requirement to validate the relevant gastrin radioimmunoassay to a standard compatible with good clinical laboratory practice and FDA guidelines.

The specific aims were:

- To validate the reliability and accuracy of our gastrin RIA
- To characterise the expression of gastrin-regulated genes in preneoplastic conditions of the stomach and oesophagus.
- To identify if a significant relationship exists between serum gastrin concentration and expression of gastrin regulated genes in preneoplastic conditions of the stomach
- To identify the expression profile of putative biomarkers of gastrin-responsiveness in BO biopsies
- To validate identified putative biomarkers of gastrin-responsiveness in calibrated cell lines of gastric cancer transfected with the CCK2R.

Chapter 2

Materials and Methods

2.1 Sample collection and storage

2.1.1 Patient Recruitment

2.1.1.1 Series 1

Subjects were selected from a cohort of ~1,400 patients, aged 18 and over, who had clinical indications for undergoing diagnostic upper gastrointestinal endoscopy. Control subjects were selected for investigation if they were *H. pylori* negative and exhibited no endoscopic or histological evidence of upper gastrointestinal neoplasia or preneoplastic pathology (atrophic gastritis, gastric intestinal metaplasia, or Barrett's oesophagus). Barrett's or gastric preneoplastic (atrophy, intestinal metaplasia or both) patient groups included subjects that were *H. pylori* negative and exhibited endoscopic and histological evidence of the relevant condition. Further exclusion criteria included diabetes mellitus, coma or hemodynamic instability, being moribund or having terminal malignancy, cirrhosis (Child B or C), abnormal clotting or bleeding diasthesis, inability to give informed consent, contraindication to endoscopy, pregnancy, HIV, hepatitis B or C infections.

Subjects underwent diagnostic gastroscopy in the Gastroenterology Unit at the Royal Liverpool University Hospital. Endoscopic pinch biopsies of gastric corpus and antrum (2–4 of each) were obtained for histology; *H. pylori* status was determined on the basis of serology, antral urease test (Pronto Dry; Medical Instrument, Solothurn, Switzerland), and antral and corpus histology. The study was approved by the Liverpool Local Research Ethics Committee and by the Royal Liverpool and Broadgreen University Hospitals NHS Trust, and all patients gave written, informed consent.[211]

2.1.1.2 Series 2

Subjects were recruited from a list of patients having already been identified at previous endoscopy as having Barrett's oesophagus (confirmed by histopathology) of a continuous length of at least 2 cm and were already attending the hospital for a routine surveillance endoscopy. Exclusion criteria for the study were the same as those defined in 2.1.1.1. Potential patients were presented with a participant information sheet and consent form upon their arrival in clinic and given 10-15 minutes to read the

documents before being approached by a member of the research team prior to endoscopy and details of the project explained and any questions answered. If the patient was willing to participate in the study the consent form was completed.

	Whole Group	IM Only	Mixed Metaplasia	Dysplasia (LGD,HGD)	Cancer
Number of patients	54	36	11	5	2
N male	45	28	12	4	2
N female	9	8	0	1	0
Mean age	62	62	58	62	66
Mean gastrin (pM)	104	107	82	119	89

Table 2.1 – **Breakdown of Barrett's biopsy sub-groups.** All Barrett's biopsies underwent histopathological examination and were organised into various sub-groups for data analysis. Biopsies were grouped based on the most severe histological diagnosis (from least to most severe: IM, mixed metaplasia, dysplasia and finally cancer).

At the time of endoscopy, if the patient had consented, up to 20 mL of blood was taken from the patient with up to 6 Barrett's pinch biopsies taken for research purposes in addition to those required for routine histopathology. The study was approved by the Liverpool Local Research Ethics Committee and by the Royal Liverpool and Broadgreen University Hospitals NHS Trust or University of Szeged ethics committee.

In both studies (series 1 and 2) patients had the right to withdraw consent at any time, upon which all biological samples would be destroyed and any derived data deleted. A breakdown of Series 2 patient information and gastrin concentration, grouped by histological findings is shown in table 2.1.

2.1.2 Human blood samples

Human venous blood samples for serum were drawn into 7.5 mL Sarstedt S-Monovette® tubes with clotting activator (Sarstedt, Germany) and stored on ice immediately until centrifuged (2500 x g, 10 min, 4°C) and serum separated. Venous blood samples for plasma were drawn into 9 mL Sarstedt S-Monovette® (Sarstedt, Germany) tubes with K2 EDTA gel at a concentration of 1.6 mg/mL of blood and stored on ice immediately until centrifuged (2500 x g, 10 min, 4°C) and plasma separated. Serum or plasma were stored frozen at -20°C.

2.1.3 Human tissue samples

2.1.3.1 Gastric biopsies

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 for histopathological assessment and from the corpus (8 biopsies) for subsequent determination of putative biomarkers by quantitative polymerase-chain reaction (qPCR).[294]

2.1.3.2 Oesophageal biopsies

Enrolled subjects underwent routine upper GI gastroscopy using the same equipment defined in 2.1.3.1, and biopsies were taken of Barrett's mucosa.

2.1.3.3 Processing of biopsy material

Specimens for histology were 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 incorporating descriptive diagnoses and scores for the modified Sydney classification,[107] Padova classification[295] and modified Vienna classification.[296,294]

Biopsies for putative biomarker analysis by qPCR were placed immediately in 1 mL RNAlater® RNA stabilising buffer (ThermoFisher Scientific, UK) and stored on ice before incubation at 4°C for 24 h. Samples were then transferred to a fresh 1 mL of RNAlater® and stored at -20°C until use.

2.2 Radioimmunoassay

2.2.2 Production of stripped plasma

Human stripped plasma, to be used as a diluent for human plasma samples and standards, was generated from a frozen sample of 500 mL of outdated blood bank human plasma (Baxter healthcare, UK) was defrosted and stripped of endogenous peptides by incubation with 50 g activated charcoal (Sigma-Aldrich) at 4°C for 30 min and then centrifuged (3000 x g, 4°C, 10 min). The supernatant was initially filtered through glass fibre filter paper (Whatman GF/B, Sigma-Aldrich) to remove larger particulates of charcoal then filtered through Sep-Pak C18 cartridges (Waters, UK) that had been primed with 10 mL acetonitrile 50% v/v in distilled water and washed with 10mL 0.02 M sodium barbitone buffer pH 8.2 to remove very fine charcoal particulates and hydrophobic solutes. The plasma was then aliquoted and stored at -20°C.

2.2.3 Determination of circulating gastrin concentration

Circulating gastrin concentrations were analysed in duplicate by radioimmunoassay (RIA). Samples were diluted 1:20 in Veronal buffer (0.02 M sodium barbitone), containing 0.5 g/L sodium azide and 0.1% bovine serum albumin (Jackson immunoresearch, USA) adjusted to pH 8.4, then incubated in the presence of 100 µL [^{125}I]Tyr^{12} radiolabelled G17 peptide (Lot. No. CP21770, Perkin Elmer customs and radiotherapeutics division, Poland) and 100 µL antibody for 48 h at 4°C. Three antibodies were assessed for specificity to different gastrin peptides – L2,[297] L6[50] and 1295[298] (all produced by the University of Liverpool, UK) at concentrations of 1:300,000, 1:40,000 and 1:100,000 respectively. Ultimately antibody L2 was utilised for total serum gastrin concentration quantification as a result of validation experiments performed in chapter 3.

Post-incubation, the free radiolabel was separated from antibody-bound by addition of 100 µL dextran-coated charcoal (5 g activated charcoal, 250 mg dextran (Sigma-

Aldrich), 250 mg skimmed milk powder (Marvel International Food Logistics Ltd., UK) and 50 mL distilled H_2O) and centrifugation at 1976 x g for 10 min at 4°C. The supernatant was decanted and the radioactivity of both supernatant and pellet counted for 1 min using a Packard Bell RIAstar gamma counter. The ratio of bound to free (B/F) label in the sample minus the non-specific binding of the radiolabel was calculated.

Quantification of unknown gastrin concentration was determined by comparing the B/F for samples to that of a standard curve included in each assay. Standard curve was generated via the serial dilution of synthetic human gastrin peptides covering a final concentration range of 0.1-100 pM with the addition of a volume of charcoal-stripped human plasma identical to sample volume in unknown tubes to account for any matrix effect.

A number of progastrin derived peptides were used for antibody validation including synthetic human non-sulphated G17 (Bachem, UK), G34 (Pharmaceuticals, USA), G17-CFP (Pepsyn, University of Liverpool, UK) and G17-Gly (Pepsyn, University of Liverpool, UK). A number of different synthetic human non-sulphated G17 peptides were also tested to determine which supplier produced the peptide with the greatest specificity for our antibodies – suppliers included Bachem, UK; Phoenix Pharmaceuticals, USA; Sigma-Aldrich, UK and Anaspec, Belgium. The G17 peptide produced by Sigma-Aldrich exhibited the greatest specificity towards our antibodies in experiments presented in chapter 3 and thus was the standard used for all patient circulating gastrin quantification.

2.3 Enzyme-linked immunosorbent assays (ELISA)

2.3.1 H. pylori status

Serum samples collected for the series 2 cohort were tested for the presence of antibodies to *H. pylori* using a commercial *H. pylori* ELISA kit (Biohit HealthCare, UK) according to manufacturer's instructions. Serum samples were diluted 1:200 on manufacturer's recommendation using the supplied diluent and vortexed. To the plate 100 µL of diluent buffer (blank), supplied calibrator solutions and supplied positive and negative control samples were added in duplicate as well as patient samples; samples were then sealed and incubated for 30 min at 37°C. The solution was then

removed by inverting the plate and firmly tapping on absorbent tissue, and the plate was washed three times with 350 µL of supplied wash buffer per well, ensuring all wash buffer was removed between washes by firmly tapping the inverted plate onto absorbent tissue. To each well, 100 µL of conjugated antibody solution was added and the plate incubated at 37°C for 30 min before removal, after which the washing step was repeated. Next, 100 µL of supplied substrate solution was added to each well and incubated in the dark at room temperature for 30 min before 100 µL of provided stop solution was added to complete the reaction. The absorbance of the well was then measured at 450 nM on a Tecan GENios plate reader using manufacturer supplied software. Using a calculation provided by the manufacturer to convert absorbance to enzyme immune units (EIU) combined with the calibrator curve run in-plate, sample absorbance was transformed with an EIU result of <40 representing a negative result and >40 representing positive.

2.4 Gene expression analysis

2.4.1 RNA extraction from biopsies

Biopsies were removed from -20°C storage and emptied onto absorbent paper to absorb the RNAlater® in which they had been stored, then placed in 1mL TRIzol® reagent (ThermoFisher Scientific) and homogenised for 15-20 sec. Once homogenised, 200µL of chloroform (VWR, UK) was added to each sample and the samples were vigorously shaken by hand for 15 sec before being allowed to settle for 5 min at room temperature and centrifuged (15 min, 12,000 x g, 4°C).

The clear aqueous phase was carefully removed by pipetting into a new Eppendorf tube, to which 500 µL of isopropanol was added (Sigma-Aldrich), the tubes were then again vigorously shaken by hand for 15 sec settling for 5 min at room temperature. Samples were then centrifuged (10 min, 12,000 x g, 4°C), and the supernatants poured off and discarded. To the remaining RNA pellet, 500µL of ice-cold 75% ethanol (diluted with DEPC treated H_2O from VWR) was added before briefly vortexing and centrifuging (5 min, 12,000 x g, 4°C).

The supernatant was again discarded and to the pellet 500 µL ice-cold 100% ethanol (VWR) was added before briefly vortexing and centrifuging (5 min, 12,000 x g, 4°C).

The supernatant was then discarded and the pellets were allowed to air dry for 30 min at room temperature inside a clean PCR hood.

Once dry, 50 µL DEPC treated DNase/RNase-free distilled water (ThermoFisher Scientific) was added to the RNA pellets which were then re-suspended by placing onto a shaker for 15 min. The RNA concentration was then quantified using a Nanodrop 2000c.

2.4.2 Reverse transcription of RNA to cDNA

Based on the RNA concentration, a volume equivalent to 4 µg RNA was reverse transcribed. To this, 2 µL of oligo dT primer (Promega, UK) was added and the total volume made up to 39 µL with DEPC treated water. The tubes were then briefly microfuged and incubated at 70°C for 5 min before being allowed to cool to 40°C.

Once the tubes had cooled to room temperature 12 µL 5 x AMV buffer (Promega), 5 µL 12.5 mM DNTPs (Sigma-Aldrich), 1 µL RNAsin (Promega) and 3 µL AMV reverse transcriptase (Promega) was added. The tubes were then pulsed using a microfuge and incubated at 42°C for 1 h, following which they were incubated at 80°C for 5 min before being cooled on ice. The samples were then divided into 4.3 µL aliquots and stored at -20°C ready for qPCR.

2.4.3 Singleplex qPCR of biopsy cDNA

Real time qPCRs were carried out using an Applied Biosystems AB7500 system with manufacturer supplied software (version 1.4.1). Reagents used were PrecisionPLUS low ROX mastermix (PrimerDesign, UK) and Taqman™ technology based primer/probe pairs. Primers and probes were designed using Primer Express v3.0 to be intron spanning and were purchased from Eurogentec (Belgium) and sequences are displayed in table 2.2.

Primer and probe sequences used were as follows:

Gene Designation	Sequence	
GAPDH	Forward	GCT CCT CCT GTT CGA CAG TCA
	Reverse	ACC TTC CCC ATG GTG TCT GA
	Probe	CGT CGC CAG CCG AGC CAC A-FAM
CGA	Forward	GAT ACC GAG GTG ATG AAA TGC A
	Reverse	TCC TTC AGT AAA TTC TGA TGT CTC AGA
	Probe	CCA GCC CCA TGC CTG TCA GCC-FAM
HDC	Forward	CCC TGA GCC GAC GGT TT
	Reverse	GTA CCA TGT CTG ACA TGT GCT TGA
	Probe	CTC TGT TAA ACT CTG GTT CGT GAT TCG GTC C- FAM
MMP-1	Forward	CCA ACA ATT TCA GAG AGT ACA ACT TAC AT
	Reverse	TGA AGG TGT AGC TAG GGT ACA TCA AA
	Probe	TTG CAG CTC ATG AAC TCG GCC ATT C-FAM

Table 2.2 – **Forward, reverse and probe sequences used for qPCR.**

The qPCR mixture was prepared using 4 µL (267 ng) cDNA, 50 µL 2 x mastermix, 3 µL 10 µM forward primer, 3 µL 10 µM reverse primer, 4 µL 5 µM probe and 36 µL of DEPC-treated water to total 100 µL. Then, 25 µL of PCR mixture was pipetted in triplicate into clear 96 well plates (Starlab UK), sealed and exposed to a thermal profile of one cycle at 50°C for two minutes, one cycle at 95°C for 10 minutes then 40 cycles comprising 15 seconds at 95°C and one minute at 60°C with fluorescent data collection at the end of the 60°C period.

Each plate contained a no template control, three quality controls and a standard curve in order to quantify mRNA abundance. Results were only considered accurate if the

three quality controls were within 15% of expected mean values, PCR amplification efficiency was between 90-110% and the correlation coefficient of the standard curve was >0.97.

2.4.4 Multiplex qPCR of biopsy cDNA

Real time qPCRs were carried out using an Applied Biosystems AB7500 system with manufacturer supplied software (version 2.3). Reagents used were PrecisionPLUS low ROX mastermix (PrimerDesign, UK) and Taqman™ primer/probe pairs. Primers and probes were designed using Primer Express v3.0 to be intron spanning and were purchased from Eurogentec (Belgium) except those for COX-2 (ThermoFisher scientific, UK), CCK2R and SHH (PrimerDesign).

Primer and probe sequences used that feature in method 2.3.3 above were retained for multiplex reactions, however the GAPDH probe was reformulated to the same sequence with a conjoined TAMRA dye rather than FAM to avoid spectral overlap and allow accurate multiplexing with FAM labelled probes. Primer and probe sequences used in multiplexed qPCR reactions are shown in table 2.3.

Gene Designation	Sequence	
MMP-7	Forward	GGA TGG TAG CAG TCT AGG GAT TAA CT
	Reverse	GGA ATG TCC CAT ACC CCA AGA A
	Probe	CCT GTA TGC TGC AAC TCA TGA ACT TGG C-FAM
COX-2	Proprietary sequences designed by ThermoFisher, assay ID:Hs00153133_m1	
CCK2R	Forward	TGA CTC TGG GAT GCT CCT AGT
	Reverse	TGG TCA GAG GTA TGA GAT TAG GC
	Probe	ACC TCA CAG TGA CCC TTC CCA ATC AGC-FAM
TFF1	Forward	GCC CAG ACA GAG ACG TGT ACA G
	Reverse	CGT CGA AAC AGC AGC CCT TA
	Probe	CTG GTG TCA CGC CCT CCC AGT GT-FAM
TFF2	Forward	GGG TCC CCT GGT GTT TCC
	Reverse	GAG ACC TCC ATG ACG CAC TGA
	Probe	CCC CCT CCC AAA GCA AGA GTC GG-FAM
SHH	Forward	GAA GAG GAG GCA CCC CAA AA
	Reverse	CCT TAA ATC GCT CGG AGT TTC TG
	Probe	ACC CCT TTA GCC TAC AAG CAG TTT ATC CCC A-FAM

Table 2.3 - **Forward, reverse and probe sequences used for multiplex qPCR**.

Primers were diluted from a stock concentration of 100 µM to 5 µM with probe diluted to 4 µM in DEPC-treated H_2O then, using the combined diluted primer/probe pairs, the qPCR mixture was prepared using 8 µL (8 ng) cDNA, 40 µL 2 x mastermix, 4 µL combined GAPDH probe/primer mix, 4 µL combined target probe/primer mix and 36 µL of DEPC-treated water to total 92 µL. Then, 20 µL of PCR mixture was pipetted in triplicate into clear 96 well plates (Starlab UK), sealed and exposed to a thermal profile of one cycle at 95°C for 10 minutes, then 40 cycles comprising 20 seconds at 94°C, 10 seconds at 59°C and one minute at 60°C with fluorescent data collection at

the end of the 60°C period. Each plate also included a no template control to rule out possible contamination of PCR reagents.

2.4.5 Optimisation of multiplex qPCR

Initially, the same qPCR reaction mixture employed in the singleplex qPCR assay was attempted for use in multiplex assays with the volume of DEPC-treated H_2O adjusted to account for extra primer/probe volume. However, this resulted in uneven amplification of cDNA as shown in figure 2.4.5.1.

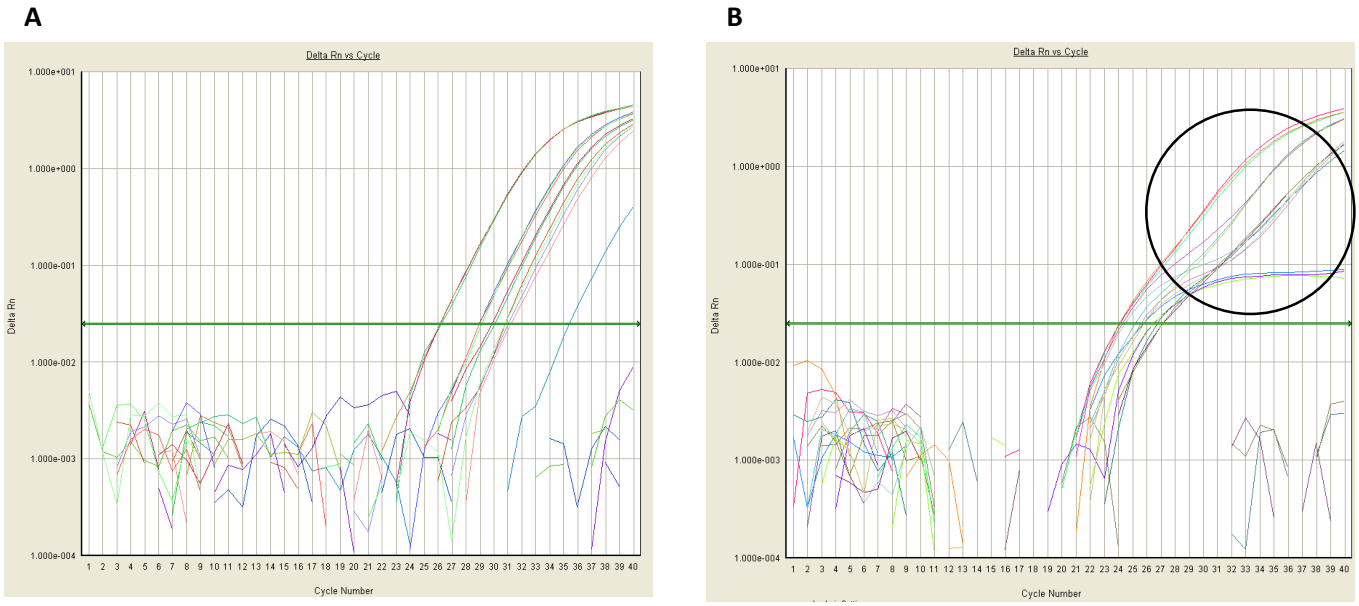

Figure 2.4.5.1 – **A comparison of the qPCR amplification curves generated via a singleplex qPCR and a multiplexed qPCR reaction before optimisation.** Amplification curves generated utilising a primer/probe pair for CgA using the same starting cDNA samples. Curves generated using the singleplex method (A) show smooth logarithmic amplification curves plateauing as expected towards the final few cycles of amplification. Curves generated using a multiplexed qPCR reaction mix including a GAPDH primer/probe pair similar to that of the singleplex mixture however (B) exhibit uneven amplification in some samples towards the end of the reaction (circled above).

This observation cast doubt over the validity of such qPCRs and was suspected to be the result of either spectral overlap of the qPCR FAM probes or due to insufficient concentrations of reagents within the mastermix to facilitate logarithmic amplification of two cDNA sequences. This led to the formulation of new qPCR probes with dyes that did not feature a spectral overlap and the altering of reagent volumes in the qPCR

reaction mixture to ensure sufficient reagents were present to carry out the reactions. Post optimisation the qPCRs exhibited the expected smooth logarithmic amplification curves as shown in figure 2.4.5.2.

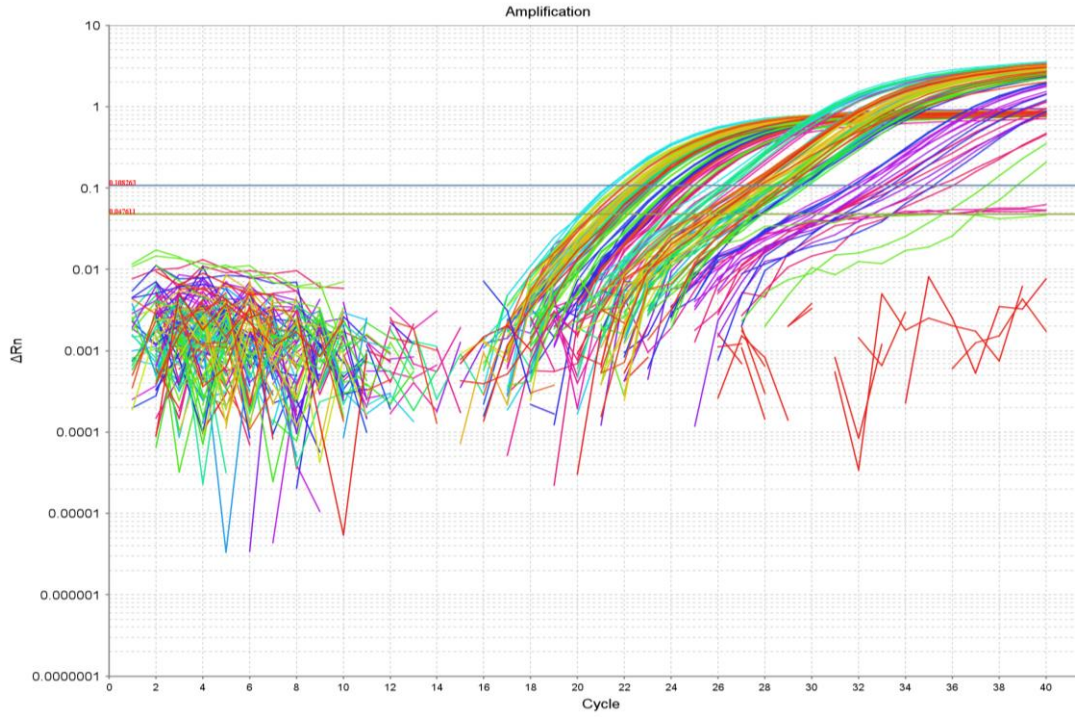

Figure 2.4.5.2 – **Successful logarithmic amplification of both target sequences post-optimisation of multiplex qPCR protocol.** As with figure 2.4.5.1B, figure 2.4.5.2 represents multiplexed GAPDH and CgA qPCRs using real patient biopsy cDNA samples. Difference in screenshot appearance derives from different versions of Applied Biosystems analysis software used as detailed in sections 2.4.3 and 2.4.4.

2.4.6 Validation of multiplex qPCR results compared to singleplex

Considering the necessary changes in qPCR protocol between singleplex and multiplex reactions outlined in 2.4.4 and 2.4.5, it was crucial to compare the results of both protocols to identify whether comparisons could be made between data acquired via each method. Comparing the results obtained using the quality control samples included in a set of singleplex and multiplex reactions the mean co-efficient of variation between methods was calculated at 1.05% with expected quality control values well within the acceptable range for an assay (within the 15% accepted variance between assays).

2.5 MicroRNA expression analysis

2.5.1 Extraction of microRNA from serum

Cell free microRNAs were extracted from human patient samples using the Qiagen™ miRNeasy serum/plasma kit (Qiagen, UK) according to the manufacturer's instructions. In brief, 200 µL of serum was added to 1 mL QIAzol lysis reagent (Qiagen) aspirated three times and incubated at room temperature for 5 min. To each sample, were then added 3.5 µL *C. elegans* miR-39 miRNA mimic as a reverse transcription control at a concentration of 1.6 x 10^8 copies per µL, then 200 µL chloroform. Samples were vortexed at max speed for 15 sec and incubated again at room temperature for three min. The samples were then centrifuged for 15 min at 12,000 x g and 4°C, at which point 600 µL of the top clear layer of each sample was carefully removed by pipetting and transferred to a fresh Eppendorf tube, the rest of the sample was discarded. To the 600 µL sample, 900 µL of 100% ethanol was added and mixed by aspirating three times by pipette; after which the sample was passed through a supplied miRNeasy spin column 700 µLs at a time by centrifugation at 8000 x g for 30 sec at room temperature (two passes required for full sample). The flow through was discarded, then 700 µL of supplied buffer RWT was passed through the column by centrifugation (8000 x g, 30 sec), again flow through was discarded before 500 µL of supplied buffer RPE was passed through the column in an identical fashion.

2.5.2 Reverse transcription of serum extracted microRNA

Extracted microRNAs were reverse transcribed using the Qiagen™ miScript II RT kit (Qiagen) according to the manufacturer's instructions. In brief, microRNA trapped within the columns provided with the extraction kit was eluted into 14 µL DEPC treated DNase/RNase-free distilled water and quantified using a Nanodrop 2000c. Samples were normalised to 60ng RNA per reverse transcription reaction and the following added: 4 µL 5x miScript hiSpec buffer 2 µL 10x miScript Nucleics Mix, 2 µL miScript reverse transcriptase mix and DEPC-treated H_2O to 20 µL.

Samples were then briefly mixed before incubation at 37°C for 60 min in a thermomixer, at which point the samples were then incubated at 95°C for 5 min.

2.5.3 Reverse transcription of biopsy extracted microRNA

Biopsy-derived miRNAs were reverse transcribed using the same method as in 2.4.2, however the original RNA was extracted using method 2.3.1 not 2.4.1 as with serum.

2.5.4 qPCR of microRNA

Real time qPCRs were carried out using an Applied Biosystems AB7500 system with manufacturer supplied software (version 2.3). Reagents used were Qiagen miScript SYBR green mastermix (Qiagen) and Qiagen miScript primer assay primer pairs. Primers were designed in-house by Qiagen, UK and as such remain proprietary so sequences cannot be listed here.

The qPCR reactions were prepared using 10 µL (10 ng) miRNA, 50 µL mastermix, 10 µL miScript assay primer mix and 30 µL DEPC-treated H_2O. Then, 25µL of PCR mixture was pipetted in triplicate into white 96 well plates (Starlab), sealed and then exposed to a thermal profile of one cycle at 95°C for 15 minutes then 40 cycles comprising 15 seconds at 94°C, 30 seconds at 55°C and 35 seconds at 70°C with fluorescent data collection at the end of the 70°C period.

Each plate contained two sets of identical miRNA samples – one being analysed for endogenous control (RNU6B) abundance and the other for abundance of miRNA of interest, so all normalisation was performed using data derived from the same plate, mastermix preparation and qPCR run to minimise variance. Each plate also included a no template control to rule out possible contamination of PCR reagents.

2.5.5 Representation of qPCR Data

All qPCR Data presented within this thesis, unless otherwise stated, have been converted to fold difference relative to the relevant control group (controls for series 1 biopsies, serum gastrin <30 pM for series 2 biopsies/serum) which is depicted as having a mean fold change of 1.0 ± SEM. Transformation of raw Ct values to fold change was performed by taking the Ct value of the housekeeping gene (GAPDH) and subtracting it from the Ct value of the target sequence -producing a ΔCt value. Then the mean ΔCt value of the control group was subtracted from the mean ΔCt value of the groups with elevated gastrin to produce the ΔΔCt value. The ΔΔCt value was

converted to fold change by use of the equation 2^-(ΔΔCt) to account for the logarithmic function of qPCR.

SEM was calculated from raw mean Ct data where variance was expressed as a percentage of the original value and scaled to represent the same percentage variation when applied to the fold change value.

2.6 Cell culture

2.6.1 Recovery of frozen cell lines

Cells were removed from liquid nitrogen stored in cell culture freezing medium (ThermoFisher scientific) and thawed in a water bath at 37°C before plating in 20 mL cell-appropriate media in a T-75 culture flask (ThermoFisher scientific).

2.6.2 Cell maintenance

AGS (CRL-1739, American Type Culture Collection, USA) and AGS-GR (AGS cells stably transfected with the CCK2R under puromycin selection [72]) cells were maintained in 10 mL Ham's F-12 medium (Sigma-Aldrich) supplemented with 10% FBS (ThermoFisher scientific) and 1% (wt/vol) penicillin/streptomycin (Sigma-Aldrich) referred to as full media (FM). Serum-free (SF) media consisted of all components of FM except 10% FBS. Cells were grown at 37°C in a 5% v/v CO_2 atmosphere with medium changes every 48 h.

HGT cells kindly donated by Dr. C. Laboisse (INSERM U239, Hôspital Bichat, Paris, France)[299] were stably transfected with the CCK2R using the same method as the AGS cells referenced above. HGT-GR cells were maintained in 10mL RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS and 1% (wt/vol) penicillin/streptomycin referred to as full media (FM). Serum-free (SF) media consists all components of FM except 10% FBS. Cells were grown at 37°C in a 5% v/v CO_2 atmosphere with medium changes every 48 hours.

2.6.3 Treatment protocol

Cells were plated at a density of $5x10^6$ cells per flask in FM and returned to the incubator for one day to allow for adherence to the flask. The next day FM was removed and cells washed twice with 10 mL sterile PBS (ThermoFisher scientific)

before 10 mL SF media was added. To cells being treated with G-17, a volume of synthetic human G-17 (Sigma-Aldrich) diluted in 0.05 M ammonium bicarbonate buffer (AmBic) (BDH Chemicals, UK) was added equivalent to the final concentration required per flask. Cells were then returned to the incubator and incubated for 24 hours before removal of media/cells.

2.6.4 Removal and protein purification of treated media

After 24 h treatment cell media was removed from the culture flasks via a pipette, transferred to a 15 mL Falcon™ centrifuge tube (FisherScientific, UK) and stored immediately on ice. Tubes were then centrifuged at 800 x g for 7 min at 4°C before the top 9 mL of media was carefully transferred to a new centrifuge tube, discarding the last 1 mL as a possible source of cell contamination of media/cell fragments. To the new tubes, 25 µL of StrataClean resin (Agilent technologies, UK) was added and the tubes vortexed for 1 min to ensure complete mixing before centrifugation (2000 x g, 3 min, 4°C). Media were then removed and discarded and the resin pellet resuspended in 0.5 mL AmBic buffer, transferred to a clean Eppendorf tube and vortexed to ensure thorough washing of the resin. The tube was then centrifuged (2000 x g, 2 min, 4°C) to re-pellet resin, supernatant removed and the washing process repeated three times before a final resuspension of resin in 80 µL AmBic before storage at -80°C.

2.6.5 Preparation of whole cell lysates

After removal of media cells were washed twice with 10 mL sterile PBS and 100 µL RIPA lysis buffer added to the flask (25 mM Tris-HCL pH 7.6, 150mM NaCl, 1% w/v NP-40, 1% w/v sodium deoxycholate and 0.1% w/v sodium dodecyl sulphate) containing 1% v/v protease inhibitor set III, EDTA-free (Calbiochem, Germany) and 1% v/v phosphatase inhibitor cocktail set III, EDTA-free (Calbiochem). Cells were manually scraped from the flask base in the presence of this buffer and then the cell-containing buffer solution removed and pipetted into Eppendorf tubes, sonicated for 5 min and incubated on ice for 30 min. The lysates were then centrifuged (12000 x g, 4°C, 30 min) and the supernatants stored at -80°C.

2.7 Western blots

2.7.1 Protein quantification

To ensure equal loading in western blotting, protein concentrations were quantified using modified Lowry DC protein assays (Amersham pharmacia, UK). Bovine serum albumin (Jackson immunoresearch) was used to establish a standard curve spanning 0.125-2 µg/mL in triplicate in 96 well Nunc-Immuno Maxisorp™ plates (Sigma-Aldrich). Similarly, triplicates of 5 µL of sample diluted in RIPA lysis buffer (1:10) were added followed by the addition of 25 µL reagent A and 200 µL of reagent B. The samples were incubated at room temperature for 30 min and absorbance was measured at 450 nm on a Tecan GENios plate reader using manufacturer supplied software. Cell media protein concentration was unable to be quantified due to the presence of the resin purification beads combined with a very low concentration of secreted protein, thus for normalisation an equal volume of cell media sample was used for each treatment condition when blotting.

2.7.2 SDS polyacrylamide gel electrophoresis

Proteins were resolved by discontinuous sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). A separating gel of 4-12% (TIMPS) or 12% (MMPs) w/v sodium dodecyl polyacrylamide (ThermoFisher scientific) was inserted into an XCell surelock™ mini-cell electrophoresis assembly (ThermoFisher scientific) according to the manufacturer's instructions and immersed in 500 mL 1 x NuPAGE MOPs running buffer and 1% NuPAGE antioxidant (ThermoFisher scientific) with 2 gels run simultaneously in the same assembly. Each gel was loaded with 5 µL MagicMark™ XP western protein standard (ThermoFisher scientific) to provide molecular weight standards and to monitor electro-blotting progression. Cell media samples were reduced by combining 20 µL cell media with 8 µL NuPAGE LDS sample buffer (ThermoFisher scientific), 3.2 µL NuPAGE reducing agent (ThermoFisher scientific) and 0.8 µL H_2O. Cell lysate samples were reduced by combining a volume equivalent to 20 µg total protein with the same reducing mix, varying H_2O volume to match the total volume of that used for cell media reduction (32 µL). Once samples were prepared for reduction they were incubated at 100°C for 5 min after which 25 µL of each sample was carefully pipetted into the wells of the gel. The gel was then run at a constant voltage of 200v for 50 min.

2.7.3 Electrophoretic transfer

Proteins were electrotransferred from the gel onto Amersham Protran 0.45 nitrocellulose membranes (GE healthcare life sciences, UK) in 1 x NuPage transfer buffer and 1% NuPAGE antioxidant (ThermoFisher scientific) at a constant voltage of 30 v for 1 h. Membranes were then washed in 10 mL Tris-buffered saline containing 0.1% Tween 20 (TBS-T) (Sigma-Aldrich) on a rocking platform for 10 min, repeating three times. They were then blocked for 1 h with 10 mL blocking buffer (TBS-T containing 5% w/v skimmed milk powder).

2.7.4 Application of antibodies

Primary antibody was diluted in 5 mL blocking buffer and applied to membranes overnight on a rocking platform at 4°C. On the next day, membranes were washed three times in TBS-T on a rocking platform and 5 mL horseradish peroxidase (HRP)-conjugated secondary antibodies were applied, diluted in the same buffer as primary antibodies, for 1 h on a rocking platform. Membranes were then washed again three times in TBS-T on a rocking platform. Primary and secondary antibody information is listed in table 2.4.

2.7.5 Development of blots

Membranes were semi-dried with Whatman™ filter paper (GE Healthcare Life Sciences) before having a thin layer of Clarity™ ECL substrate (Bio-Rad, UK) pipetted on top and then incubated for 5 min protected from light. They were then exposed and images captured using a ChemiDoc XRS imaging system (Bio-Rad).

2.7.6 Densitometry evaluation of band intensity

The intensity of bands was quantified using BioRad Imagelab software version 5.2.1 and for cell lysate samples normalisation was performed relative to the intensity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Antibody	Species	Dilution	Supplier	Cat. No
GAPDH	Mouse	1:1000	Meridian life science, USA	H86504M
TIMP-1	Goat	1:100	R&D Systems, UK	AF970
TIMP-3	Mouse	1:500	R&D Systems, UK	MAB973
MMP-1	Goat	1:500	R&D Systems, UK	AF901
MMP-2	Goat	1:200	R&D Systems, UK	AF902
MMP-3	Rabbit	1:1000	Abcam, UK	AB52915
MMP-7	Rabbit	1:1000	University of Liverpool, UK	L522(G)
Anti-mouse	Goat	1:10,000	Sigma-Aldrich, UK	A4416
Anti-goat	Rabbit	1:10,000	Sigma-Aldrich, UK	A5420
Anti-rabbit	Goat	1:10,000	ThermoScientific, UK	31460

Table 2.4 - **Primary and secondary antibodies, their dilutions, suppliers and catalogue numbers used for western blot analysis.**

Chapter 3

Validation of Circulating Gastrin Assay

3.1 Introduction

As detailed in section 1.3.1, multiple biologically active peptides can be produced from the *gastrin* gene, including the C-terminally amidated gastrins, G17 and G34, along with N-terminally extended forms G17-Gly, G34-Gly and G17-CFP.[58] Currently the most well understood forms of gastrin, in relation to biological activity on tissues, are the amidated gastrins and thus the first step in investigating potential biomarkers of gastrin stimulation on tissues is the availability of an accurate and specific assay of circulating gastrin peptides. To this end, the present study made use of a gastrin radioimmunoassay since (a) there are doubts concerning the accuracy of commercially available ELISAs,[300] (b) the laboratory had previous experience with the technique,[50] and (c) a range of specific antibodies to gastrin were available from previous studies. Importantly, however, these antibodies had been in storage for approximately 40 years and, in spite of changes in RIA technology, had not been validated for many years. The re-validation of the gastrin RIA was therefore considered essential.

It is known that *H. pylori* infection,[301] PPI usage[302] and age[302] can influence circulating gastrin concentration.[303] It was therefore considered important to take this into account in designing the study. Two series of samples were available. The first (i.e. series 1) had been archived some years previously and the relevant clinical data was available, including histological reports. The second (i.e. series 2) was collected during this study and in this cohort all patients were already being treated with PPIs. *H. pylori* status for series 1 patients had already been performed and data were available whereas for series 2 patients current or previous infection by *H. pylori* bacteria was determined by serology during the course of the study.

Samples for series 2 were collected across two study centres – the Royal Liverpool University Hospital (RLUH) and the Gastroenterology Unit of the University of Szeged (UoSz), so the development of a gastrin and patient profile for both cohorts is crucial in identifying any regional differences (change in rates of *H. pylori* prevalence for example) that may skew the data and prevent them from being considered a singular cohort.

3.1.1 Objectives

Specific objectives of this chapter were:

- To validate the gastrin RIA.
- Using this assay, determine the circulating gastrin profile in patients of series 1 and 2.

3.2 Materials and methods

3.2.1 Determination of circulating gastrin concentration

3.2.1.1 Validation of antibodies

Three different gastrin antibodies were assessed for specificity to gastrin peptides in radioimmunoassay (RIA) – namely antibodies L2,[297] L6[50] and 1295[298] at dilutions of 1:300,000, 1:40,000 and 1:100,000 respectively.

3.2.1.2 Radioimmunoassay

Circulating gastrin concentration was quantified by RIA according to the protocol outlined in section 2.2.3, by comparing the ratio of free/bound radiolabel in patient samples to a 10 point standard curve generated using synthetic G17. A range of gastrin peptides (figure 3.2.1) were studied to determine antibody specificity.

G34 - QLGPQGPPHLVADPSKKQGPWLEEEEEAYGWMDF-NH_2

G17 - QGPWLEEEEEAYGWMDF-NH_2

G17-Gly - QGPWLEEEEEAYGWMDFG

G17-CFP - QGPWLEEEEEAYGWMDFGRRSAEDEN

Figure 3.2.1 **– Amino acid sequences of gastrin peptides used for antibody validation.**[304]

3.2.2 Production of stripped plasma

Human stripped plasma, to be used as a suitable diluent matrix for human samples and standard curve standards, was generated using the protocol detailed in methods section 2.2.2.

3.2.3 Determination of H. pylori infection status

Infection status was determined using a Biohit *H. pylori* IgG ELISA kit via the protocol detailed in methods section 2.3.1.

3.3 Results

3.3.1 Specificity of gastrin antibodies

3.3.1.1 Antibody L2 is specific for C-terminally amidated gastrins

To identify the specificity of antibody L2, concentration curves were established using G34, G17, G17-Gly and G17-CFP synthetic peptides. Within a concentration range of 0.1-100 pM antibody L2 only exhibited specificity for the major biologically active forms of gastrin (G17 and G34); inhibition of antibody binding of C-terminally extended G-17 only occurred at a concentration of >1 nM (figure 3.3.1).

3.3.1.2 Antibody L6 is specific for amidated G17

To identify the specificity of antibody L6, concentration curves were established using G34, G17, G17-Gly and G17-CFP synthetic peptides. Within a concentration range of 0.1-100 pM antibody L6 only exhibited specificity for amidated G-17; inhibition of antibody binding of G34, or of C-terminally extended G-17 only occurred at a concentration of >0.1 - 1 nM (figure 3.3.2).

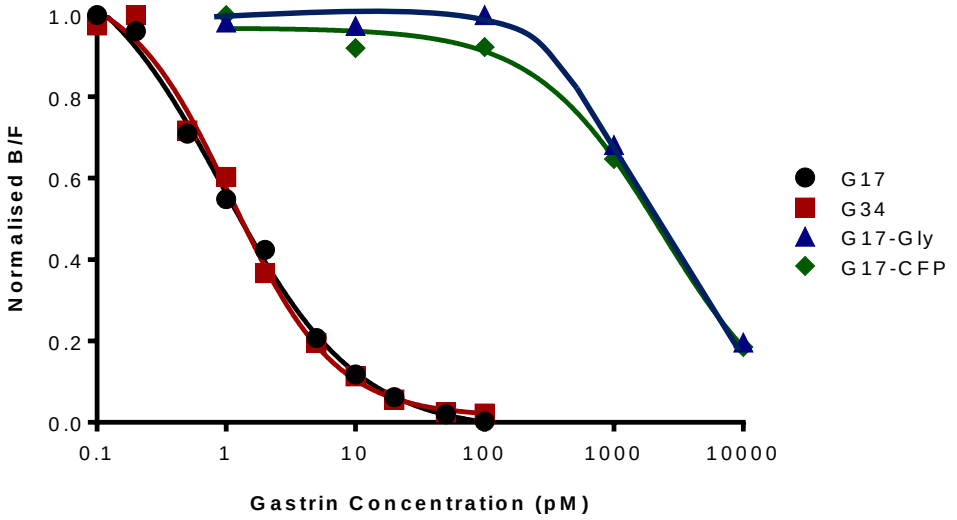

Figure 3.3.1 – **Antibody L2 is specific for C-terminally amidated gastrins.** Antibody L2 exhibited specificity towards amidated G-17 and G-34 (0.1-100 pM). Inhibition of binding due to C-terminally extended gastrin only occurred at concentrations >1 nM. N=1.

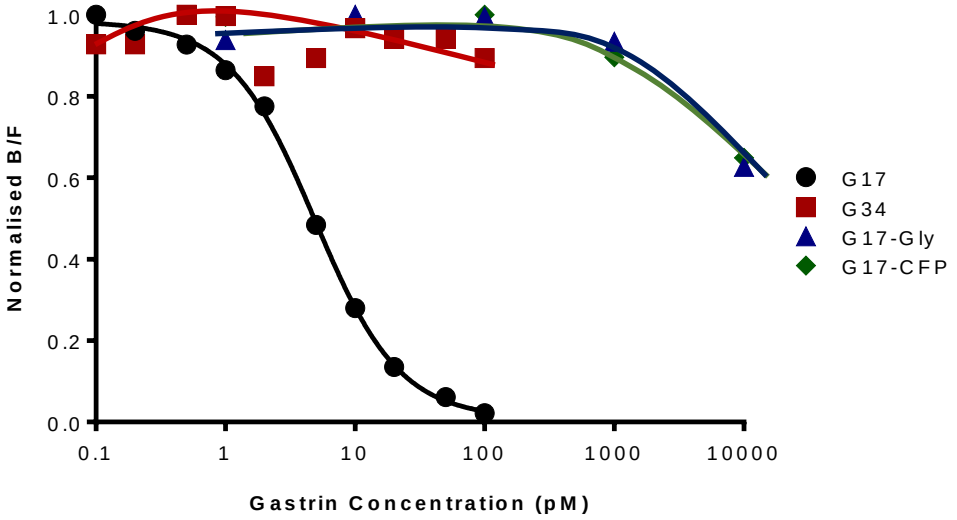

Figure 3.3.2 – **Antibody L6 is specific for amidated G17.** Antibody L6 exhibited specificity towards amidated G-17 (0.1-100 pM). Inhibition of binding due to C-or N-terminally extended, gastrin forms only occurred at a concentration >1 nM. N=1.

3.3.1.3 Antibody 1295 is specific for the N-terminus of G17

To identify the specificity of antibody 1295, concentration curves were established using G34, G17, G17-Gly and G17-CFP peptides. Within a concentration range of 0.1-100 pM antibody 1295 exhibited specificity for only G17 and its C-terminally extended forms -Gly and -CFP (figure 3.3.3).

3.3.1.4 Summary of antibody specificity

Comparing the affinity of our three gastrin antibodies, normalised to 1.0 for G17: L2 was specific for G17 and G34, L6 was specific for G17 and 1295 exhibited specificity for G17, G17-Gly and G17-CFP (table 3.3.1).

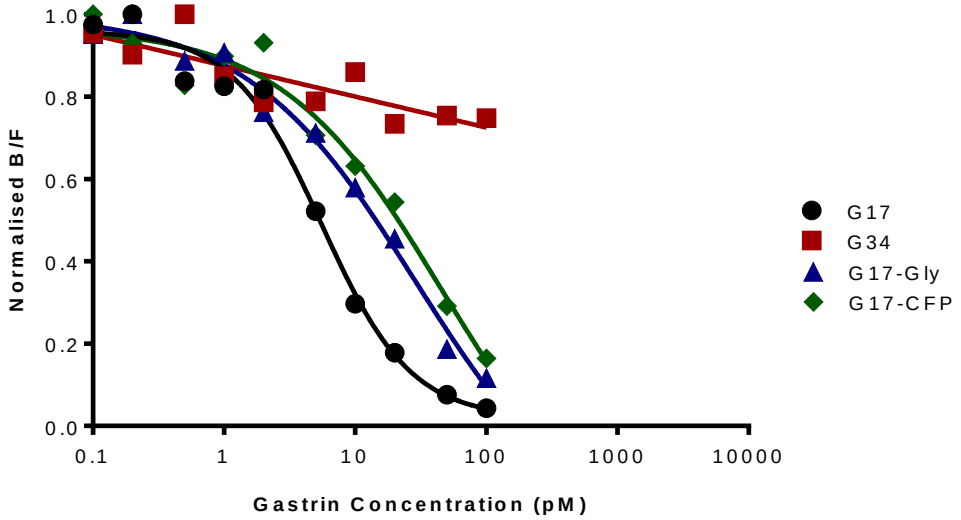

Figure 3.3.3 – **Antibody 1295 is specific for the N-terminus of G17.** Antibody 1295 exhibited specificity towards G17 and its C-terminal extended forms, but not G34, across the range 0.1-100 pM. N=1.

	L2	1295	L6
G17	1.0	1.0	1.0
G34	0.86	<0.01	<0.01
G17-Gly	<0.01	0.19	<0.01
G17-CFP	<0.01	0.12	<0.01

Table 3.3.1 – **Relative potency of four peptides for three antibodies.** The data are normalised to 1.0 based on the IC50 for G17 binding compared to other gastrin peptides for each antibody.

3.3.2 Effect of matrix on antibody binding

3.3.2.1 Human plasma does not influence binding of antibody L2

To ensure that the matrix of human samples (i.e. serum or plasma) does not non-specifically influence the affinity of antibody L2 for G17, standard curves were compared after preparation in assay diluent versus diluent containing human plasma stripped of endogenous gastrin. The introduction of stripped plasma caused a negligible change in sensitivity of antibody L2 to G17 over the 0.1-100 pM concentration range (figure 3.3.4).

3.3.2.2 Human plasma reduces sensitivity of antibody L6

To ensure that the matrix of human samples does not non-specifically influence the affinity of antibody L6 for G17, standard curves were compared after preparation in assay diluent versus diluent containing human plasma stripped of endogenous gastrin.

The introduction of stripped plasma caused a decrease in antibody affinity shown by a slight shift to the right of the standard curve over the 0.1-100 pM concentration range (figure 3.3.5).

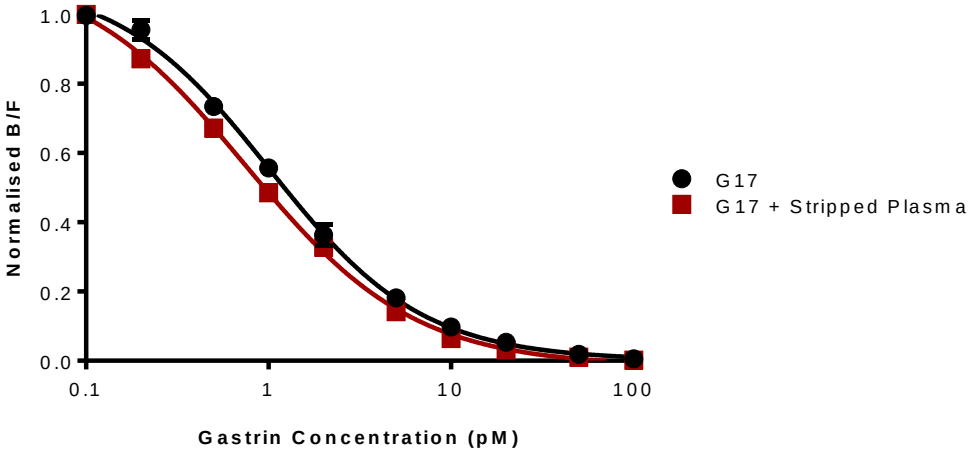

Figure 3.3.4 – **Human plasma does not significantly influence binding of antibody L2.** The introduction of stripped plasma caused a negligible change in sensitivity of antibody L2 to G17 over the 0.1-100 pM concentration range. Mean ±SEM, N=3.

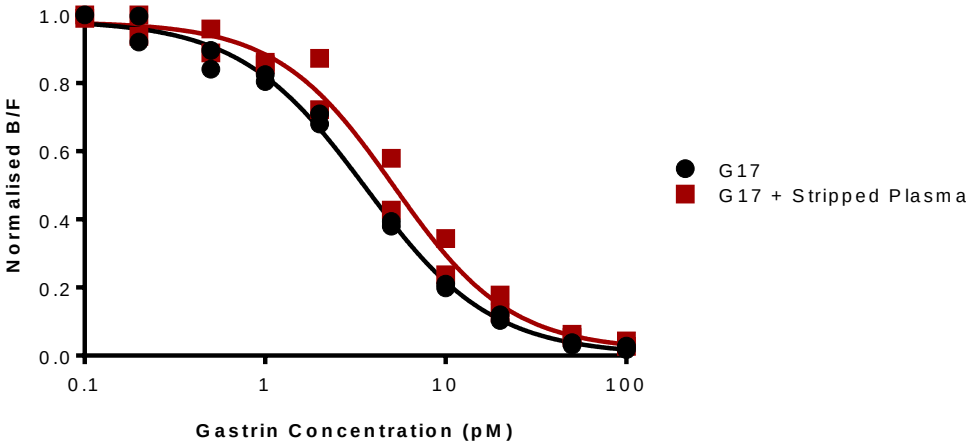

Figure 3.3.5 – **Human plasma reduces sensitivity of antibody L6.** When stripped human plasma was included in the G17 standard curve (0.1-100 pM) the curve was slightly shifted to the right. N=2.

3.3.2.3 Human plasma reduces sensitivity of antibody 1295

To ensure that human plasma does not non-specifically influence the affinity of antibody 1295 for G17, standard curves were compared after preparation in assay diluent versus diluent containing human plasma stripped of endogenous gastrin.

The introduction of stripped plasma caused a decrease in antibody affinity shown by a shifting to the right of the standard curve over the 0.1-100 pM concentration range (figure 3.3.6).

3.3.2.4 Summary of matrix effects

Thus, human plasma had a non-specific inhibited binding of antibodies L6 and 1295 to G17 while with antibody L2 there was a slight increase in binding in the presence of matrix (table 3.3.2).

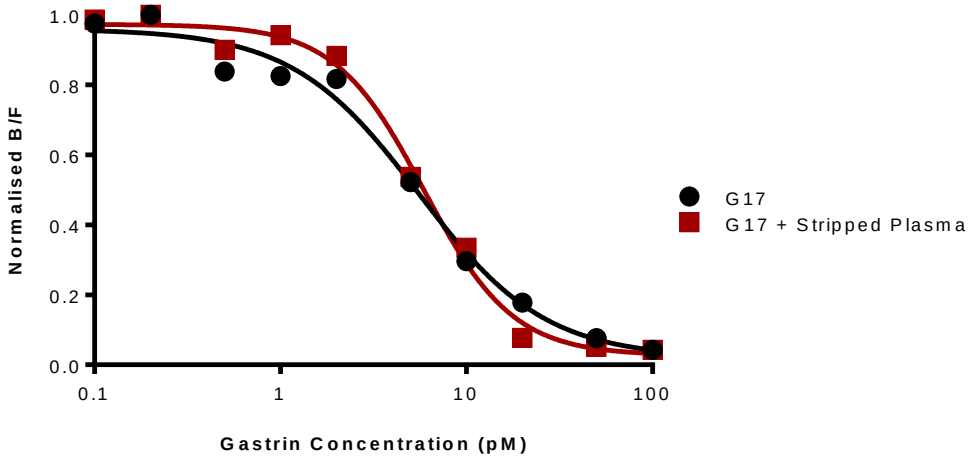

Figure 3.3.6 – **Testing the effect of human plasma on binding of antibody 1295 to gastrin-17.** Antibody 1295 exhibited a slight decrease in sensitivity towards G17 when human plasma was included across the 10-100 pM standard curve range, with a decrease in sensitivity across the 0.1-5 pM range. N=1.

	L2	1295	L6
IC_{50} G17 (pM)	1.4	5.51	3.36
Normalised IC_{50} G17	1.0	1.0	1.0
IC_{50} G17 + Stripped Plasma (pM)	1.2	6.05	4.2
Normalised IC_{50} G17 + stripped plasma	0.85	1.10	1.25

Table 3.3.2 – **A comparison of the relative affinity of each antibody for G17 in the presence of stripped human plasma.** Adding stripped plasma to the standard curve caused a loss in sensitivity (i.e. a shift to the right of the curve) increasing the IC_{50} value for antibodies L6 and 1295. Conversely, adding stripped plasma to an L2 standard curve caused an increase in sensitivity of the antibody for G17. Normalised to 1.0 at maximal inhibition of antibody binding.

3.3.3 Selection of G17 standards

As part of validating the gastrin radioimmunoassay, three commercially available G17 peptides were compared to identify which produced the most sensitive concentration curve for quantification.

3.3.3.1 Sigma-marketed G17 exhibits greatest sensitivity to antibody L2

Despite all peptides producing a near identical concentration curve, the Sigma-produced G17 peptide exhibited slightly higher sensitivity at the lowest gastrin concentrations tested. The Sigma-produced peptide was thus selected for quantification of all patient gastrin concentrations moving forward (figure 3.3.7).

3.3.3.2 Assay variability

Inter-assay variation was calculated based on repeat measurements of the same patient sample across multiple L2 assays, intra-assay variation was calculated using six patient samples from series 1 within the same assay and the limit of quantification was determined by reading the smallest potential gastrin quantity from the standard curve before it plateaued and become unreliable for quantification, and then multiplying by the dilution factor for patient samples (table 3.3.3). Non-specific binding of antibody to radiolabel was accounted for in the binding ratio calculation, however was below 10% for all assays and typically less than 5%.

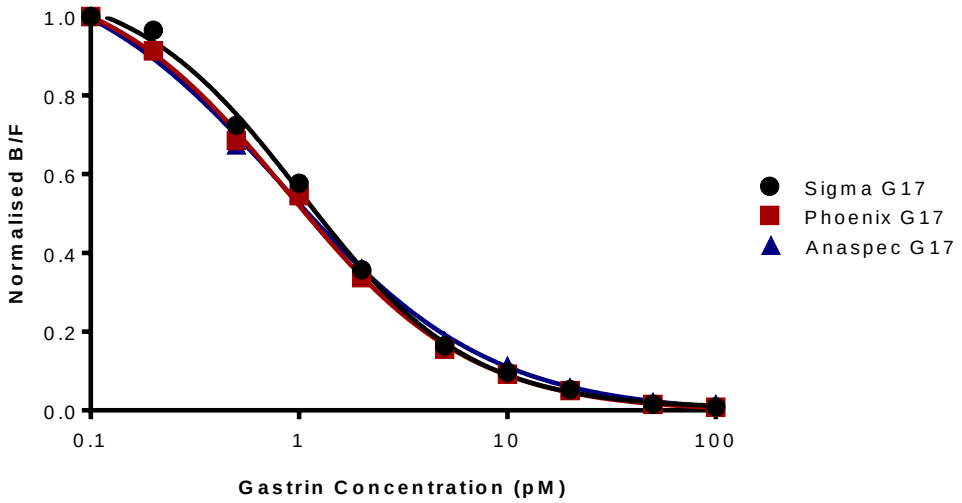

Figure 3.3.7 – **Three commercially available G17 standards exhibited similar activity with antibody L2.** Synthetic human G17 was sourced from three manufacturers. The concentration curves were virtually identical. N=1.

	Antibody L2
Inter-assay variation	6.3%
Intra-assay variation	15.7%
Limit of detection	2 pM

Table 3.3.3 – **Precision of L2 gastrin radioimmunoassay.** Measures of inter- and intra- assay variation for the L2 gastrin radioimmunoassay along with the average limit of detection for patient samples.

3.3.4 Patient groups analysed for study

Throughout this study two sets of patient groups were analysed. Series 1 refers to a number of control and Barrett's patients selected from an archive of over 1300 patient samples for which gastric biopsies and matching serum samples were available.

Within the archive there were a total of 922 patient samples exhibiting no GI pathology suitable for use as controls. Of these, serum and cDNA from biopsy were available for 114 patients from which a subset of 85 samples was selected based on previously determined qPCR data to form a control group.

In the whole archive there were 55 patients with Barrett's oesophagus, three of whom also had oesophageal cancer and so were omitted creating a 52 patient Barrett's cohort.

Series 2 refers to a group of Barrett's patients for which Barrett's mucosa biopsies and matching serum samples were obtained.

3.3.5 Gastrin profile of series 1

3.3.5.1 Comparison between series 1 control and Barrett's patients

Across control and Barrett's patients from the series 1 data set average age was ± 10 years, with a higher proportion in the Barrett's cohort being male than in the control cohort. Fasting circulating gastrin concentration in the Barrett's group was approximately two-fold higher than in the control group (table 3.3.4).

Individual data points displayed together as a box plot with central bars indicating the median, upper and lower bars representing the 75th and 25th percentiles respectively and whiskers indicating range (fig 3.3.8) exhibited a broadly similar overall range of circulating gastrin concentrations with a tendency to fewer very low gastrin concentrations in the Barrett's group and proportionately more in the range 30 -100 pM.

	Mean Age	Circulating Gastrin Concentration (pM)	Male (N)	Female (N)	*H. pylori* positive (N)
Control	54 ± 2.0	42.2 ± 5.6	36	50	0
Barrett's	64 ± 1.6	86.9 ± 15.4	31	22	7

Table 3.3.4 – **Comparison between series 1 control and Barrett's patients.** Across all control and Barrett's patients from series 1, average age was ± 10 years, with a higher proportion in the Barrett's cohort being male than in the control cohort. Circulating gastrin concentration within the Barrett's cohort was significantly higher than that of the control group. Values represented as mean ± SEM.

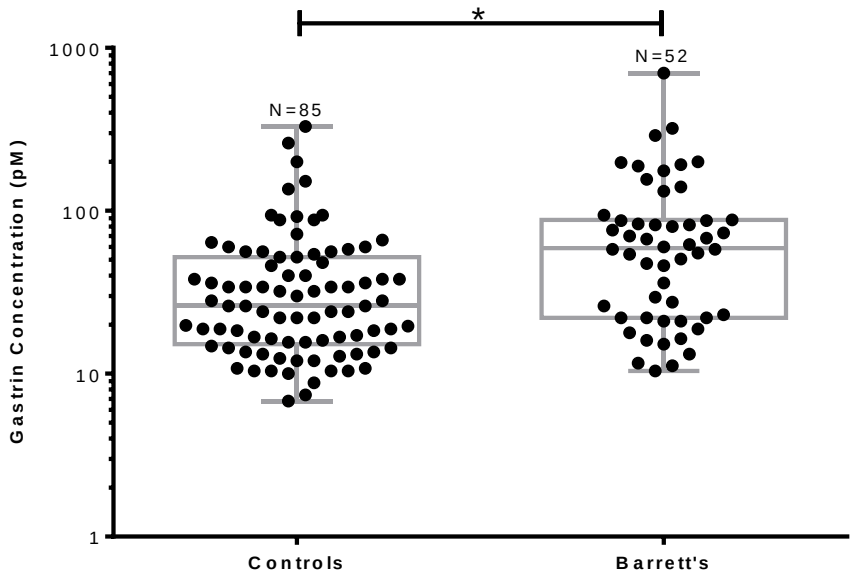

Figure 3.3.8 – **Gastrin concentration is significantly higher in series 1 Barrett's Patients.** There was significantly higher circulating gastrin concentration in Barrett's patient's compared with controls. Unpaired t-test t-with Welch's correction $p<0.05$.

3.3.5.2 Gastrin concentration in series 1 patients is significantly higher with PPI usage

Circulating gastrin concentration in both control and Barrett's patients was significantly higher in patients treated with PPIs compared with those who were not. However Barrett's patients on PPIs exhibited gastrin concentrations significantly higher than control patients on PPIs (fig 3.3.9).

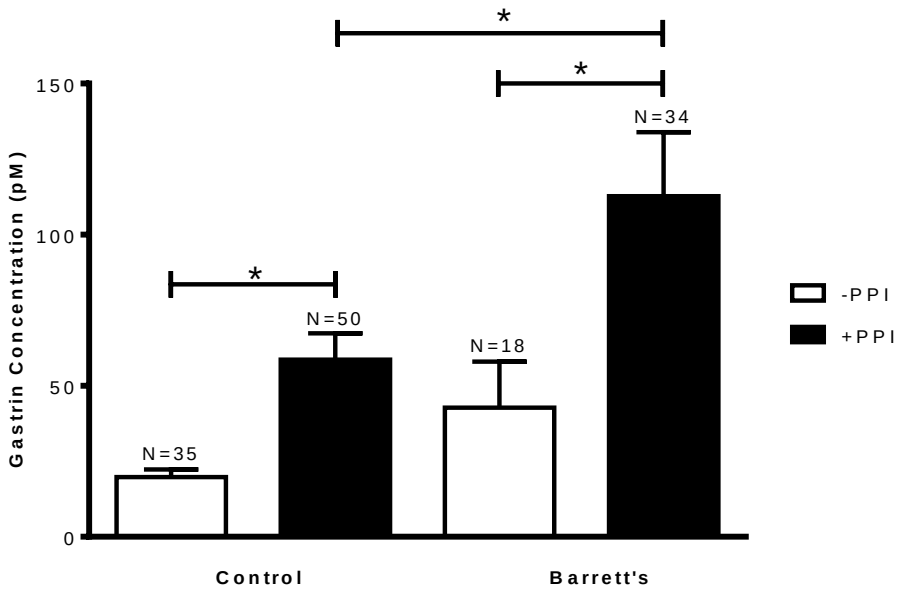

Figure 3.3.9 – **Gastrin concentration in series 1 patients is significantly increased by PPI usage.** Circulating gastrin concentration in control and Barrett's patients was significantly higher when treated with PPIs compared with patients not on PPIs. Mean ± SEM, $p<0.05$, one-way ANOVA and t-test with Welch's correction.

3.3.5.3 Gastrin concentration is significantly higher in series 1 Barrett's patients when matched for age, sex and H. pylori status

In view of the differences between controls and Barrett's patient groups, in particular the fact that there were seven *H. pylori* positive patients in the Barrett's cohort and none in the control cohort, it was considered important to repeat the comparison on groups matched for age, sex and *H. pylori* status to eliminate them as potentially skewing factors. A new matched control group was formed, with the majority of samples derived from the previous 86 patient control group where appropriate and from the remaining 922 control samples if matches could not be found. For gastrin concentration comparison between groups, PPI status was ignored however the number of patients taking PPIs was equal in both groups. Mirroring the data shown in figure 3.3.9, mean circulating gastrin concentration remained significantly higher in the Barrett's patient group compared with the age, sex and *H. pylori* matched control group. The Barrett's patient group also exhibited a greater range of gastrin concentrations than patients in the control group (fig 3.3.10).

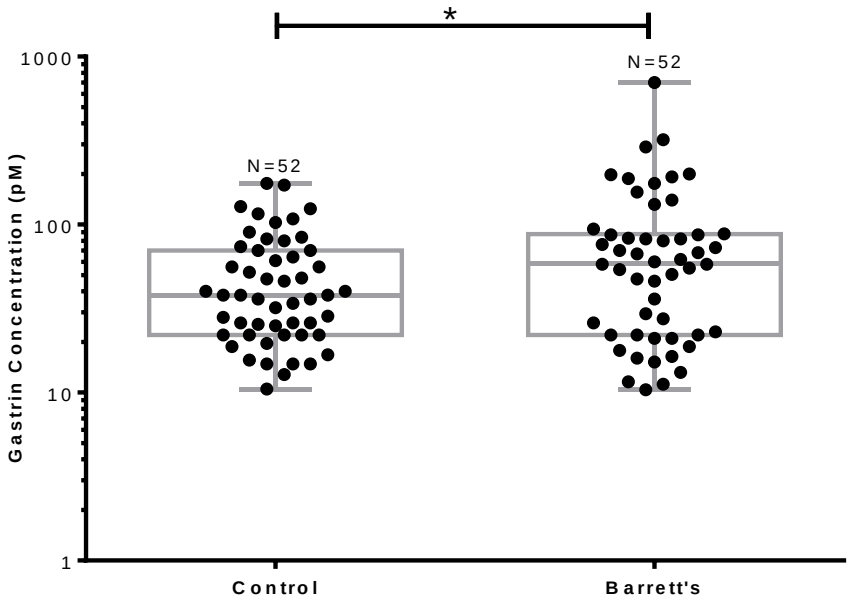

Figure 3.3.10 – **Gastrin concentration is significantly higher in series 1 Barrett's patients matched for age, sex and *H. pylori* status.** Average circulating gastrin concentration in Barrett's patients is significantly higher when compared with controls matched for age, sex and *H. pylori* status. Central bars indicate median, upper and lower represent 75th and 25th percentiles respectively and whiskers indicate range. Analysis by unpaired t-test with Welch's correction, $p<0.05$.

3.3.5.4 Gastrin concentration in series 1 patients is significantly increased with PPI usage when matched for age, sex and H. pylori status

Even when the most common factors with the potential to influence circulating gastrin concentration were removed by rigorously matching the control group, circulating gastrin concentration in Barrett's patients remained significantly higher in those treated with PPIs compared with either control or Barrett's patients not treated with PPIs (fig 3.3.11 and table 3.3.5).

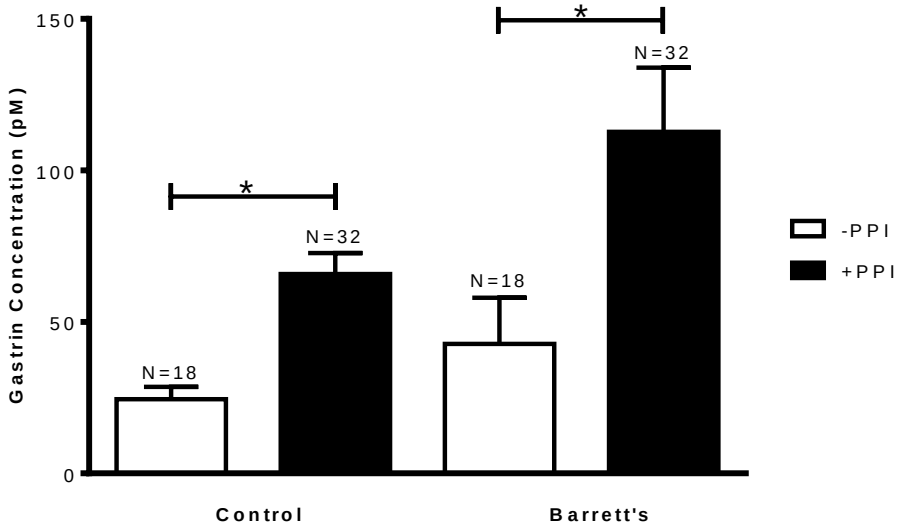

Figure 3.3.11 – **Gastrin concentration is significantly increased in series 1 Barrett's patients with PPI use when matched for age, sex and *H. pylori* status.** There were significantly higher circulating gastrin concentrations in Barrett's patients on PPIs compared with either controls or Barrett's patients not on PPIs. Mean ± SEM, $p<0.05$, one-way ANOVA.

	Mean Age	Mean Circulating Gastrin Concentration (pM)	Male N number	Female N Number
Control	64 ± 1.6	50.7 ± 5.5	31	22
Barrett's	64 ± 1.6	86.9 ± 15.4	31	22

Table 3.3.5 – **Comparison between series 1 control and Barrett's patients matched for age, sex and *H. pylori* status.**

3.3.6 Gastrin profile of series 2 patients

3.3.6.1 Circulating gastrin concentration in series 2 patients was similar across research centres

Barrett's biopsies composing series 2 were collected from two separate hospitals, the Royal Liverpool University hospital and the Gastroenterology Unit of the University of Szeged, Hungary. Circulating gastrin concentration was compared between institutions to ensure samples were comparable between patient populations. No significant difference was identified between samples collected in Liverpool or Hungary (fig 3.3.12 and table 3.3.6).

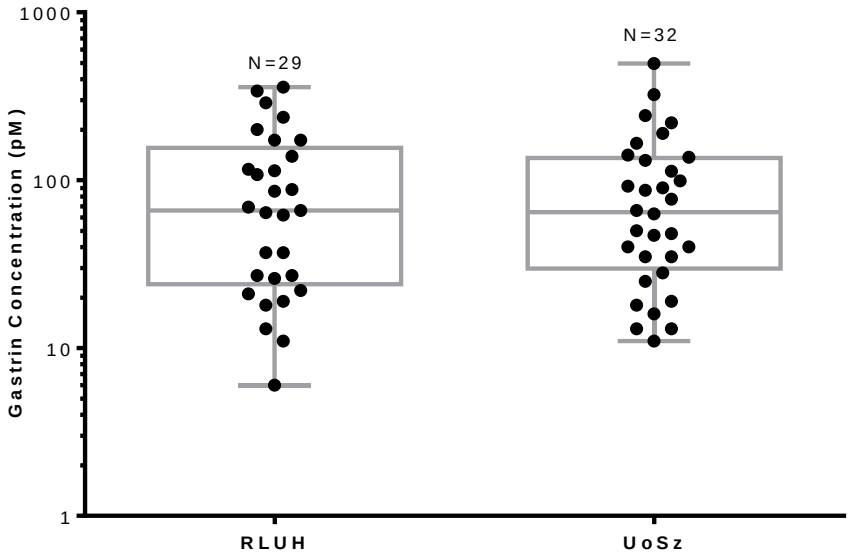

Figure 3.3.12 – **Circulating gastrin concentration in series 2 patients was similar across research centres.** Series 2 samples were collected from the Royal Liverpool University hospital and the Gastroenterology Unit of the University of Szeged, Hungary. There was no significant difference between samples collected in either location. Central bars indicate median, upper and lower represent 75th and 25th percentiles respectively and whiskers indicate range. Analysis by unpaired t-test, $p<0.05$.

	Mean Age	Mean Circulating Gastrin Concentration (pM)	Male N number	Female N Number
RLUH	68 ± 1.5	102 ± 18.9	19	10
UoSz	58 ± 2.4	99 ± 18.8	31	1

Table 3.3.6 – **Comparison between series 2 RLUH and UoSz recruited patients.** Across both cohorts, average age was ± 10 years, with a higher proportion of patients in both cohorts being male. Circulating gastrin concentration across cohorts exhibited no significant difference.

3.3.6.2 Circulating gastrin in series 2 Barrett's patients spans the dynamic physiological range

In order to interpret the circulating gastrin concentrations in a physiological context, a dose-response curve for gastrin-stimulated acid secretion was adapted from Blair *et. al* 1987.[305] Individual circulating gastrin concentrations are shown along the x-axis for all series 2 Barrett's patients. Note that they cover the range below, within and above the dynamic range for stimulation of acid secretion (fig 3.3.13).

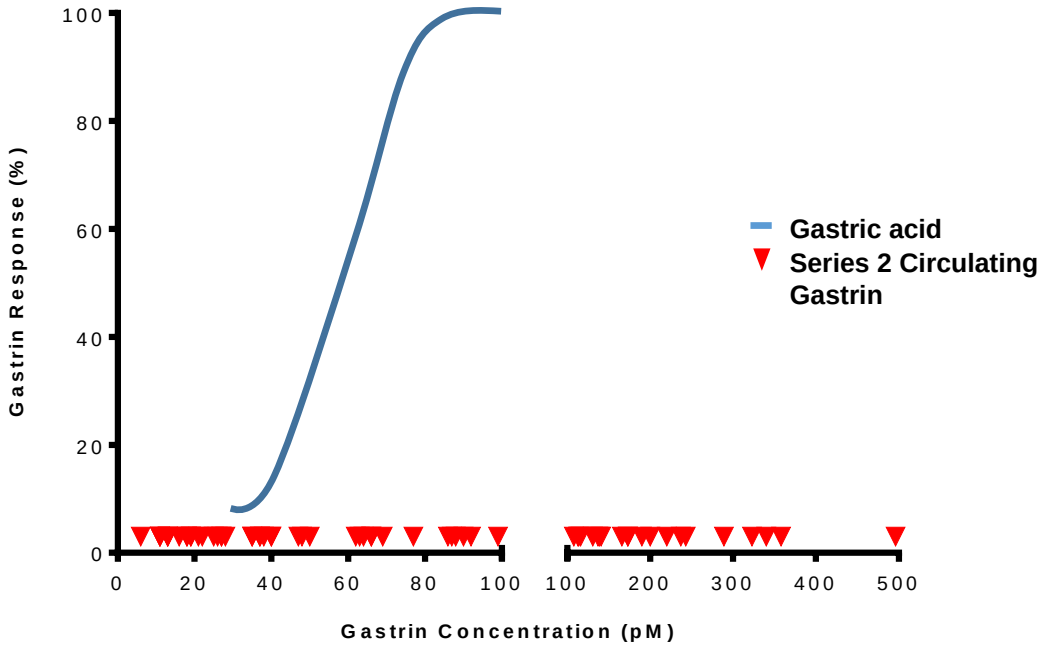

Figure 3.3.13 – **Circulating gastrin in series 2 Barrett's patients spans the dynamic physiological range.** A dose-response curve for gastrin-stimulated acid secretion was adapted from Blair *et. al* 1987.[305] Individual circulating gastrin concentrations are shown along the x-axis. Note that they cover the range below (N=18), within (N=22) and above (N=21) the dynamic range for stimulation of acid secretion.

3.3.6.3 Distribution of circulating gastrin concentration of series 2 patients

On the basis of the data from figure 3.3.13, it was considered reasonable to divide subjects into three groups based on circulating gastrin concentrations matched to unstimulated acid secretion (<30 pM), within the dynamic range (between 30-100 pM) and supra-maximal (>100 pM).

Approximately similar numbers of patients had a fasting gastrin concentration that was within the dynamic range for acid secretion (N=22), compared with supra-maximal for acid secretion (N=21) or below the range for stimulation of acid secretion (N=18) (fig 3.3.14).

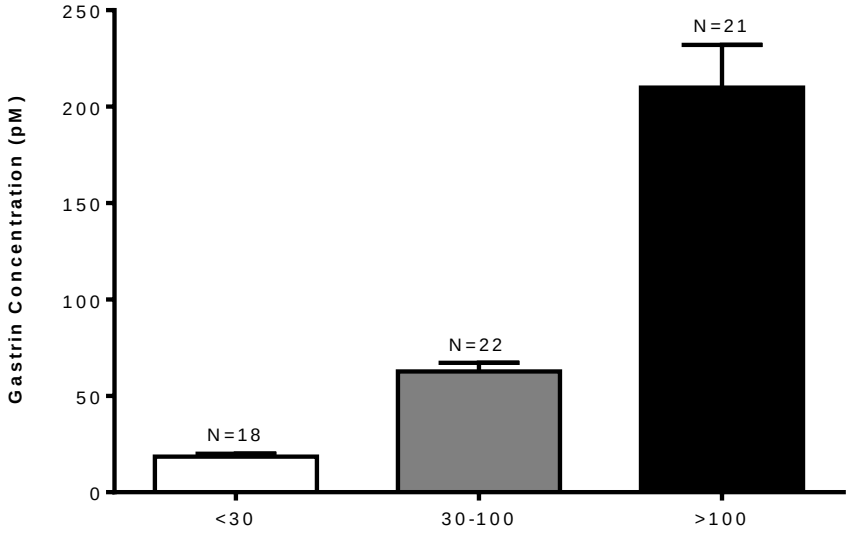

Figure 3.3.14 – **Distribution of circulating gastrin concentration of series 2 patients.** Using the data from figure 3.3.13, the circulating gastrin concentrations of series 2 patients were linked to level of gastric acid secretion and separated into three groups based on below the threshold for stimulation of acid secretion (<30 pM), within the dynamic range (between 30-100 pM) or supra-maximal for acid secretion (>100 pM).

3.3.7 H. pylori infection status of series 2 patients

With the aim of ensuring patient samples obtained from both institutions were comparable, *H. pylori* status was determined by ELISA using patient serum samples. Across both RLUH and UoSz obtained biopsies, the majority of samples tested negative for *H. pylori* antigen.

H. pylori positive samples made up 21% of RLUH samples and 23% of UoSz samples, showing no significant difference in infection rate between samples collected from either institutions (fig 3.3.15). Average circulating gastrin concentration in *H. pylori* negative patients when pooling both research centres was 12 pM, with *H. pylori* positive patients exhibiting a gastrin concentration of 86 pM.

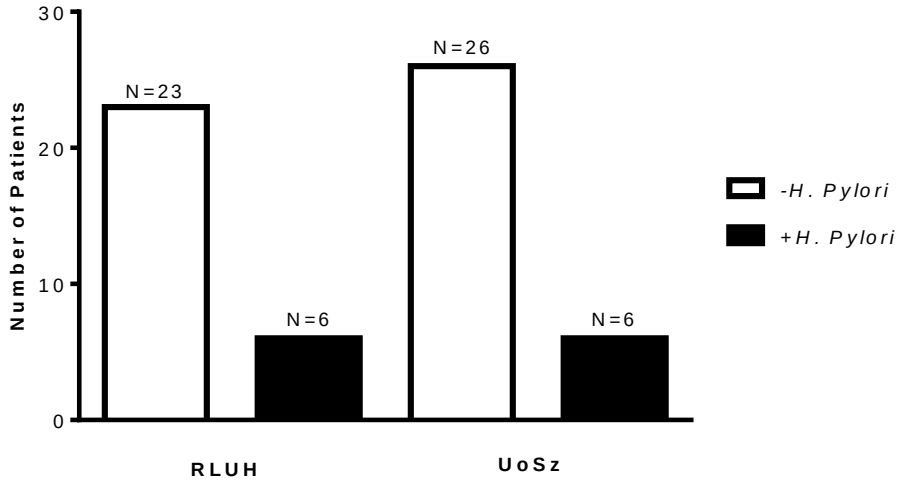

Figure 3.3.15 – ***H. pylori* infection status of Series 2 patients.** Across both RLUH and UoSz obtained biopsies, the majority of samples tested negative for *H. pylori* antigen in serum. *H. pylori* positive samples made up 21% of RLUH samples and 23% of UoSz samples, exhibiting no significant difference in infection rates between samples collected from either institution.

3.4 Discussion

Currently, the majority of gastrin diagnostic measurements in blood (serum or plasma) are performed using commercially available ELISA kits.[306] Our decision to use RIA instead was influenced by two factors: the availability of a long term in-house technique and a recent review of all commercially available options, both RIA and ELISA, which identified inaccurate measurements in over half of commercial ELISA kits.[300]

The novel aspect of this work is the re-validation of three >40-year old antibodies. There are important similarities and differences between the present and previous findings.[50,297] Overall, the specificity of three antibodies was as expected. Antibody L2 exhibited specificity for the C-terminal of both G17 and G34 but not any N-terminally extended peptides; L6 for intact G17, and 1295 for the N-terminus of G17. However, there were important differences between antibodies in the effects of matrix that meant the sensitivity of assays using L6 and 1295 were decreased, while that of L2 was slightly increased. At the outset it was hoped that the availability of three different antibodies would make it possible to establish the peptide profile of circulating gastrin forms in Barrett's disease. In the event, the low sensitivity of L6 and 1295 in serum meant that this was not possible. The present study was therefore based on antibody L2; it is worth stressing that the specificity of L2 corresponds to that of most other antibodies used for routine determination of circulating gastrin concentration. It seems likely that difference in matrix effects in the present study compared with previous ones is attributable to changes in assay technique and in particular the radiolabelled peptide employed and the method of separation of antibody bound and free label.

There has been renewed concern recently surrounding the reliable validation of antibodies used in assays. In particularly the basic characterisation of the specificity and sensitivity of commercial and in-house developed antibodies is performed less frequently than it should be.[307] This has led to what some have called a "reproducibility crisis" in fields depending on antibody-based research methods. One result has been researchers wasting time and money only to discover that the antibodies they used were inappropriate under their particular experimental conditions.[308]

Current guidelines issued by the Food and Drug Administration (FDA) in America for formally validating a ligand binding assay, state that researchers must demonstrate that antibodies are selective, specific and reproducible within the context in which they are used.[309,310] More specifically, there are strict standards governing selectivity, accuracy and precision, calibration curve, sensitivity, reproducibility and stability of reagents used.

Selectivity of our antibodies was determined by using a panel of gastrin peptides of different lengths including samples of peptides from different suppliers, the effect of matrix on antibody binding versus standard assay diluent and the non-specific binding of antibody to radiolabel was taken into account into the binding ratio calculation and was less than 10% in all assays and generally about 5%.

Mean coefficient of variation of our L2 assay was determined by using six runs of multiple patient samples from series 1 within the same assay and was calculated as 15.7%, well within the 20% specified within the guidelines.[310] Mean inter-assay precision, utilising the same patient sample repeated across each L2 assay (N=7), had a co-efficient of variation of 6.3%, again well within guidelines for assay validation. The inter-assay variability is also a measure of the reproducibility of the assay, as it covers multiple experimental runs on different days, with different batches of radiolabel.

The calibration curve used for gastrin quantification exceeds the specifications laid out in FDA guidelines, namely it is a 10-point curve (recommended 5) fitted with a 5-parameter logarithmic sigmoidal curve prepared within an appropriate matrix (stripped plasma). The lower and upper limits of quantification varied slightly between calibration curves present in each assay, however they can be broadly determined to be a dilution-corrected gastrin concentration of 2 and 400 pM respectively. This also covers the sensitivity of the assay, as the smallest concentration readable would be 2 pM in a patient sample.

Stability of the assay was taken into account again across multiple assays utilising different batches of radiolabel tested between freeze-thaw cycles of both label and peptide and tested between freeze-thaw cycles of QC patient sample tested in each

run. The buffer reagents were made up fresh each run and the antibody was kept at 4°C so was not at risk of freeze-thaw related degradation.

What my work shows is that our L2-based gastrin assay for circulating gastrin has been rigorously tested to FDA standards and rather than being a liability, the antibody is well characterised, validated and may be utilised for decades for accurate quantification of gastrin. Performing this validation work prior to the rest of the work presented in this thesis was considered vital as all biomarker analyses were performed in relation to gastrin concentration, requiring a rigorous quantification of gastrin concentration for each patient to avoid a "reproducibility crisis".

Uniquely, our work aims to link circulating gastrin concentration within samples to the classically known physiological output of gastrin, gastric acid secretion from parietal cells. Many published research articles work with circulating gastrin concentration and its relationships to disease conditions such as neuroendocrine tumour formation[311] and gastric cancer development[116], but very few consider gastrin concentration in the context of the dynamic range for acid secretion.[74]

The data shows that within our initial series 1 control and Barrett's unmatched patients, treatment with PPIs was associated with a significantly higher circulating gastrin concentration in both groups, as expected. Significantly higher circulating gastrin concentration with PPI treatment remained in both groups even when using a control group matched for other variables known to influence circulating gastrin concentration such as *H. pylori* infection status,[301] age,[302] and sex[312] effectively accounting for their contribution to gastrin concentration in the analysis. Analysis of the mean circulating gastrin concentration in series 2 patients between *H. pylori* positive and negative samples revealed higher gastrin concentration in *H. pylori* positive samples, as expected from previously published work in the field, and highlights the importance of accounting for *H. pylori* status in our series 1 analysis.

We observed a trend towards higher circulating gastrin concentration in Barrett's patients without PPI treatment compared to controls without PPI treatment (significant in the unmatched group) and a greater increase in circulating gastrin in Barrett's patients when treated with PPIs compared to the PPI induced increase in control

patients. Higher circulating gastrin concentration in Barrett's patients with PPI treatment compared to controls is in line with currently published research on long-term PPI treatment,[74] however many of those patients exhibited some degree of ECL cell hyperplasia which our Barrett's gastric samples did not. Currently, gastric research in Barrett's patients remains overlooked and no research has been published to explain why, in Barrett's patients without ECL cell hyperplasia, circulating gastrin concentration without PPI treatment would be higher than in controls or why Barrett's patients would respond with a greater increase in gastrin concentration to PPI treatment than controls. This represents a novel finding in our research and offers potential for further study.

The main findings of this results chapter are that a) all of our tested gastrin antibodies are still specific even after approximately 40 years of storage, b) antibody L2 proved the best candidate for total circulating gastrin assay validation, which was performed to FDA standards, c) the gastrin profiles for both control and Barrett's patients are similar with respect to changes in response to PPI treatment, however Barrett's patients consistently exhibit higher circulating gastrin concentration than control patients regardless of PPI status.

Chapter 4

Putative biomarker expression in gastric biopsies

4.1 Introduction

To begin the identification of putative biomarkers of gastrin activity, initial work sought to define the response of putative biomarkers in the best characterised targets of gastrin – the ECL and parietal cells of the stomach - in control and Barrett's subjects and to determine their response to PPI treatment and associated hypergastrinaemia.[194] Putative ECL cell markers of gastrin activity, HDC and CgA, have previously been employed as biomarkers of the direct action of gastrin on ECL cells in patients and so represent the best candidates for use in tracking any differences in response between the control and Barrett's stomach with PPI treatment and associated hypergastrinaemia.[185, 293] Included in our analysis of gene expression within the two patient groups was quantification of the abundance of CCK2R transcripts to determine whether there might be changes in sensitivity to circulating gastrin between patient groups and PPI treatment status. In addition to putative biomarkers of ECL cell activity, there is evidence that gastrin regulates the expression of enzymes responsible for tissue remodelling, specifically MMP-1,[211] which potentially relates to changes in the cellular microenvironment conducive to cancer development and thus could have potential relevance as an indirect putative marker of gastrin regulated transformative responses.

Somewhat surprisingly, much still remains to be discovered about how common treatments such as PPI therapy influence the expression of HDC, CgA, CCK2R or MMP-1 in the normal stomach and the stomach of Barrett's patients. As identified in the previous chapter, in our patient samples there was a difference in the magnitude of response in circulating gastrin concentration to PPI treatment in Barrett's patients when compared to matched controls. This implies that gastric cellular responses in Barrett's patients may differ with PPI treatment compared to normal subjects as may the transcript abundance of gastrin-regulated putative biomarkers. Thus, the first objective of the work in this chapter was to characterise the expression of putative gastrin-regulated biomarkers in the stomach of control and Barrett's patients and their response to treatment with PPIs.

Preneoplastic changes in the stomach, notably those that include glandular atrophy, are associated with increased circulating gastrin concentrations.[313] A second objective of the work in this chapter was therefore to compare the abundance of putative gastrin-

regulated transcripts in control subjects and patients with defined preneoplastic changes in gastric architecture as detailed in figure 4.1.1.

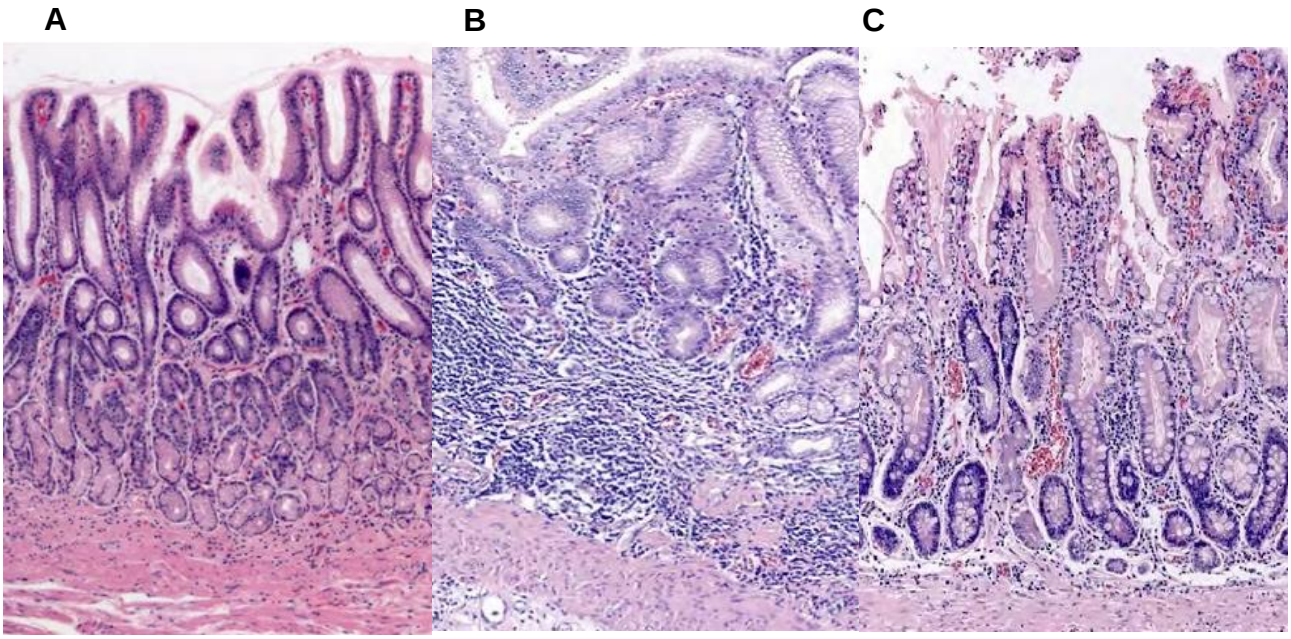

Figure 4.1.1 –**Micrographs of gastric biopsies stained with a Haematoxylin and eosin stain.** Panel A depicts an example of normal gastric mucosa, panel B depicts gastric mucosa with atrophy leading to extensive loss of glands and panel C depicts intestinal metaplasia of the gastric mucosa.[314]

4.1.1 Objectives

Specific objectives of this chapter were:

- To determine how putative biomarkers of gastrin responsiveness change in relation to circulating gastrin in control and Barrett's patient gastric biopsies.
- To compare the biomarker expression profile of control and Barrett's patients with and without PPI treatment.
- To characterise the expression of putative gastrin-regulated biomarkers in gastric biopsies exhibiting preneoplastic morphology.

4.2 Materials and methods

Dr Andy Moore and Dr Sentil Murugesan had previously recruited patients, obtained patient information, blood samples and biopsies for the patients in series 1. Gastric biopsies were retrieved from -20°C long-term storage and RNA was extracted and converted to cDNA via the protocols detailed in section 2.4. For some samples, archived cDNA had already been prepared by Dr Islay Steele for previous studies and in these cases this material was used. If biopsies had previously had RNA extracted for previous studies but no cDNA remained, original RNA (again prepared by Dr Islay Steele) was retrieved from -80°C long-term storage and reverse transcribed.

Biopsies analysed in the first part of this chapter were processed via qPCR using the singleplex method outlined in methods section 2.4.3 using the primer/probe sets described within that section.

Biopsies analysed in the second part of this chapter were processed via qPCR using the multiplex method outlined in methods section 2.4.4 using the primer/probe sets referenced in both section 2.4.3 and 2.4.4.

4.3 Results

4.3.1 Putative biomarker expression in normal stomach

4.3.1.1 Relative CgA abundance is significantly higher with PPI treatment

Relative abundance of CgA mRNA, normalised to GAPDH mRNA abundance, in series 1 gastric biopsies was significantly higher in both control and Barrett's subjects treated with PPIs compared with untreated. However, control patients exhibited a greater increase in CgA abundance with PPI treatment than Barrett's (3.78-fold increase vs. 2.08-fold) (fig 4.3.1A).

When control and Barrett's patients were matched based on their age, sex and *H. pylori* status, relative CgA abundance remained significantly higher in both groups with PPI treatment (fig 4.3.1B).

Comparing the relationship between relative CgA abundance and circulating gastrin concentration in combined control and Barrett's gastric biopsies, a Spearman's rank correlation identified a significant, relationship between the two, r=0.44 df=137 p<0.05 (fig 4.3.1C). When analysed individually, a significant relationship between circulating gastrin and CgA abundance remained in both groups (control r=0.49 df=84 p<0.05; Barrett's r=0.31 df=51 p<0.05).

In order to determine whether the sensitivity to circulating gastrin in control and Barrett's patients was similar, a control group was selected that was matched to the Barrett's groups for similarity in circulating gastrin concentrations (±2 pM). Within both groups relative CgA abundance was significantly higher with PPI treatment (fig 4.3.1D).

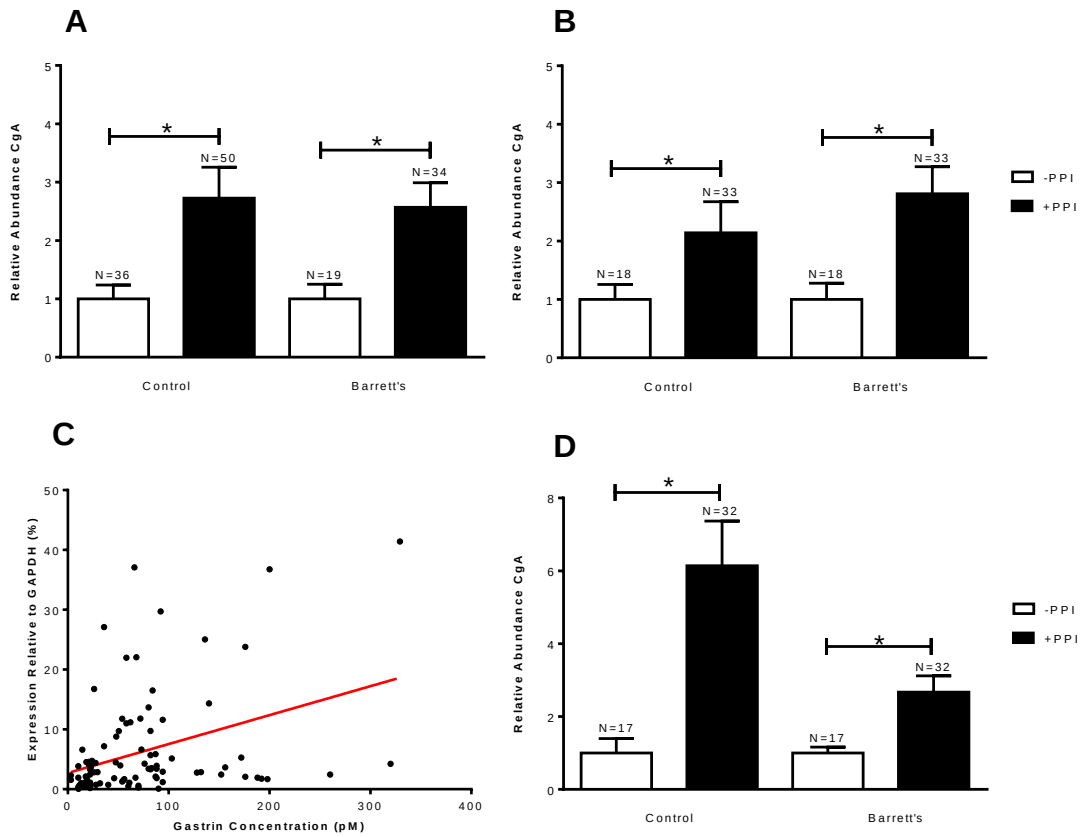

Figure 4.3.1 – **Relative CgA abundance is significantly higher with PPI treatment.** Relative abundance of CgA mRNA, expressed normalised to GAPDH mRNA abundance, was significantly higher with PPI treatment in both groups (A). Relative CgA abundance with PPI treatment remained significantly higher in both patient groups when controls were age, sex and *H. pylori* status matched (B). Across all samples a significant relationship existed between relative CgA expression and circulating gastrin concentration (C). Significantly higher relative CgA expression with PPI in groups matched for circulating gastrin (D). Mean ±SEM, $p<0.05$, unpaired two-tailed t-test with Welch's correction.

4.3.1.2 Relative CCK2R abundance is significantly lower with PPI treatment

Abundance of the CCK2R was analysed in gastrin-matched control and Barrett's patient gastric biopsies to identify any loss of sensitivity to endogenous gastrin within the stomach which may impact the abundance of gastrin sensitive biomarkers. Unexpectedly, in both control and Barrett's patient groups (fig 4.3.2), patients treated with PPIs exhibited significantly lower receptor transcript abundance, relative to GAPDH mRNA abundance, with both patient groups exhibiting a similar degree of change.

Comparing the relationship between relative CCK2R abundance and circulating gastrin concentration in gastrin matched combined control and Barrett's gastric biopsies, Spearman's rank correlation identified a significant relationship between the two, r=0.48 df=96 p<0.05. When analysed individually, a significant relationship between circulating gastrin and CCK2R abundance remained in both groups (control r=0.50 df=47 p<0.05; Barrett's r=0.50 df=47 p<0.05).

4.3.1.3 Relative HDC abundance is significantly higher with PPI treatment

Within the unmatched cohort of control and Barrett's patient biopsies HDC mRNA abundance, relative to GAPDH mRNA abundance, was significantly higher with PPI treatment in both groups compared with untreated subjects (fig 4.3.3A). However, when samples were gastrin-matched only the Barrett's group exhibited higher relative HDC abundance with PPI treatment. Despite this, there was a clear upward trend in HDC abundance in the control patient group treated with PPIs compared with untreated although the variance was higher (fig 4.3.3B).

Comparing the relationship between relative HDC abundance and circulating gastrin concentration in unmatched control and Barrett's gastric biopsies, Spearman's rank correlation identified no significant relationship between the two. In gastrin matched samples however, Spearman's rank identified a significant relationship between the two r=0.29 df=101 p<0.05. When analysed individually, a significant relationship was only identified between circulating gastrin and HDC abundance in the Barrett's group, r=0.30 df=47 p<0.05.

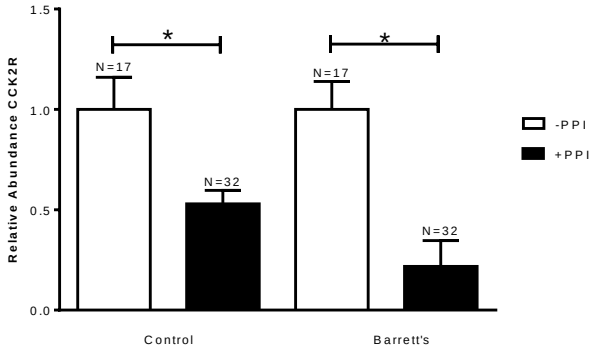

Figure 4.3.2 – **Relative CCK2R abundance is significantly lower with PPI treatment.** Significantly lower abundance of CCK2R mRNA with PPI treatment in control and Barrett's groups when matched for circulating gastrin concentrations. Mean ±SEM, $p<0.05$, unpaired two-tailed t-test with Welch's correction.

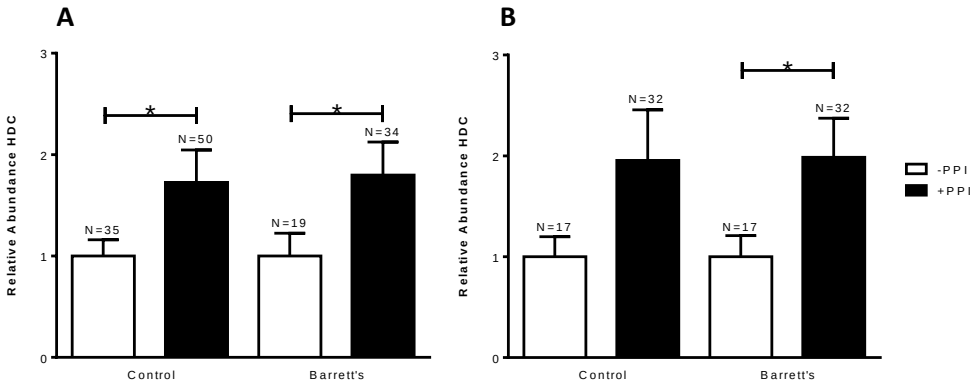

Figure 4.3.3 – **Relative HDC abundance is significantly higher with PPI treatment.** Significantly higher abundance of HDC mRNA in control and Barrett's patients treated with PPIs compared with untreated (A). In a control group with circulating gastrin concentrations matched to those of the Barrett's group, relative HDC abundance was significantly higher in the Barrett's patient group with PPI treatment (B). Mean ±SEM, $p<0.05$, unpaired two-tailed t-test with Welch's correction.

4.3.1.4 Relative MMP-1 abundance is significantly higher with PPI treatment in control biopsies

Within the unmatched cohort of control and Barrett's patient biopsies, MMP-1 mRNA abundance, relative to GAPDH mRNA abundance, was significantly higher with PPI treatment in the control group only (fig 4.3.4A). The relationship between serum gastrin and MMP-1 abundance was analysed using Spearman's rank correlation and identified a significant positive relationship (r=0.47, p<0.05) within the control group, however no significant relationship existed in Barrett's biopsies.

When controls were gastrin-matched to Barrett's samples, neither group exhibited a significant change in MMP-1 abundance with PPI treatment. Both groups did however exhibit a similar upward trend in MMP-1 abundance with PPI treatment (fig 4.3.4B).

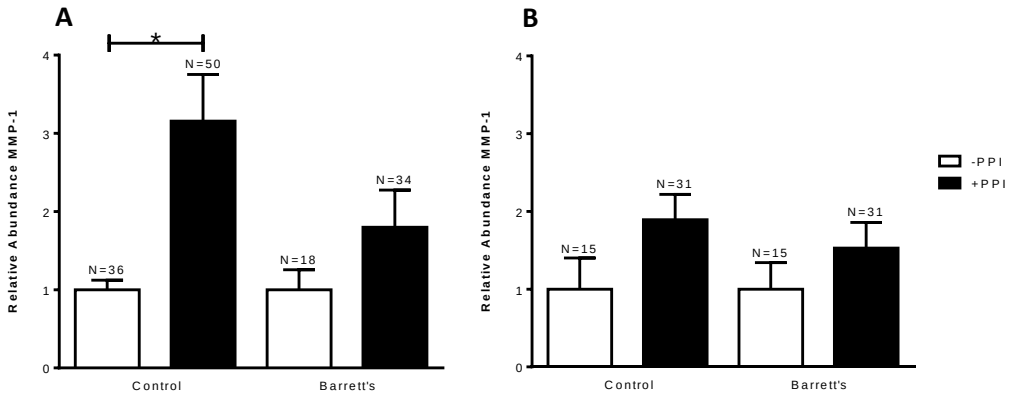

Figure 4.3.4 – **Relative MMP-1 abundance is significantly higher with PPI treatment in control biopsies.** Significantly higher abundance of MMP-1 mRNA, expressed relative to GAPDH mRNA abundance, in control patients with PPI treatment (A). No significant changes in gastrin matched controls or Barrett's patients (B). Mean ± SEM, p<0.05, unpaired two-tailed t-test with Welch's correction.

4.3.2 Gastric biomarker expression in preneoplastic biopsies

4.3.2.1 Preneoplastic patients exhibit significantly higher gastrin concentration

To identify if putative biomarker abundance was altered in preneoplastic conditions of the stomach, a number of samples were retrieved from the series 1 archive. Three groups of patients were recovered with appropriate age and sex matched controls – namely those with gastric atrophy, gastric intestinal metaplasia, and the combination of both atrophy and metaplasia.

Each patient group exhibited significantly higher circulating gastrin concentration compared to control groups, however the degree of increase in gastrin concentration was directly related to severity of progression of preneoplasia along the Correa cascade. Atrophy patients exhibited the lowest increase in gastrin concentration (fig 4.3.5A), intestinal metaplasia patients exhibited a greater increase in gastrin concentration than atrophy (fig 4.3.5B) and the combined atrophy and intestinal metaplasia group exhibited the greatest increase in serum gastrin concentration (fig 4.3.5C).

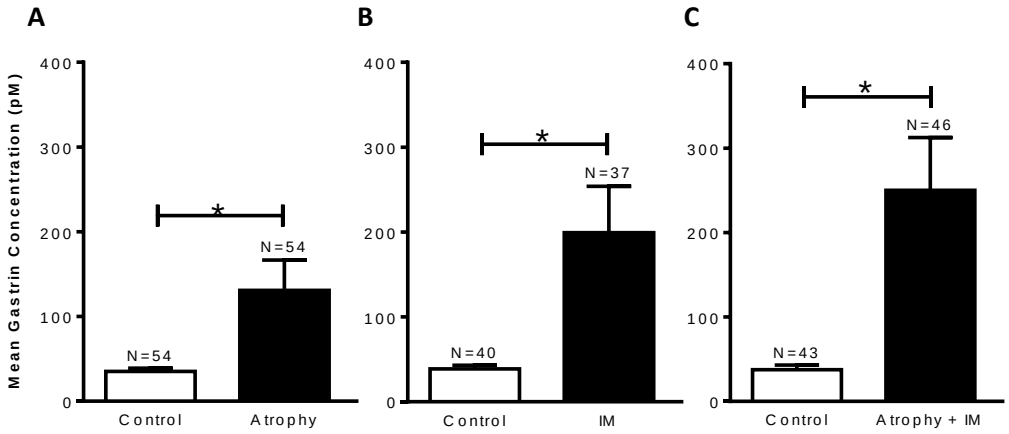

Figure 4.3.5 – **Preneoplastic patients exhibit significantly higher circulating gastrin concentration.** Significantly higher gastrin concentration observed in patients with gastric atrophy (A), intestinal metaplasia (B) and combined atrophy with metaplasia (C). Mean ± SEM, $p<0.05$ unpaired two-tailed t-test with Welch's correction.

4.3.2.2 Relative CgA mRNA abundance is significantly lower in biopsies exhibiting intestinal metaplasia

Unlike gastric biopsies obtained from control and Barrett's patients, the significantly higher gastrin concentration associated with preneoplastic changes in the stomach did not translate to a higher abundance of CgA, expressed relative to GAPDH mRNA abundance (fig 4.3.6A and C).

All preneoplastic biopsies exhibited a trend towards lower CgA abundance compared to controls, and in the intestinal metaplasia group this difference was statistically significant (fig 4.3.6B).

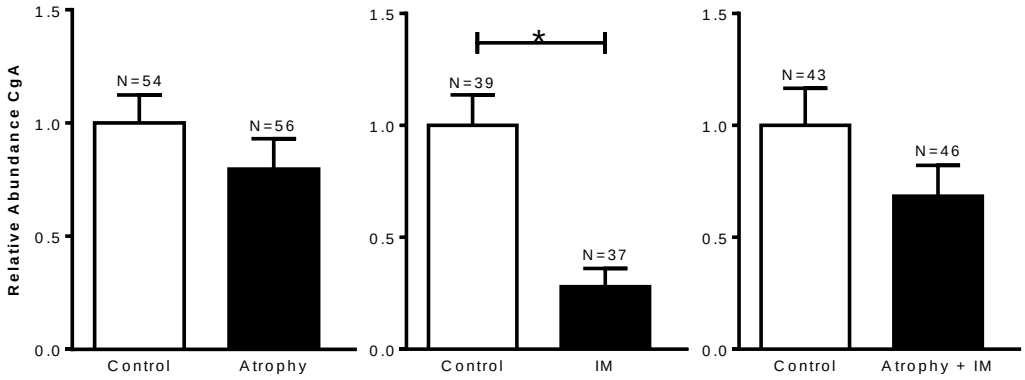

Figure 4.3.6 – **Relative CgA mRNA abundance is significantly lower in biopsies exhibiting intestinal metaplasia.** CgA abundance, expressed relative to GAPDH mRNA abundance, was significantly lower in IM patients (B) but not atrophy (A) or atrophy with IM patients (C). Mean ± SEM, $p<0.05$ unpaired two-tailed t-test with Welch's correction.

4.3.2.3 Relative CgA mRNA abundance is significantly higher in patients with gastrin >30 pM

Breaking down the preneoplastic CgA abundance results further, sub-groups were defined based on those with circulating gastrin below the dynamic range for stimulation of acid secretion (<30 pM) and those within and above that dynamic range (>30 pM). In both control (fig 4.3.7A) and preneoplastic (fig 4.3.7B) groups, relative CgA abundance was significantly higher when circulating gastrin concentration was above 30 pM. Separating by preneoplastic condition was less informative, with only the atrophy (fig 4.3.7C) and IM (fig 4.3.7D) control groups exhibiting a significant difference in abundance between gastrin groups (fig 4.3.7E).

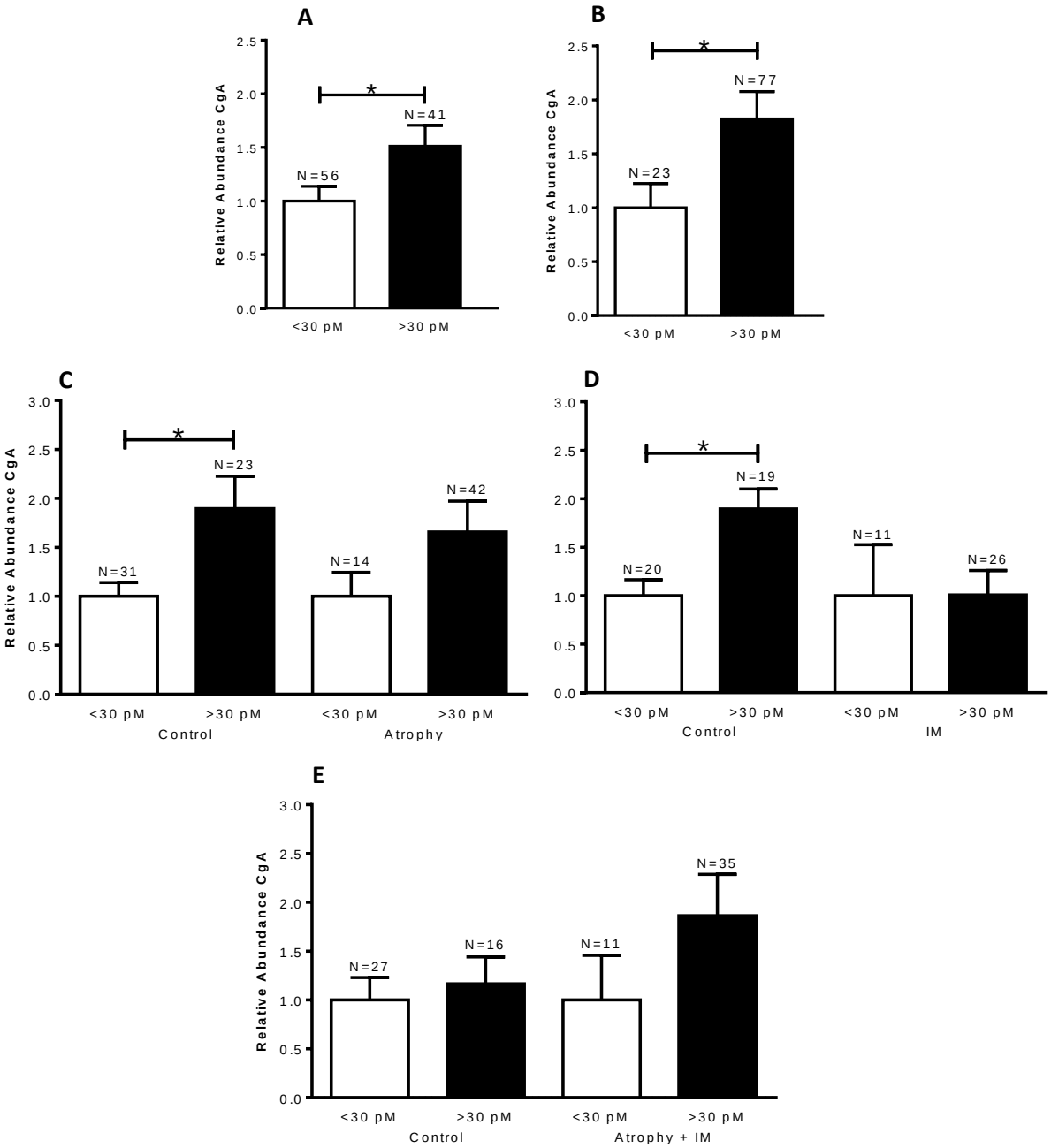

Figure 4.3.7 – **Relative CgA mRNA abundance is significantly higher in patients with gastrin >30 pM.** Relative CgA mRNA abundance was significantly higher in the control group (A) and the combined atrophy and IM patient groups (B) with circulating gastrin >30 pM. Breaking those groups down further by preneoplastic condition, atrophy and IM controls exhibited significantly higher CgA abundance compared with the <30 pM gastrin group (C, D) no significant changes were identified in disease states (C, D, E). Mean ± SEM, $p<0.05$ unpaired two-tailed t-test with Welch's correction.

4.3.2.4 CCK2R mRNA abundance is significantly lower in all preneoplastic groups

Analysing the expression of the CCK2R, all preneoplastic conditions exhibited significantly lower receptor mRNA abundance compared to control subjects that were matched to patients based on age and sex (fig 4.3.8A, B and C). Atrophy patients exhibited the least reduction in receptor abundance, intestinal metaplasia exhibited a greater reduction and combined atrophy plus intestinal metaplasia group exhibited the greatest reduction in receptor abundance.

A significant negative correlation between circulating gastrin concentration and relative CCK2R mRNA expression was identified in the control groups for gastric atrophy and IM preneoplastic control groups by Spearman's rank correlation (atrophy r=-0.48, df=52; IM r=-0.38, df=3; p<0.05) however no significant correlation was identified in the control group for atrophy + IM control group. Within patient groups however this significant correlation was lost, with no preneoplastic group exhibiting a significant relationship with circulating gastrin concentration.

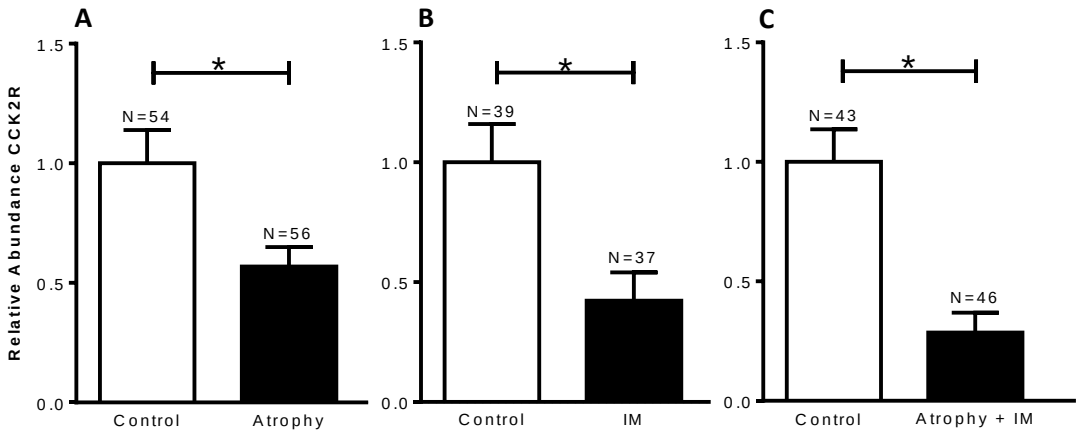

Figure 4.3.8 – **Relative CCK2R mRNA abundance is significantly lower in all preneoplastic groups.** Relative CCK2R abundance is significantly lower compared to controls in atrophy (A), IM (B) and atrophy with IM preneoplastic patient groups (C). Mean ± SEM, p<0.05 unpaired two-tailed t-test with Welch's correction.

4.3.2.5 Relative CCK2R mRNA abundance expression was significantly lower in atrophy patients exhibiting elevated gastrin

The groups of control subjects (fig 4.3.9A) and atrophy (fig 4.3.9B) patients were then sub-divided by gastrin concentration into those below the dynamic range for stimulation of acid secretion (<30 pM) and those within and above that dynamic range (>30 pM). Within both groups, CCK2 receptor abundance was not significantly different between low and high gastrin groups.

Separating by preneoplastic condition, the atrophy group exhibited significantly lower CCK2R abundance compared to controls when circulating gastrin was >30 pM (fig 4.3.9C). Within intestinal metaplasia and combined atrophy and intestinal metaplasia groups, CCK2R abundance exhibited no significant changes in either control or preneoplastic patients based on low vs high gastrin concentration (fig 4.3.9D, E).

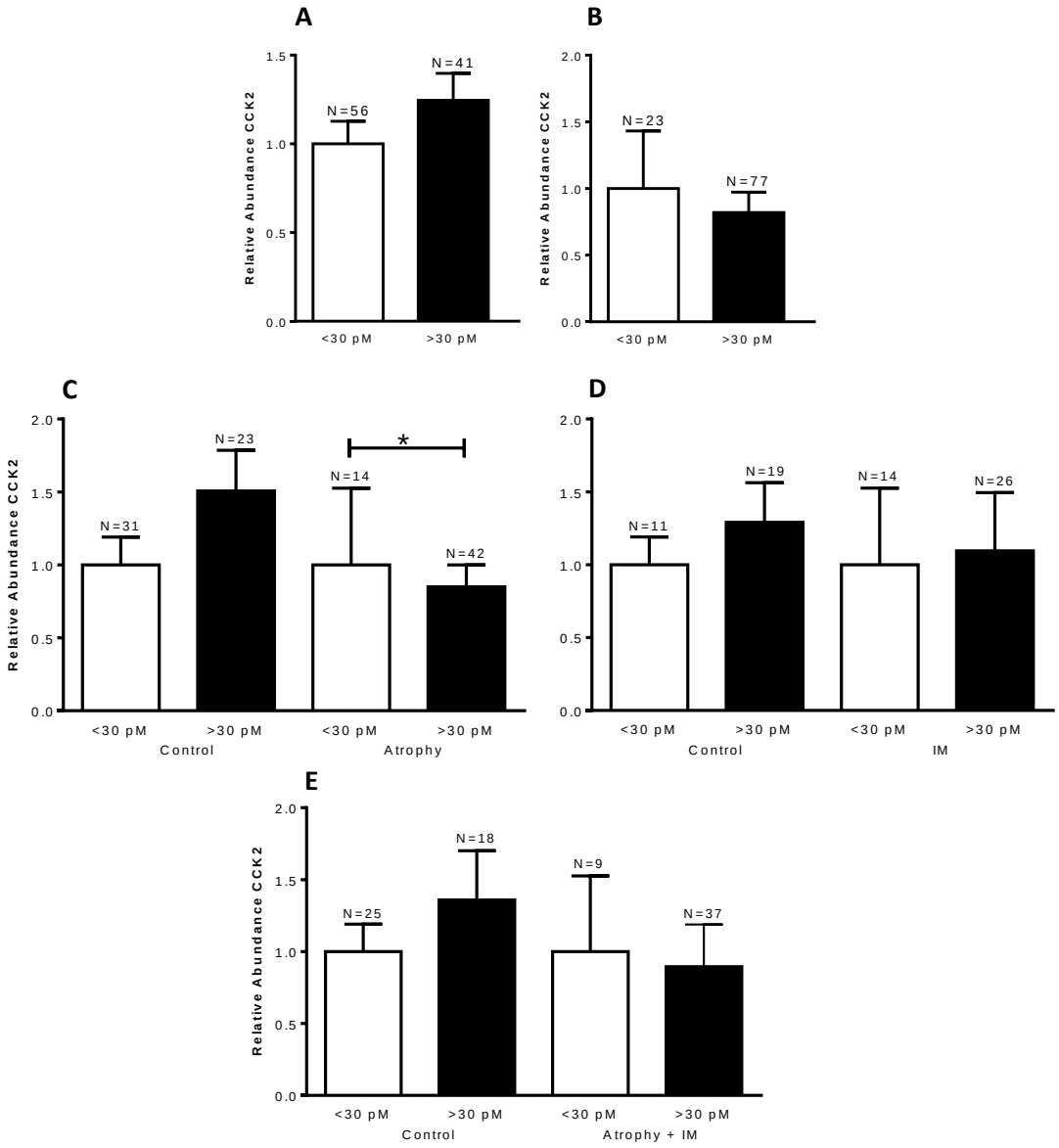

Figure 4.3.9 – **Relative CCK2R mRNA abundance was significantly lower in atrophy patients exhibiting elevated gastrin.** Relative CCK2R abundance was not significantly different in controls (A) or atrophy (B) when grouped by gastrin concentration. Broken down by preneoplastic condition, CCK2R abundance exhibited significantly lower abundance only within atrophy patients (C) with no significant change in IM (D) or combined atrophy with IM (E). Mean ± SEM, $p<0.05$ unpaired two-tailed t-test with Welch's correction.

4.4 Discussion

In this chapter, I have characterised the expression profile of putative biomarkers of gastrin-responsiveness (CgA, HDC, MMP-1, CCK2R) in the stomach of both normal (*H. pylori* negative, histologically verified), and Barrett's patients and shown how this profile is affected by PPI treatment and associated hypergastrinaemia. We have also characterised the putative biomarker expression profiles for CgA and CCK2R in preneoplastic conditions of the stomach. This work is novel, as previously abundance of these transcripts appears to have not been investigated in control subjects or in gastric biopsies of Barrett's or preneoplastic patients.

4.4.1 Changes in abundance of CgA

The first biomarker selected for testing, CgA, was chosen because of its well characterised use as a serum-based biomarker released by many neuroendocrine cells including neuroendocrine tumours (NETS).[315] A large meta-analysis recently found significantly increased circulating CgA to be a highly specific and sensitive biomarker for the detection of NETs.[315] However there is some dispute as to the usefulness of serum based CgA assays due to a number of factors. Firstly, CgA is extensively processed and modified post secretion in a pattern which varies individually with tumour type and origin, consequently circulating CgA is heterogeneous and the concentrations of any one fragment are, inevitably, highly variable. Thus, immunoassays employing antibodies with limited specificity (often unknown) can be difficult to interpret. Secondly, circulating CgA concentrations may be increased in renal and cardiac disease as well as non-endocrine malignancies so again interpretation of the data can be difficult.[316] Not surprisingly then, circulating CgA assays in common forms of hypergastrinaemia including atrophic gastritis[203] have proven to be unreliable for diagnosis and studies have shown that significant hypergastrinaemia does not necessarily correlate with significantly higher circulating CgA.[317] Despite these limitations we did perform some preliminary investigation into circulating CgA concentration in both normal and Barrett's patients with and without PPI treatment which can be found in appendix I.I.

Our approach attempts to circumvent these problems by measuring the relative abundance of the CgA mRNA at the tissue transcriptional level. This has allowed us to focus specifically on changes in the tissue of interest, in this case the stomach.

A similar approach has been used previously to assess the efficacy of the novel gastrin antagonist netazepide[185] in treating type 1 NETs utilising biopsies and qPCR and before that in a study which combined the use of ECL carcinoid biopsies and northern blots.[195] The novelty of this study is the extension of these techniques (use of biopsy and mRNA abundance quantification) to the normal stomach and preneoplastic conditions associated with high circulating gastrin.

Previously, a few studies using rat stomachs found that expression of CgA at the tissue mRNA level was significantly higher with PPI treatment by both qPCR[318] and standard PCR and western blotting.[200] Treatment of the rats with PPIs induced significantly higher mRNA expression regardless of whether the rats were fasted or not, which was inhibited by CCK2R antagonists suggesting that CgA mRNA abundance is gastrin linked. However, a literature search provided no evidence that this question had been investigated in humans.

In agreement with previously published work in the rat, patients treated with PPIs exhibited significantly higher CgA mRNA abundance in gastric biopsies compared to appropriate controls. Moreover, when samples were matched based on their age, sex and *H. pylori* status, or for circulating gastrin concentration, the elevation of CgA mRNA abundance was similar between both Barrett's and control subjects treated with PPIs, suggesting that CgA abundance is indeed linked to circulating gastrin concentration in both the control and Barrett's stomach.

In biopsies from patients exhibiting preneoplastic changes divided on the basis of circulating gastrin below (<30 pM) or above (>30 pM) the reference range, CgA mRNA abundance was not significantly different, highlighting the loss in the ECL cell population in atrophy, IM and atrophy with IM patients. In fact, patients exhibiting IM exhibited no change in CgA mRNA abundance with increased circulating gastrin concentration whereas in patients exhibiting gastric atrophy there was still a trend towards increased CgA abundance with high gastrin, presumably as a result of some ECL cell population remaining.

Breaking down further into low (<30 pM), medium (30-100 pM) and high (>100 pM) gastrin concentrations for each condition and patient group proved unhelpful, with too low an N number in the >100 pM group to perform reliable analysis.

4.4.2 Changes in abundance of CCK2R

The abundance of the main gastrin receptor, CCK2, in gastric samples is not well studied. Kimura *et. al.* reported a significant increase in receptor mRNA abundance over the course of treatment with PPIs in rats. However, controlled human studies regarding the effect of PPI treatment on gastric CCK2R expression appear not to have been published. The question is interesting, given that recently the CCK2R has become a novel therapeutic target with studies reporting CCK2R antagonists in NSAID-associated gastric ulceration,[319] type 1 gastric neuroendocrine tumours[185] and pancreatic cancer precursor legions[179]. Understanding more about how gastrin influences receptor expression may help build a better understanding of how useful novel anti-gastrin treatment methods (such as CCK2 antagonists and anti-gastrin antibodies) will be in the prevention of gastrin-responsive malignancies.

Our work identified that PPI treatment was associated with a significant reduction in the abundance of CCK2R mRNA, possibly a result of adaptation to extended periods of hypergastrinaemia. This complicates the aim of detecting possible biomarkers to track the effect of gastrin on Barrett's tissue, as changes in CCK2R abundance may impact on the sensitivity of cells to gastrin and in turn have an effect on any possible biomarker expression. The decrease in CCK2R abundance could compensate for any increase in suspected biomarker expression, meaning any putative biomarkers would need to be sufficiently sensitive to changes in gastrin to overcome the reduced receptor expression. Further study on the issue with much larger datasets is now required to explore variations in CCK2R abundance across patients and ranges of circulating gastrin concentration.

The significantly lower CCK2R abundance exhibited in preneoplastic patient biopsies was to be expected as a result of loss of CCK2R expressing ECL cells and parietal cells. As previously mentioned, loss of parietal cells and the intrinsic factor secreted by them can result in pernicious anaemia due to an inability to absorb vitamin B_{12} from the diet. Some preliminary work was performed analysing serum B_{12} concentration in control and corpus atrophy patients, detailed in appendix I.II.

Breaking down further into low (<30 pM), medium (30-100 pM) and high (>100 pM) gastrin concentrations for each condition and patient group proved unhelpful, with too low N number in the >100 pM group to perform reliable analysis.

4.4.3 Changes in abundance of HDC

The regulation of the histamine generating enzyme, HDC, has been extensively studied in relation to changes in circulating gastrin concentration.[197, 320-323] In the stomach of adult rats, extended fasting resulted in a reduction in both serum gastrin concentration and corpus HDC mRNA abundance whereas PPI-induced hypergastrinaemia induced significantly higher mRNA abundance.[324,325] Introduction of a selective CCK2 receptor antagonist blocked the higher HDC mRNA abundance associated with elevated circulating gastrin.[324] These data support the idea that gastrin may play a regulatory role in the expression of HDC mRNA and as such, HDC mRNA abundance changes may be useful as a putative biomarker of gastrin responsiveness in GI tissues.

Therefore, it was not surprising that the present data indicate that significantly elevated circulating gastrin concentration, in this case induced by PPI treatment, correlates with a significantly higher abundance of HDC mRNA. This held true in both control and Barrett's patients.

4.4.4 Changes in abundance of MMP-1

Previous work has identified a role of gastrin in stimulating MMP-1 expression from gastric epithelial cells[211] implicated in cell migration which again may prove useful a putative biomarker of gastrin activity on cells of the GIT.

Within our gastric samples, only control patients exhibited statistically higher MMP-1 mRNA abundance with PPI treatment and its associated increase in serum gastrin concentration. Further studies of this are need, however, given the large variance in MMP-1 mRNA expression and the relatively lower sample size for the gastrin-matched Barrett's and control samples. As a possible biomarker of gastrin activity on CCK2 expressing cells, despite there being some indication of a trend towards increased abundance, it would seem that MMP-1 is less sensitive to changes in gastrin concentration than either CgA or HDC, making them preferable biomarkers to use moving forward.

4.4.5 Conclusion

Our work is novel in several ways; firstly it is based on histologically verified normal human gastric samples where the main variable is treatment with PPIs and associated hypergastrinaemia; secondly, we have similar data from the stomachs of Barrett's patients again where the main variable is treatment with PPIs and associated hypergastrinemia; thirdly we have similar biomarker abundance data from a variety of preneoplastic conditions of the stomach increasing in severity of cancer risk with which to compare to controls. Nevertheless, these are all cross-sectional studies, and in the future, there are opportunities for longitudinal studies of the system.

In summary, the main findings of this chapter are that a) in gastric biopsies derived from normal and Barrett's patients PPI treatment is associated with significantly higher relative CgA abundance in both groups, significantly lower relative CCK2R abundance in both groups, significantly higher relative HDC abundance in the Barrett's patient group and only control biopsies exhibited a significant change in relative MMP-1 abundance with PPI treatment; b) patients with preneoplastic conditions of the stomach exhibited significantly higher serum gastrin concentration with the magnitude of gastrin concentration increase relative to progression of the condition along the Correa cascade, gastric preneoplastic conditions exhibit significantly lower relative CCK2R mRNA abundance whilst CgA mRNA abundance was significantly lower in biopsies exhibiting intestinal metaplasia.

Identification of a relationship between gastrin concentration and CCK2R abundance was unexpected and may have an impact on putative biomarkers of gastrin-responsiveness in Barrett's oesophagus, the next stage of the study therefore sought to identify whether a similar gastrin-CCK2R relationship existed in Barrett's oesophageal biopsies and if so whether it might influence putative biomarker abundance.

Chapter 5

Putative biomarker expression in Barrett's oesophagus biopsies

5.1 Introduction

Gastrin has previously been identified as an inducer of gene expression in CCK2R expressing ECL and parietal cells of the stomach.[196, 200, 323, 324] These changes in expression were mediated by CCK2R activation.[231,185] It was assumed that with the discovery of functional CCK2Rs in BO,[160] their activation would also induce similar changes in gene expression in BO. In turn, these could then be measured with qPCR to establish a gastrin-regulated gene expression profile. It was hypothesised that any changes in gene expression would be related to circulating gastrin concentration, which has previously been associated with greater risk of BO progression to ACO.[289] A literature search identified a number of potential candidates that were identified as being gastrin-regulated or de-regulated in cases of ACO or gastric cancer including potential markers of gastrin activity on endocrine cells (CgA), markers of mucosal damage (COX-2, TFF1 and 2) and markers of cellular invasion (SHH and MMPs).[200, 326-332] We also aimed to examine expression of CCK2R itself, relative to circulating gastrin concentration, as in the previous chapter studies in gastric biopsies suggested altered expression with PPI treatment and a relationship between circulating gastrin and expression of CCK2R.

Recent advancements in qPCR techniques have facilitated the study of microRNAs (miRNAs) – small non-coding RNA molecules that play a role in regulating gene expression - and has revealed their potential as biomarkers of multiple cancers.[227, 333, 334] MicroRNAs represent an ideal candidate for biomarker studies as they are relatively stable in both tissue and serum, so the potential exists for serum miRNA profiles to be used as a surrogate for assay of miRNAs in tissue. A number of publications have investigated the use of a variety of miRNA candidates as biomarkers of BO and its progression,[335-337] however none to date have been validated as sensitive enough to track progression. Likewise no comparison has been made of the expression of miRNAs in BE, either at serum or tissue level, and circulating gastrin concentration.

5.1.1 Objectives

Specific objectives of this chapter were:

- Identification of putative biomarkers of gastrin responsiveness in BO relative to the circulating concentrations of gastrin.
- Determination of the microRNA expression profile in serum and BO relative to circulating concentrations of gastrin

5.2 Methods

Biopsies of Barrett's oesophagus with matching serum samples were obtained from patients undergoing routine Barrett's surveillance at either the Royal Liverpool University hospital (RLUH) or the Szeged University hospital (UoSz), Hungary. Dr Andy Moore, Dr Ashley Bond and Prof. Mark Pritchard recruited patients and obtained blood samples and biopsies for samples obtained at the RLUH whilst Dr. András I. Rosztóczy and Prof. Tibor Wittman were responsible for the same duties at the UoSz. Biopsies from both institutions were sent to the University of Szeged for histological analysis by Prof. Laszlo Tiszlavicz and Dr. Zita Reisz. Biopsies destined for qPCR were immediately processed via the protocol outlined in section 2.1.3.3 and then frozen and stored at -20°C (RLUH) or frozen and transported on dry ice to Liverpool (UoSz).

RNA was extracted and converted to cDNA for qPCR utilising the method detailed in sections 2.4.1 and 2.4.2; qPCR was performed utilising the multiplexed method as detailed in section 2.4.4. Transcript abundance was normalised to GAPDH, and then expressed as fold difference relative to the mean abundance in the control group, defined as those patients with fasting circulating gastrin concentrations in the normal range i.e. <30 pM.

Serum and biopsy miRNA extraction, conversion to cDNA and subsequent qPCR was performed using the Qiagen suite of micro RNA processing kits (miRNeasy, miScript II RT kit, miScript SYBR green PCR kit coupled with miScript miRNA PCR assay primers) according to the manufacturer's instructions described in section 2.5. Putative mRNA and miRNA biomarkers analysed by qPCR are listed in table 5.2.1.

mRNA	miRNA
CgA	-21
MMP-1	-221
MMP-7	-222
COX-2	
CCK2R	
TFF1	
TFF2	
SHH	

Table 5.2.1 – **Putative biomarkers of gastrin responsiveness investigated in tissue and serum biopsies of patients with BO.** Specific primer sequences used for qPCR are listed in tables 2.1 and 2.2.

Data obtained by qPCR was initially analysed in all Barrett's oesophagus biopsies and is presented for this group and, separately, for the sub-group with intestinal metaplasia (IM) only. This sub-group was selected for presentation as it was the largest homologous group available within the Barrett's cohort (see table 2.1).

5.3 Results

5.3.1 The association between serum gastrin and relative putative biomarker abundance in Barrett's oesophagus biopsies

5.3.1.1 GAPDH mRNA abundance is gastrin insensitive

Previous work in the laboratory using a commercial panel of housekeeping genes had identified GAPDH as being the most robust reference point for normalisation of qPCR data in gastric biopsies.[134, 338] However, it was important to validate the use of GAPDH in Barrett's oesophagus biopsies and in particular to ensure that GAPDH expression did not change with variations in circulating gastrin concentration. When the average GAPDH expression across all multiplexed qPCRs for each biopsy sample was combined and related to circulating gastrin concentration, no significant relationship was identified (r=-0.09 df=52 p<0.05) (fig 5.3.1) indicating no association between serum gastrin and GAPDH abundance in oesophageal biopsies.

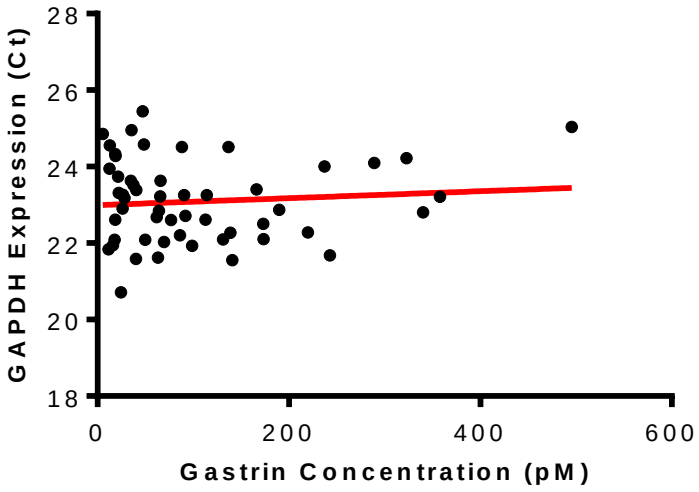

Figure 5.3.1 - **GAPDH mRNA abundance is gastrin insensitive.** Spearman's rank correlation identified no significant relationship between GAPDH abundance and circulating gastrin concentration in BO biopsies.

5.3.1.2 Relative CgA mRNA abundance is significantly higher when circulating gastrin is between 30-100 pM

Putative biomarker expression in Barrett's oesophagus biopsies was analysed across all samples and is described here in the group as a whole and, separately, in the sub-group that exhibited only intestinal metaplasia without evidence of dysplasia/cancer, or evidence of gastric metaplasia (table 2.1).

CgA mRNA abundance, relative to GAPDH abundance, within both the whole Barrett's oesophagus biopsy patient cohort (fig 5.3.2A) and samples selected for intestinal metaplasia (fig 5.3.2B) was significantly higher in patients with circulating gastrin concentrations across the post-prandial dynamic range of gastrin (30-100 pM) compared to the reference range for fasting circulating gastrin (<30 pM), or above the post-prandial range (>100 pM).

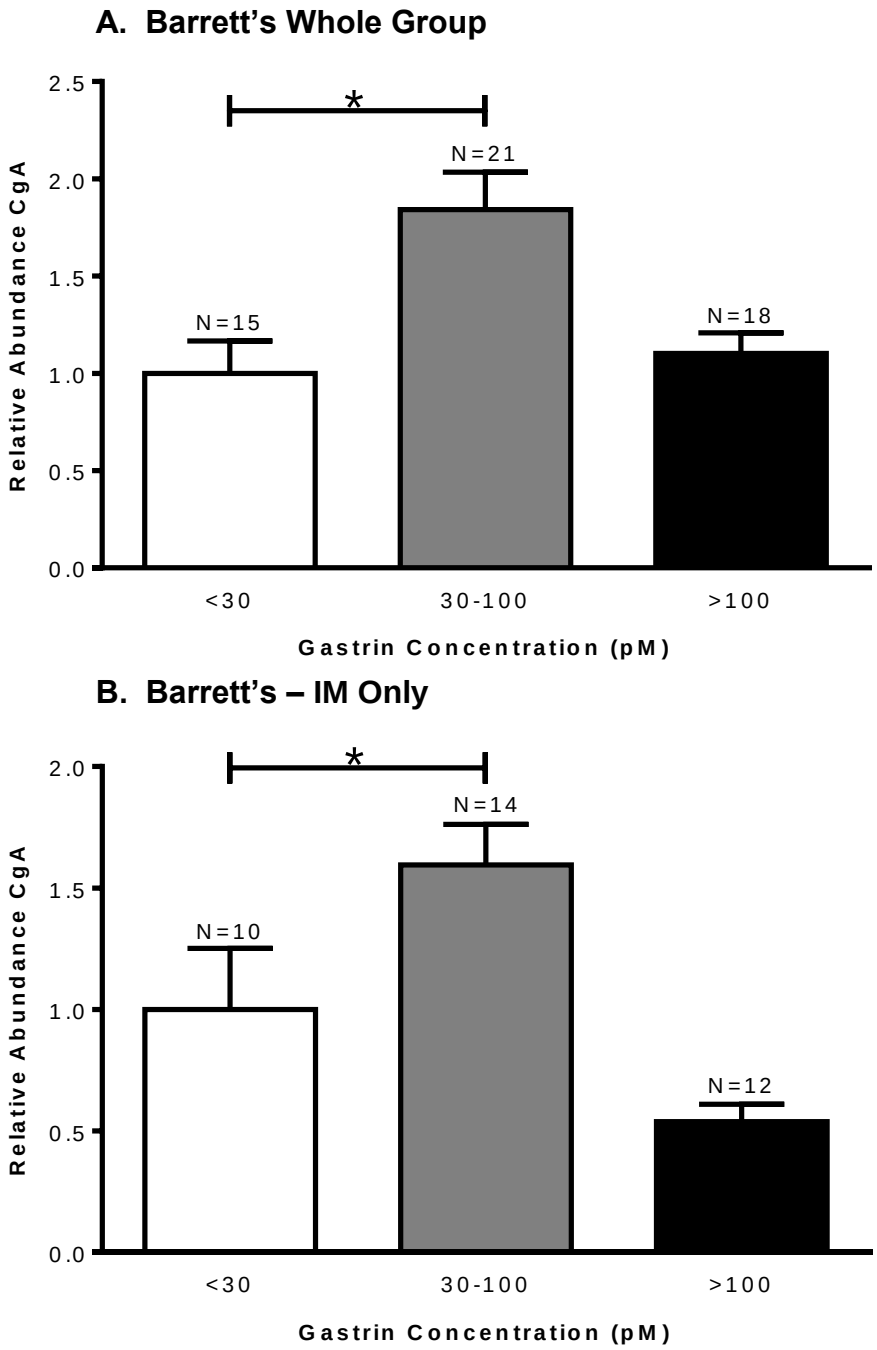

Figure 5.3.2 – **Relative CgA mRNA abundance is significantly higher when circulating gastrin is between 30-100 pM.** Relative abundance of CgA mRNA, normalised to GAPDH mRNA abundance, was significantly higher in Barrett's oesophagus biopsies of patients with circulating gastrin between 30-100 pM (A). In the subgroup selected for intestinal metaplasia, relative CgA mRNA abundance remained significantly higher in patients with circulating gastrin between 30-100 pM (B). Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.1.3 Relative MMP-1 mRNA abundance is significantly lower when circulating gastrin is >30 pM

MMP-1 mRNA abundance, relative to GAPDH abundance, within the whole Barrett's oesophagus patient cohort (fig 5.3.3A) exhibited no significant changes relative to circulating gastrin concentration.

Selecting for patients exhibiting only intestinal metaplasia (fig 5.3.3B), relative MMP-1 mRNA abundance was significantly lower at circulating gastrin concentrations within the post-prandial physiological range (30-100 pM) and above (>100 pM) compared to the reference range for fasting circulating gastrin (<30 pM).

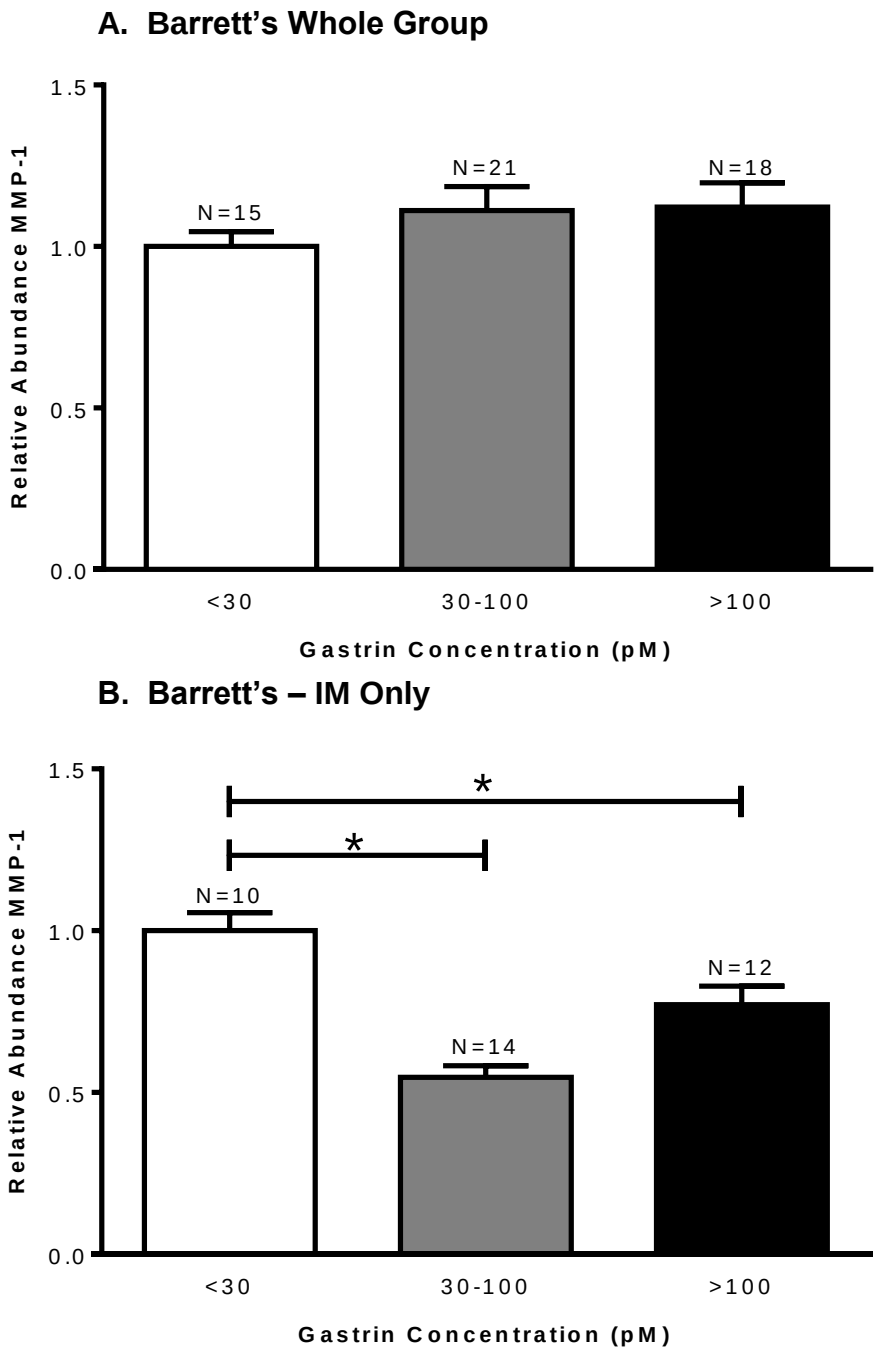

Figure 5.3.3 – **Relative MMP-1 mRNA abundance is significantly lower when circulating gastrin is >30 pM.** MMP-1 mRNA expression, relative to GAPDH, exhibits no significant change with changes in circulating gastrin concentration across all biopsies (A). Relative MMP-1 mRNA abundance was significantly lower with circulating gastrin >30 pM in the subgroup selected for intestinal metaplasia (B). Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.1.4 Relative MMP-7 abundance is significantly higher when circulating gastrin is >100 pM

MMP-7 mRNA abundance, relative to GAPDH abundance, within both the whole Barrett's oesophagus patient cohort (fig 5.3.4A) and samples exhibiting only intestinal metaplasia (fig 5.3.4B) was significantly higher when circulating gastrin concentration was above the post-prandial physiological range of gastrin (>100 pM) compared to the reference range for fasting circulating gastrin (<30 pM).

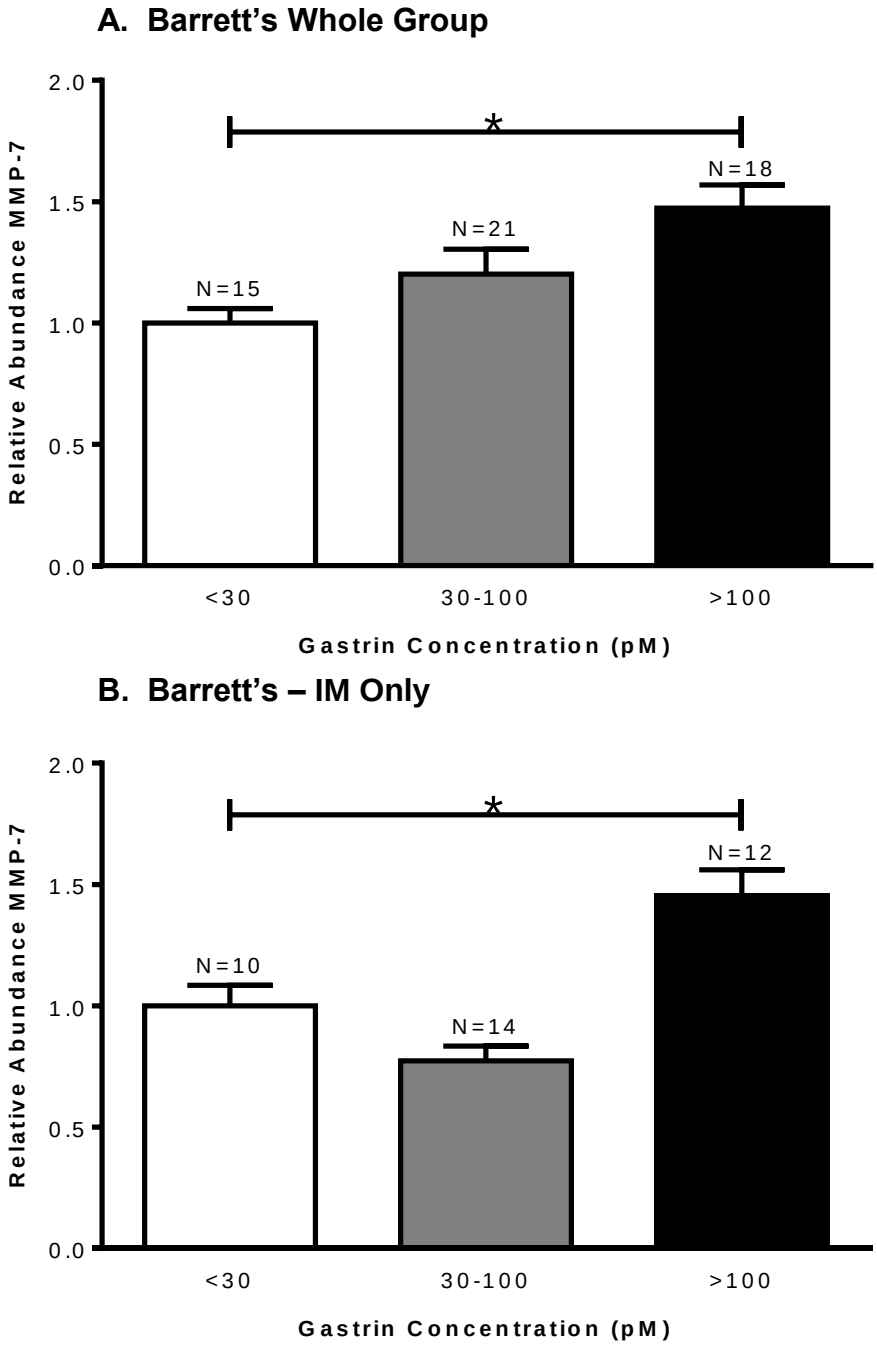

Figure 5.3.4 – **Relative MMP-7 abundance is significantly higher when circulating gastrin is >100 pM.** MMP-7 mRNA expression, relative to GAPDH, is significantly higher in biopsies of Barrett's oesophagus in patients when circulating gastrin is >100 pM (A). MMP-7 mRNA abundance is also significantly higher when circulating gastrin is >100 pM in the subgroup selected for intestinal metaplasia (B). Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.1.5 Relative COX-2 mRNA abundance is significantly higher when circulating gastrin is >30 pM

COX-2 mRNA abundance, relative to GAPDH abundance, within the whole Barrett's oesophagus biopsy cohort (fig 5.3.5A) was significantly higher when circulating gastrin concentration was within the post-prandial physiological range (30-100 pM) or above (>100 pM) compared to the reference range for fasting circulating gastrin (<30 pM). In samples selected for exhibiting only intestinal metaplasia (fig 5.3.5B) however, relative COX-2 mRNA abundance was only higher when circulating gastrin was above the post-prandial range (>100 pM) compared to fasting gastrin (<30 pM).

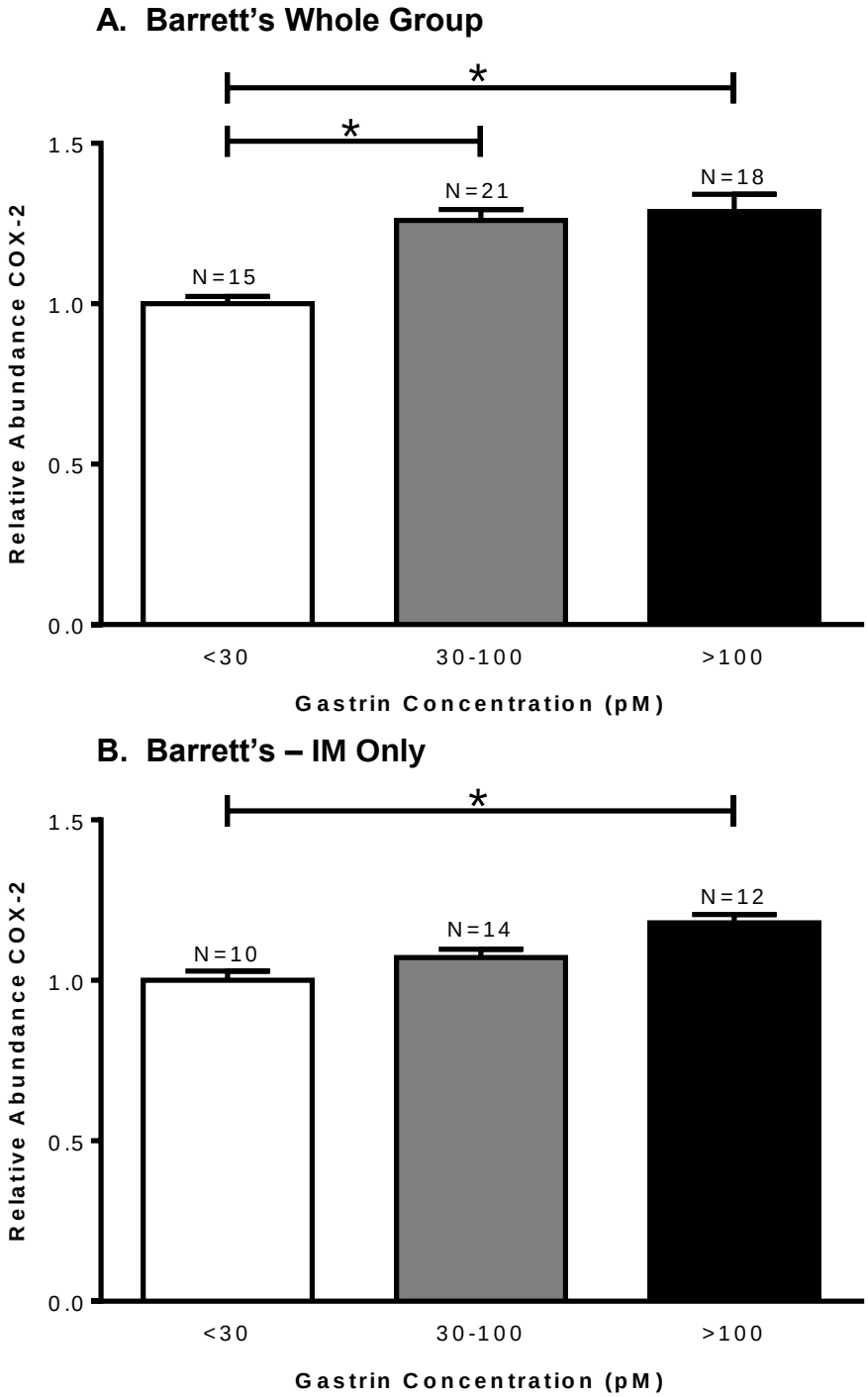

Figure 5.3.5 **– Relative COX-2 mRNA abundance is significantly higher when circulating gastrin is >30 pM.** COX-2 mRNA expression, relative to GAPDH, is significantly higher in Barrett's oesophagus biopsies in patients when circulating gastrin is >30 pM (A). COX-2 abundance is significantly higher when circulating gastrin is >100 pM in the subgroup selected for intestinal metaplasia (B). Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.1.6 Relative CCK2R mRNA abundance is significantly lower when circulating gastrin is >30 pM

In the whole Barrett's oesophagus biopsy cohort, CCK2R mRNA abundance, relative to GAPDH abundance, remained unchanged across the post-prandial physiological range of gastrin (30-100 pM) compared to the reference range for fasting circulating gastrin (<30 pM). However, with circulating gastrin concentrations above the physiological range (>100 pM) there was significantly lower CCK2R abundance (fig 5.3.6A).

In the subgroup of patients selected for only intestinal metaplasia, relative CCK2R mRNA abundance was significantly higher across the post-prandial physiological range of gastrin (30-100 pM) compared to the reference range for fasting circulating gastrin (<30 pM). However, at concentrations above the physiological range (>100 pM) relative abundance was significantly lower compared to the reference range for fasting circulating gastrin (<30 pM) (fig 5.3.6B

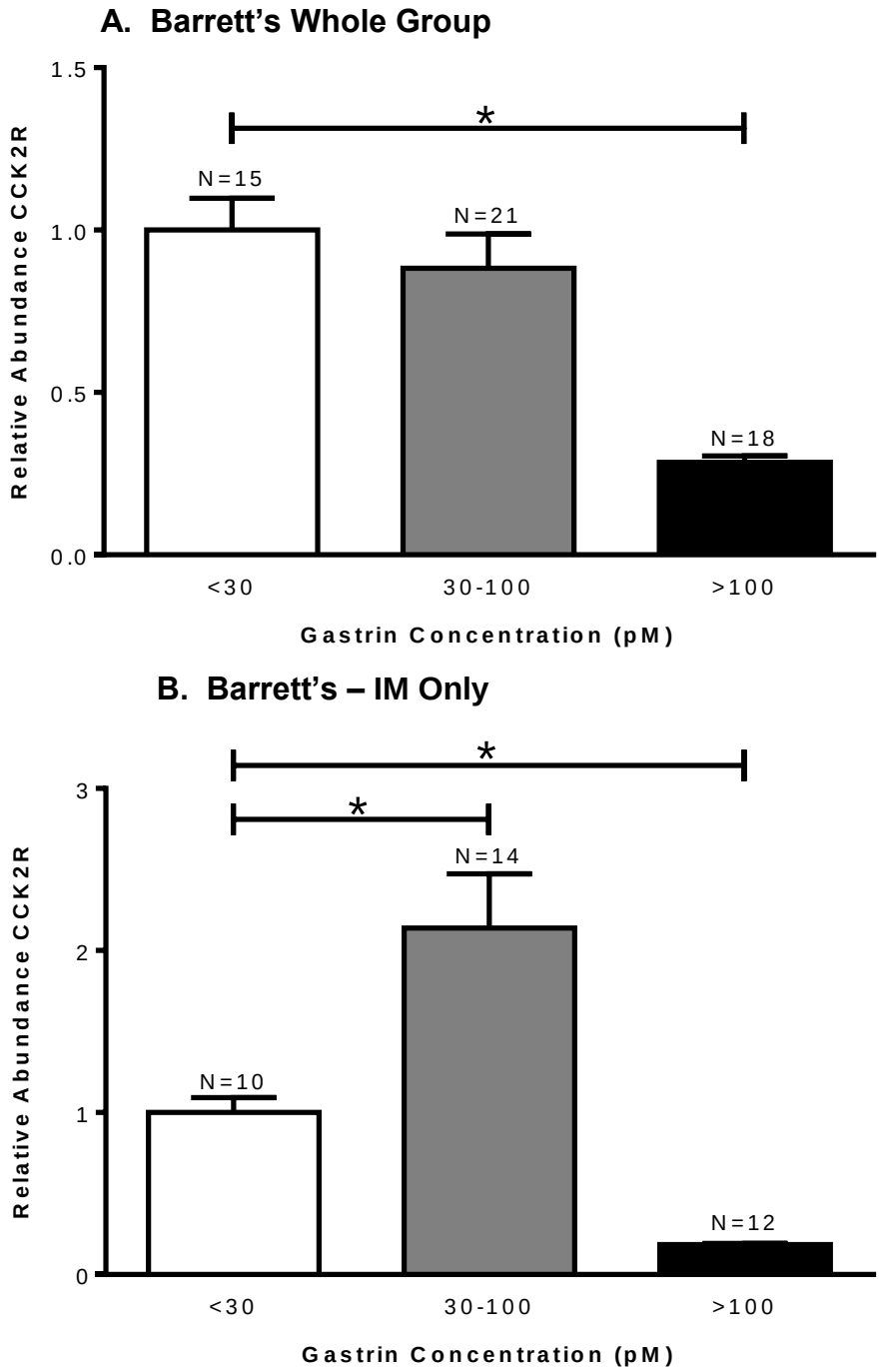

Figure 5.3.6 - **Relative CCK2R mRNA abundance is significantly lower when circulating gastrin is >30 pM.** CCK2R mRNA expression, relative to GAPDH, is significantly lower in Barrett's oesophagus biopsies when circulating gastrin is >100 pM (A). CCK2R is significantly higher when circulating gastrin is between 30-100 pM, however significantly lower when circulating gastrin is >100 pM in the subgroup selected for intestinal metaplasia (B). Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.1.7 Relative TFF1 mRNA expression in Barrett's biopsies is significantly lower when circulating gastrin is between 30-100 pM

TFF1 mRNA abundance, relative to GAPDH abundance, was only gastrin sensitive within the whole Barrett's oesophagus biopsy cohort, where abundance was significantly lower within the post-prandial physiological range (30-100 pM) of gastrin compared to the reference range for fasting circulating gastrin (<30 pM) (fig 5.3.7A). This trend however did not present in the samples selected for only intestinal metaplasia (fig 5.3.7B) in which relative TFF1 mRNA abundance was not significantly different in any gastrin groups when compared to the reference range for circulating gastrin (<30 pM).

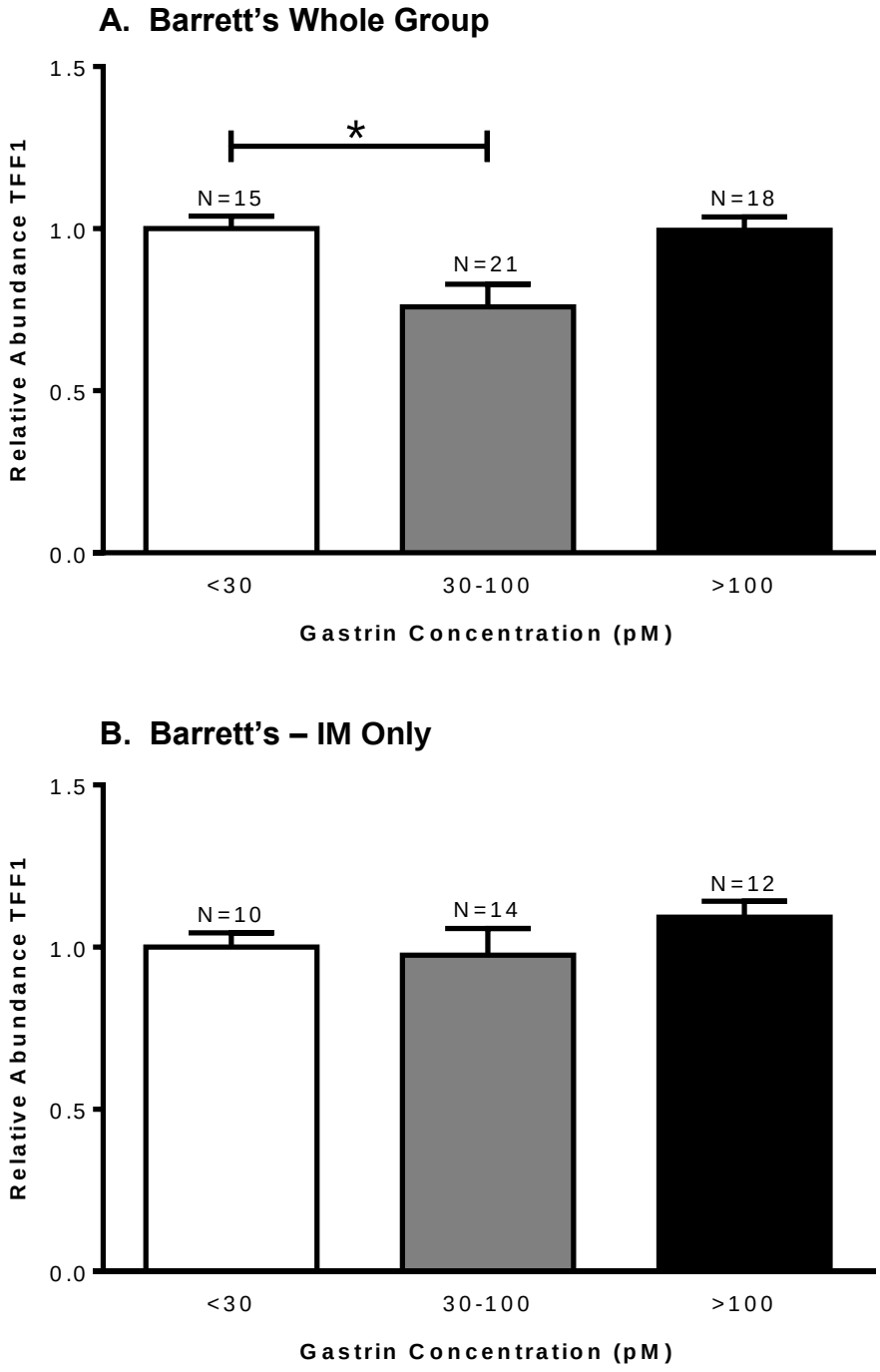

Figure 5.3.7 - **Relative TFF1 mRNA expression in Barrett's biopsies is significantly lower when circulating gastrin is between 30-100 pM.** TFF1 mRNA abundance, relative to GAPDH, was significantly lower in Barrett's oesophagus biopsies (A) however was not gastrin sensitive in the subgroup selected for intestinal metaplasia (B) Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.1.8 Relative TFF2 mRNA expression in Barrett's biopsies is largely gastrin insensitive

TFF2 mRNA abundance, relative to GAPDH abundance, was insensitive to changes in circulating gastrin concentration in both groups, with no statistically significant changes in relative abundance identified. However, across the whole Barrett's oesophagus biopsy patient cohort (fig 5.3.8A) and there did appear to be a trend towards higher relative abundance of TFF2 at circulating gastrin concentrations above the post-prandial physiological range (>100 pM) compared to the reference range for fasting circulating gastrin (<30 pM). In biopsies selected for only intestinal metaplasia (fig 5.3.8B) there also appeared a trend towards higher relative abundance of TFF2 with circulating gastrin concentration above the reference range for fasting gastrin (>30 pM).

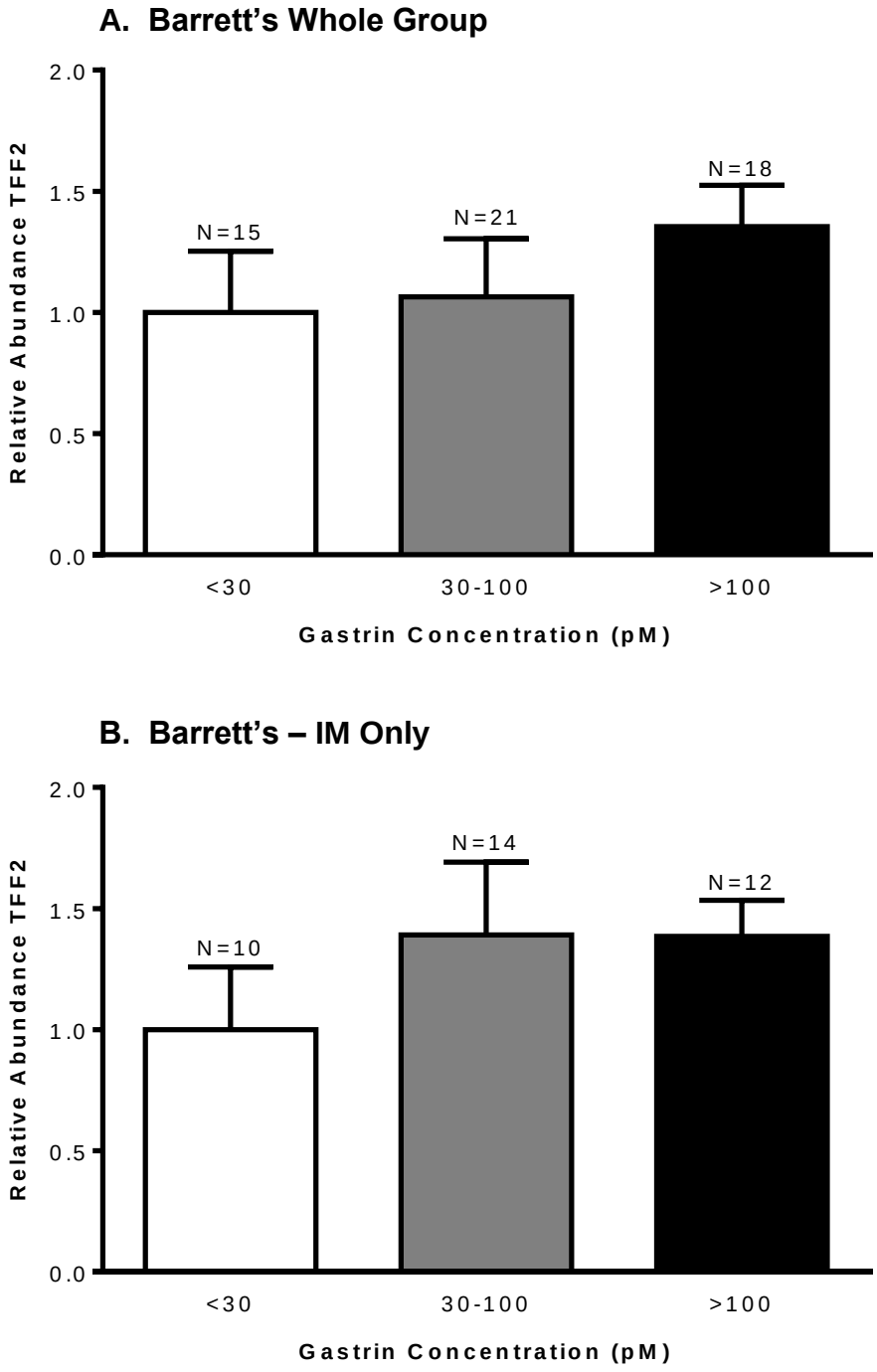

Figure 5.3.8 - **Relative TFF2 mRNA expression in Barrett's biopsies is gastrin insensitive.** TFF2 mRNA abundance, relative to GAPDH, exhibited no significant changes in Barrett's oesophagus biopsies (A) or in the subgroup selected for intestinal metaplasia (B) with circulating gastrin. Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.1.9 Relative SHH mRNA abundance was significantly lower when circulating gastrin was >100 pM

SHH mRNA abundance, relative to GAPDH abundance, within the whole Barrett's oesophagus biopsy patient cohort (fig 5.3.9A) and only samples selected for only intestinal metaplasia (fig 5.3.9B), remained unchanged across the post-prandial physiological range of circulating gastrin (30-100 pM) compared to the reference range for fasting circulating gastrin (<30 pM). However, at circulating gastrin concentrations above the physiological range (>100 pM) there was significantly lower relative abundance compared to the reference range for fasting circulating gastrin (<30 pM).

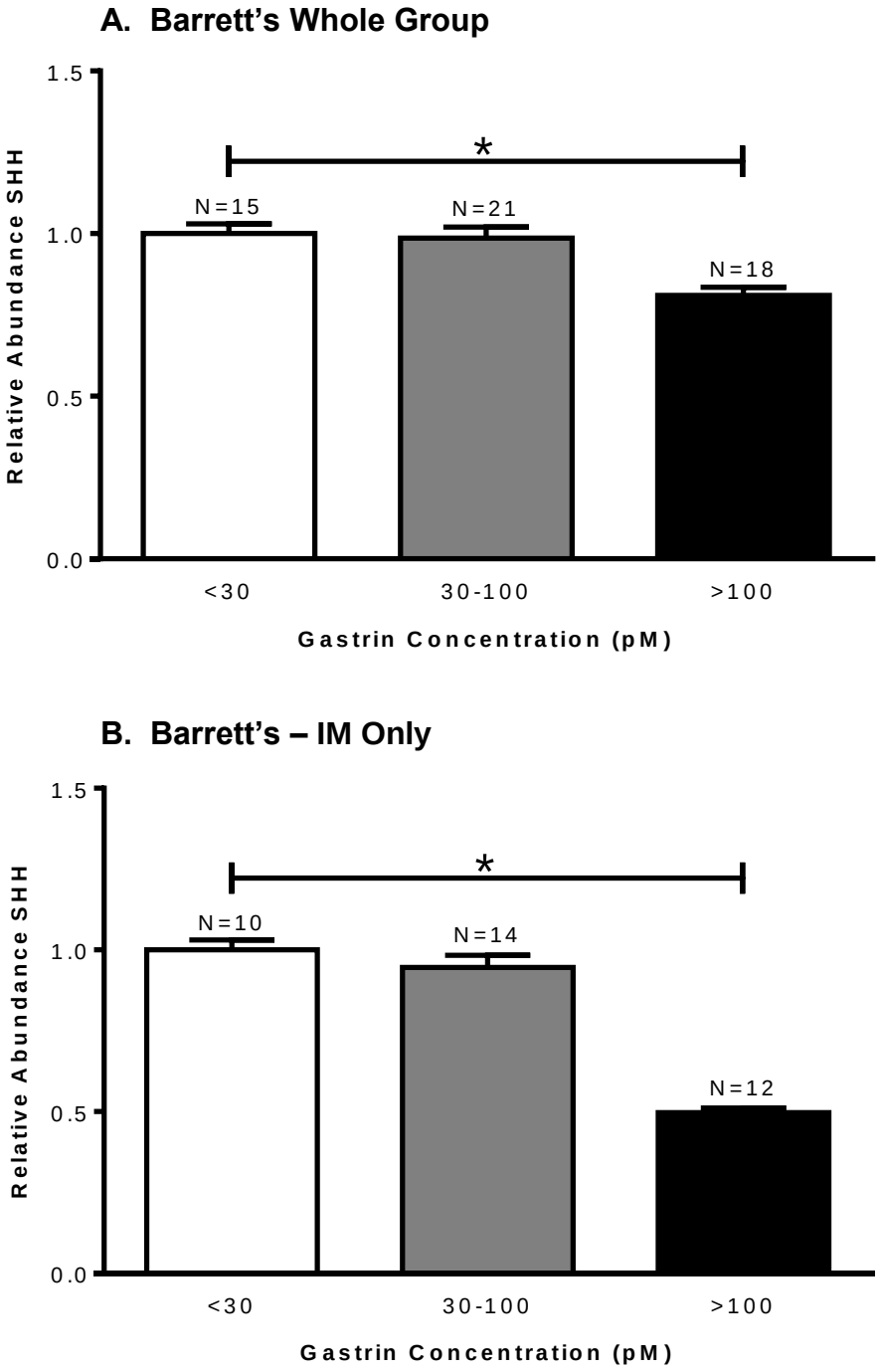

Figure 5.3.9 – **Relative SHH mRNA abundance was significantly lower when circulating gastrin was >100 pM.** SHH mRNA abundance, relative to GAPDH, was significantly lower when circulating gastrin was >100 pM in Barrett's oesophagus biopsies (A) and in the subgroup selected for intestinal metaplasia (B). Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.2 The effect of gastrin on miRNA expression in serum of Barrett's patients

5.3.2.1 RNU6B abundance in serum is gastrin insensitive

Previous work performed by Lloyd *et. al.*[230] within our department using a panel of snoRNA sequences, generated for normalisation of microRNA expression, identified RNU6B as the most abundant and stably expressed sequence to use for normalisation. As with GAPDH, it was important to validate the use of RNU6B in serum for data normalisation across samples that covered a large range of circulating gastrin concentrations (to identify any influence gastrin may have on abundance which may affect normalisation). Across all samples serum RNU6B abundance remained stable and no statistically significant relationship was identified by Spearman's rank analysis between circulating gastrin concentration and RNU6B abundance ($r=-0.24$ df=52 p<0.05) (fig 5.3.1).

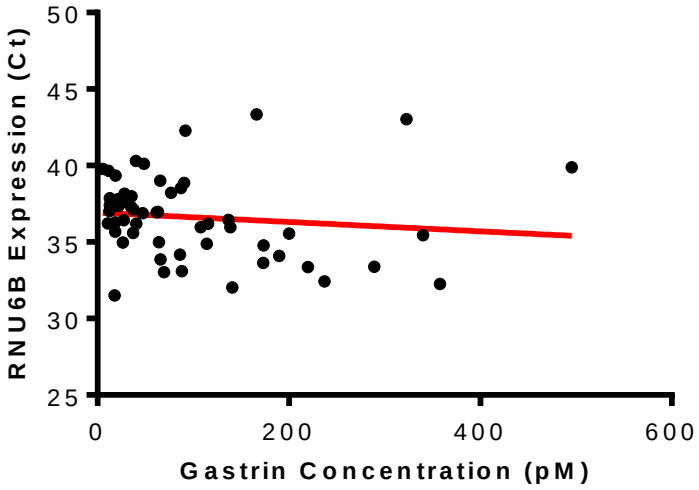

Figure 5.3.10 – **RNU6B abundance in serum is gastrin insensitive.** Spearman's rank correlation identified no significant relationship between RNU6B abundance and circulating gastrin concentration.

5.3.2.2 Relative serum miR-21 abundance is significantly higher when gastrin concentration is between 30-100 pM

Serum miR-21 abundance, relative to RNU6B abundance, within the whole Barrett's patient cohort (fig 5.3.11A) and in the sub-group selected for intestinal metaplasia only (5.3.11B) was significantly higher when circulating gastrin was in the post-prandial physiological range (30-100 pM) compared to the reference range for fasting circulating gastrin (<30 pM). At circulating gastrin concentrations above the physiological range (>100 pM) however, relative miR-21 mRNA abundance was not significantly different compared to the reference range for fasting gastrin.

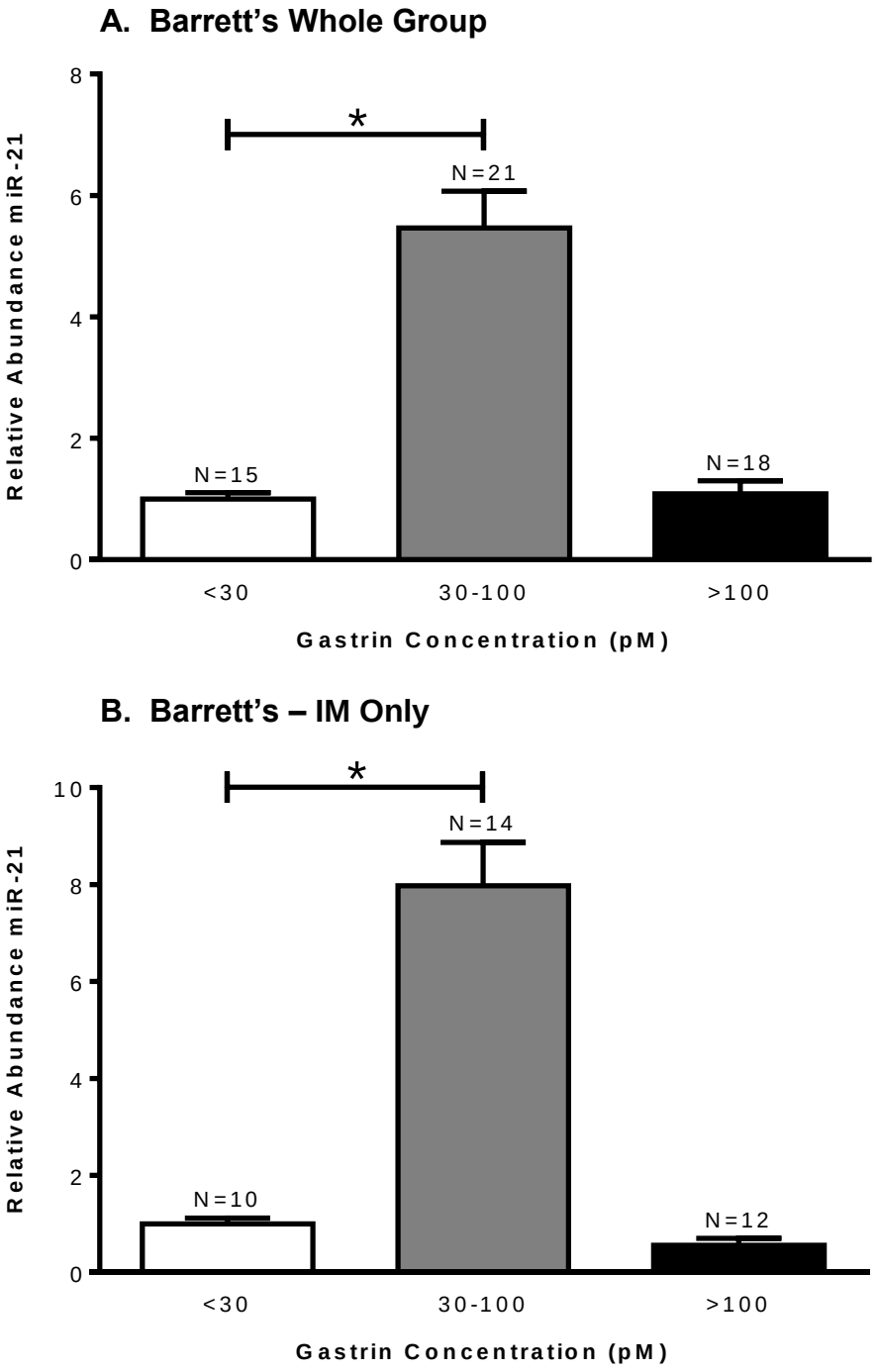

Figure 5.3.11 – **Relative serum miR-21 abundance is significantly higher when gastrin concentration is between 30-100 pM.** Abundance of miR-21 miRNA, relative to RNU6B, in serum was significantly higher when circulating gastrin was between 30-100 pM in both the whole Barrett's patient cohort (A) and in the subgroup with intestinal metaplasia only (B). Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.2.3 Relative serum miR-221 abundance is significantly higher when gastrin concentration is between 30-100 pM

Serum miR-221 abundance, relative to RNU6B abundance, within the whole Barrett's patient cohort (fig 5.3.12A) and in the sub-group selected for intestinal metaplasia only (5.3.12B) was significantly higher when circulating gastrin was in the post-prandial physiological range (30-100 pM) compared to the reference range for fasting circulating gastrin (<30 pM). At circulating gastrin concentrations above the physiological range (>100 pM) however, relative miR-221 mRNA abundance was not significantly different compared to the reference range for fasting gastrin.

Relative abundance changes of miR-221 with circulating gastrin therefore mirrors the changes seen in miR-21 (fig 5.3.11).

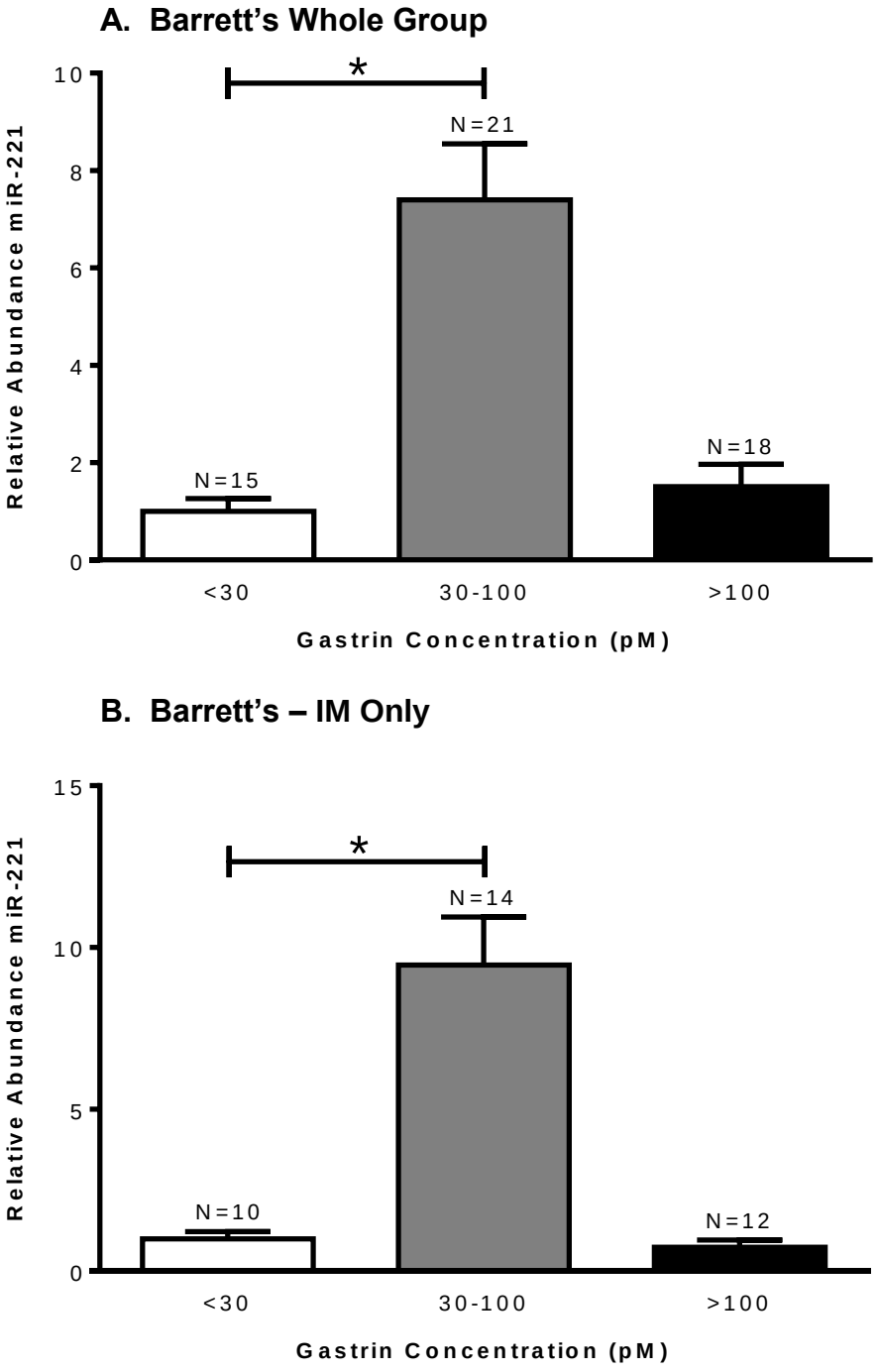

Figure 5.3.12 – **Relative serum miR-221 abundance is significantly higher when gastrin concentration is between 30-100 pM.** Abundance of miR-221 miRNA, relative to RNU6B, in serum was significantly higher when circulating gastrin was between 30-100 pM in both the whole Barrett's patient cohort (A) and in the subgroup with intestinal metaplasia only (B). Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.2.4 Serum miR-222 is significantly higher when gastrin concentration is between 30-100 pM

Serum miR-222 abundance, relative to RNU6B abundance, within the whole Barrett's patient cohort (fig 5.3.13A) and in the sub-group selected for intestinal metaplasia only (5.3.13B) was significantly higher when circulating gastrin was in the post-prandial physiological range (30-100 pM) compared to the reference range for fasting circulating gastrin (<30 pM). At circulating gastrin concentrations above the physiological range (>100 pM) however, relative miR-222 mRNA abundance was not significantly different compared to the reference range for fasting gastrin.

Relative abundance changes of miR-222 with circulating gastrin therefore mirrors the changes seen in mir-221 (fig 5.3.12) and miR-21 (fig 5.3.11).

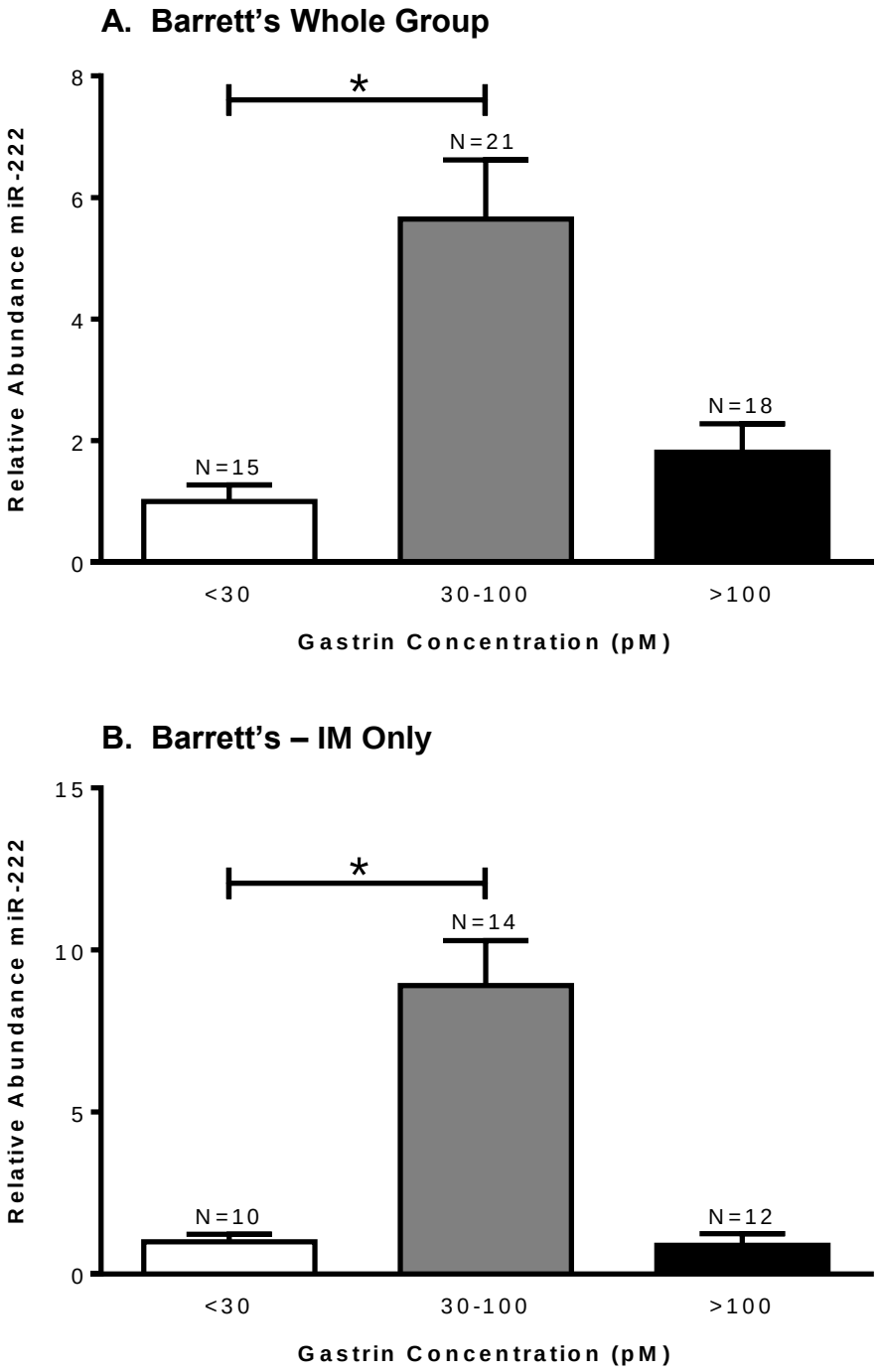

Figure 5.3.13 – **Relative serum miR-222 abundance is significantly higher when gastrin concentration is between 30-100 pM.** Abundance of miR-222 miRNA, relative to RNU6B, in serum was significantly higher when circulating gastrin was between 30-100 pM in both the whole Barrett's patient cohort (A) and in the subgroup with intestinal metaplasia only (B). Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.3 Gastrin's effect on miRNA expression in biopsies of BO

5.3.3.1 RNU6B abundance in BO biopsies is gastrin insensitive

As with GAPDH and RNU6B abundance in serum, it was important to validate the use of RNU6B in Barrett's oesophageal biopsies for data normalisation across samples that covered a large range of circulating gastrin concentrations (to identify any influence gastrin may have on abundance which may affect normalisation). Across all samples RNU6B abundance remained stable and no statistically significant relationship was identified by Spearman's rank analysis between circulating gastrin concentration and RNU6B abundance in biopsies of BO ($r=0.11$ $df=52$ $p<0.05$) (fig 5.3.14).

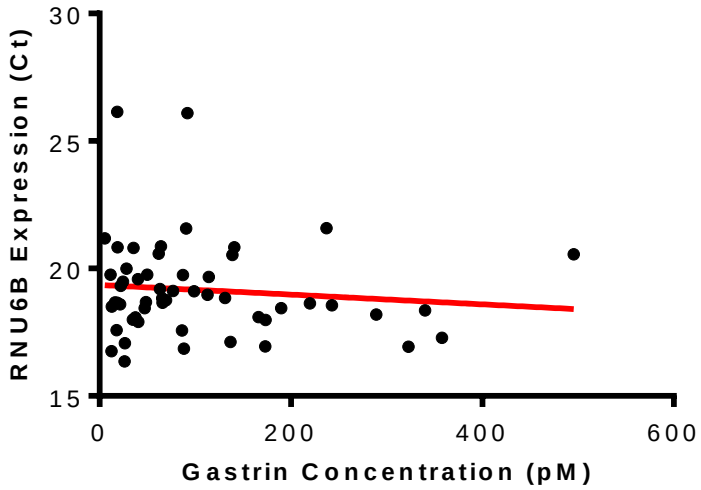

Figure 5.3.14 – **RNU6B abundance in biopsies of BO is gastrin insensitive.** Spearman's rank correlation identified no significant relationship between RNU6B abundance and circulating gastrin concentration.

5.3.3.2 Relative miR-21 miRNA abundance is significantly lower when circulating gastrin is >100 pM

Since serum miRNA could originate from any tissue in the body, it was considered crucial to also examine abundance of miRNAs at the tissue level in biopsies of Barrett's oesophagus. Tissue miR-21 miRNA abundance, relative to RNU6B abundance, within the whole Barrett's patient cohort (fig 5.3.15A) and oesophageal biopsy samples exhibiting intestinal metaplasia only (fig 5.3.15B), remained unchanged across the post-prandial physiological range of circulating gastrin (30-100 pM) compared to the reference range for fasting circulating gastrin (<30 pM). However, at circulating gastrin concentrations above the physiological range (>100 pM) there was significantly lower relative abundance compared to the reference range for fasting circulating gastrin (<30 pM).

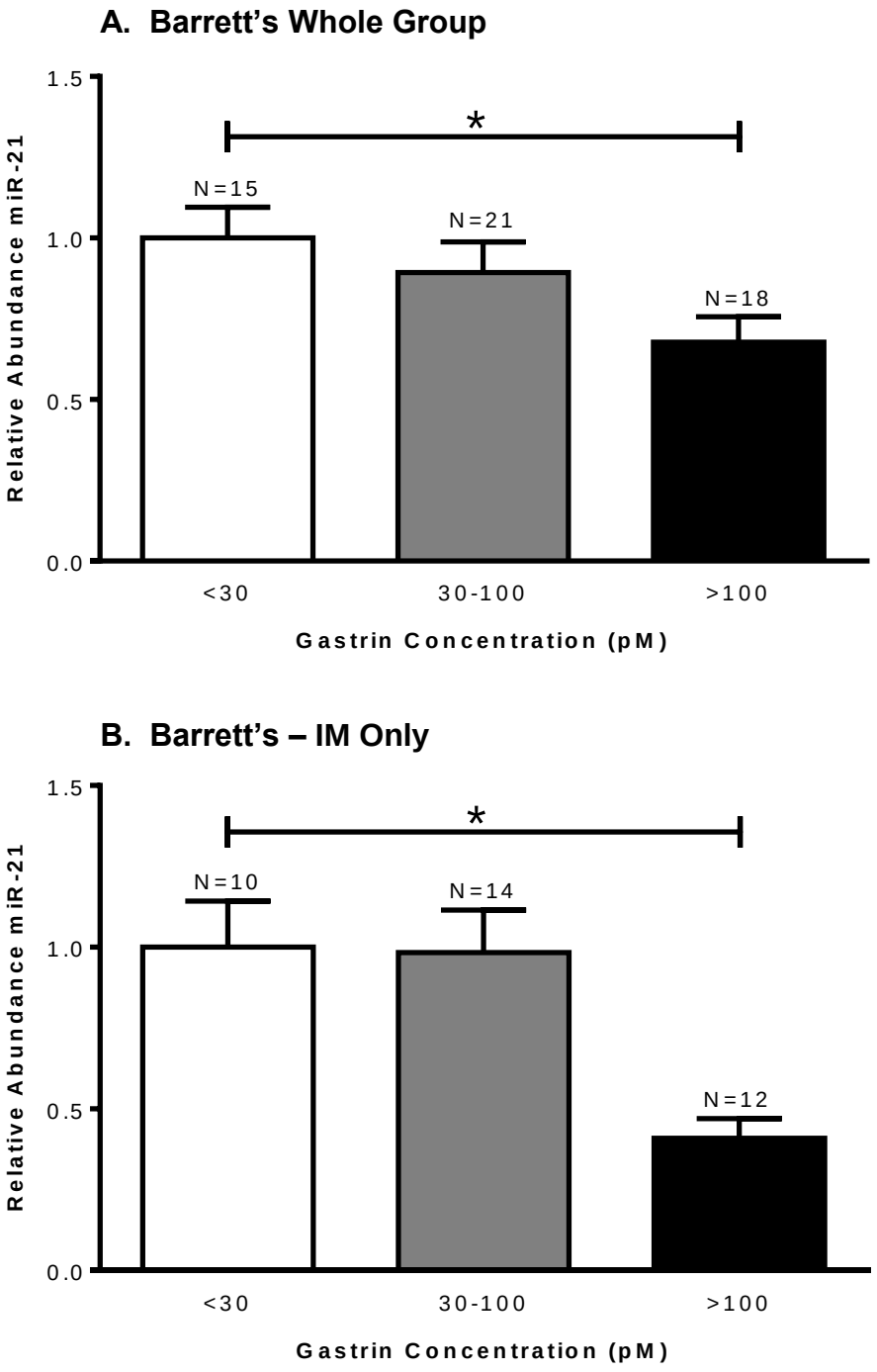

Figure 5.3.15 – **Relative miR-21 miRNA abundance is significantly lower when circulating gastrin is >100 pM.** Abundance of miR-21 miRNA, relative to RNU6B, was significantly lower when circulating gastrin was >100 pM in biopsies of Barrett's oesophagus (A) and in the subgroup selected for intestinal metaplasia (B). Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.3.3 Relative miR-221 miRNA abundance is significantly lower when circulating gastrin is >100 pM

Tissue miR-221 miRNA abundance, relative to RNU6B abundance, within the whole Barrett's patient cohort (fig 5.3.16A) and oesophageal biopsy samples exhibiting intestinal metaplasia only (fig 5.3.16B), remained unchanged across the post-prandial physiological range of circulating gastrin (30-100 pM) compared to the reference range for fasting circulating gastrin (<30 pM). However, at circulating gastrin concentrations above the physiological range (>100 pM) there was significantly lower relative abundance compared to the reference range for fasting circulating gastrin (<30 pM).

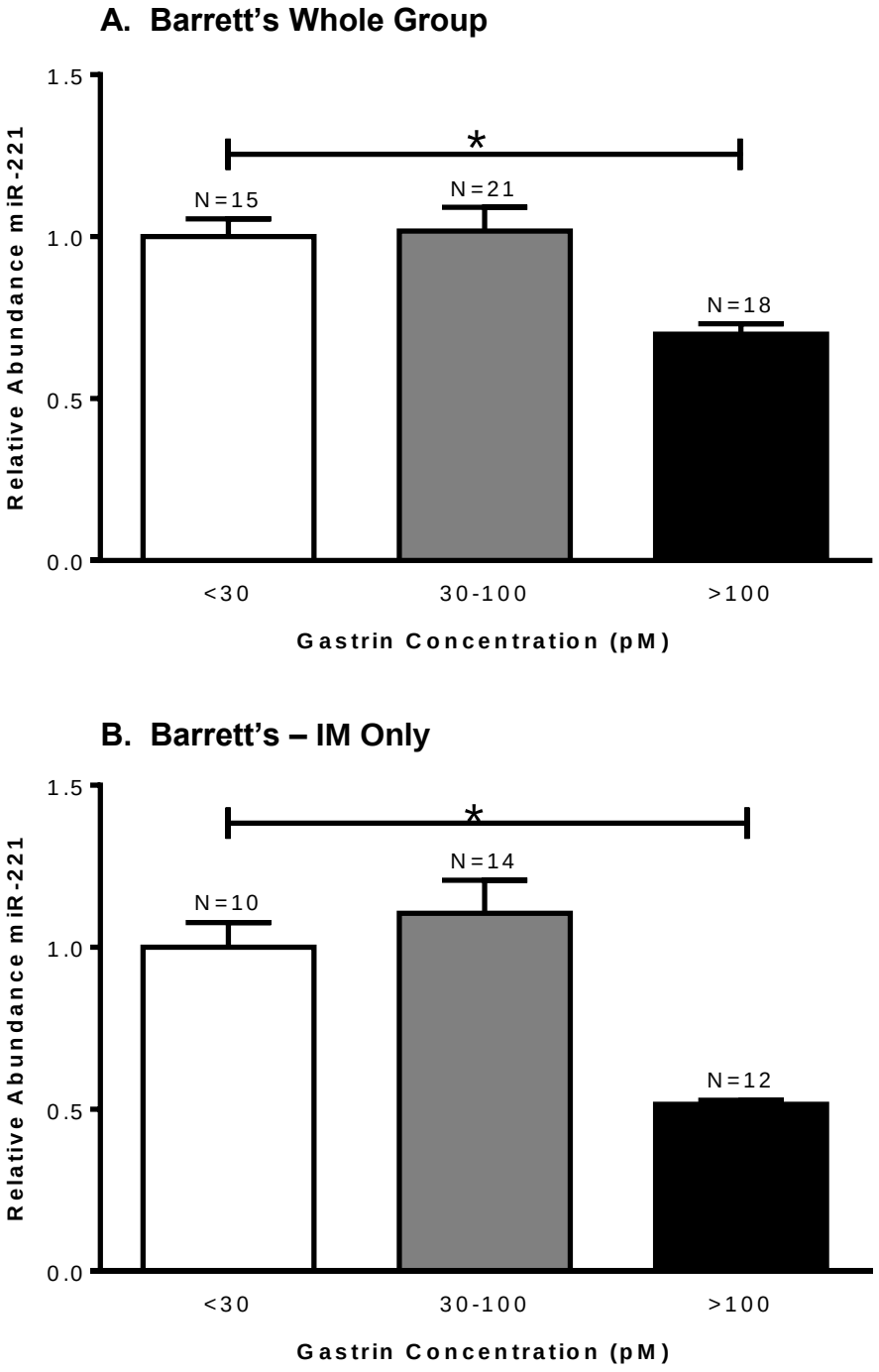

Figure 5.3.16 – **Relative miR-221 miRNA abundance is significantly lower when circulating gastrin is >100 pM.** Abundance of miR-221 miRNA, relative to RNU6B, was significantly lower when circulating gastrin was >100 pM in biopsies of Barrett's oesophagus (A) and in the subgroup selected for intestinal metaplasia (B). Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.3.3.3 Relative biopsy miR-222 abundance is significantly lower when gastrin concentration is >100 pM

Barrett's oesophagus miR-222 transcript abundance, relative to RNU6B abundance, was significantly lower in the whole Barrett's patient cohort (fig 5.3.16A) at circulating gastrin concentrations above the physiological range (>100 pM) compared to the reference range for fasting circulating gastrin (<30 pM).

Selecting for oesophageal biopsy samples exhibiting intestinal metaplasia only (fig 5.3.16B) however, exhibited no significant changes in relative abundance with circulating gastrin concentration when compared to the reference range for fasting circulating gastrin (<30 pM). Despite this, there remained a general trend towards lower miR-222 abundance with all gastrin concentrations >30 pM.

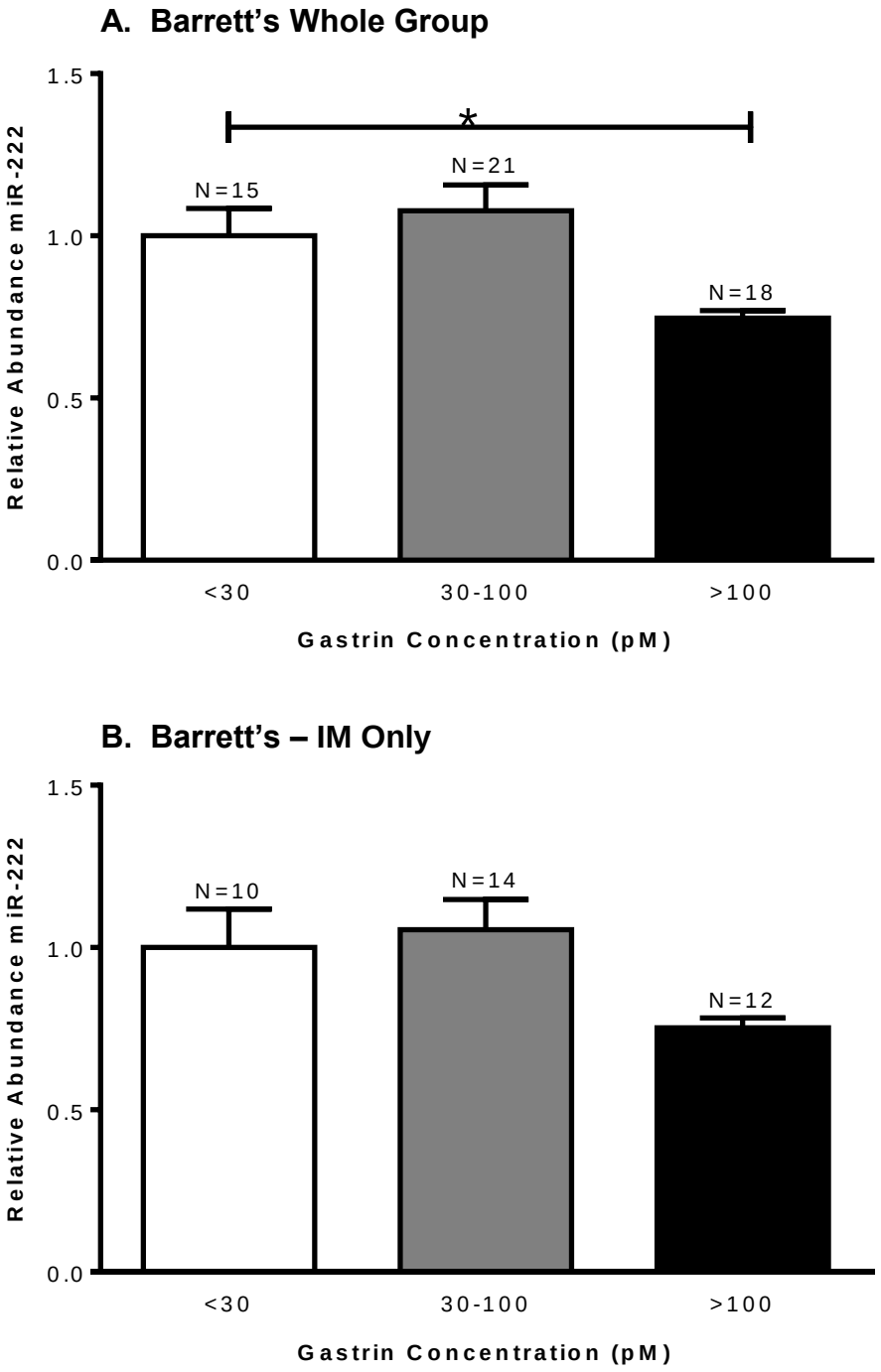

Figure 5.3.17 – **Relative miR-222 miRNA abundance is significantly lower when circulating gastrin is >100 pM.** Abundance of miR-222 miRNA, relative to RNU6B, was significantly lower when circulating gastrin was >100 pM in Barrett's oesophagus biopsies (A) and in the subgroup selected for intestinal metaplasia (B). Mean ± SEM, $p<0.05$, one-way ANOVA with Holm-Sidak correction.

5.4 Discussion

The main findings derived from this chapter are:

The expression profile of putative biomarkers of gastrin responsiveness within Barrett's epithelium is complex, with sensitivity and direction of change varying depending on gene selected and circulating gastrin concentration. Of genes for which mRNA abundance was tested (CgA, MMP-1, MMP-7, COX-2, CCK2 and SHH) all exhibited some changes in abundance when samples were separated by circulating gastrin.

The expression profile of circulating miRNA proved remarkably similar across all miRNAs investigated, miR-21, -221 and -222 were all significantly higher within the post-prandial physiological range of gastrin when compared to the to the reference range for fasting circulating gastrin. Above the physiological range of gastrin the abundance of the miRs returns to that of the reference range for fasting circulating gastrin.

The expression profile of biopsy miRNA differed dramatically to that in serum, with miR-21 showing no significant changes in response to gastrin and a very large variance within gastrin groups. The profile of tissue miRs -221 and -222 share similarities with those in serum, they both exhibit similar changes in abundance however these changes are a significant lowering of abundance as opposed to significantly higher abundance compared to the reference fasting range of gastrin.

Biomarker and miRNA profiles were separated and analysed in the context of the heterogeneity of the group. There were small sub-groups of Barrett's patients that exhibited dysplasia or gastric metaplasia but these were considered insufficient for separate analysis and presentation. There was, however, a large sub-group of patients that exhibited intestinal metaplasia and this was considered adequate for separate presentation. This is, of course, the most common form of the disease and the form associated with the development of ACO.[261] The present study was therefore based on both a heterogeneous group and a slightly smaller relatively homogeneous group.

Separation by grades of metaplasia and dysplasia has been employed before in gene expression studies[339] which concluded that cardiac and intestinal metaplasia share approximately 95% of the same gene expression profile whereas gastric remained

distinct. Within the wider literature however, rarely are Barrett's samples separated by types of metaplasia or dysplasia. Our data shows that when investigating abundance of putative biomarkers, consideration of types of metaplasia or dysplasia can make a significant difference. Four genes of interest – MMP-1, COX-2, TFF1 and CCK2R, exhibited significantly different abundance profiles in IM compared with the whole Barrett's group. This highlights the importance of rigorous histological identification of the type of metaplasia or dysplasia present in BO when investigating BO at the genetic level.

Serum miRNA abundance exhibited no significant differences between either group, which was not unexpected considering serum based miRNA abundance is non-specific to any particular tissue due to its circulating nature. Biopsy miRNA abundance however did exhibit a significant difference between sub-groups in the mir-222 group, although the trend remained the same albeit no longer significant as it was in the larger group.

Separation of samples by circulating gastrin concentration by the most well characterised physiological function of gastrin – gastric acid secretion - was performed as previously very few papers have related changes in gastrin-regulated gene expression to the physiological range of circulating gastrin.[340, 74, 341]

Comparing the abundance of putative biomarkers within Barrett's biopsies to the preneoplastic stomach biopsies analysed in Chapter 4: in the case of CgA, biopsies of BO exhibited higher abundance with gastrin above the fasting reference range (>30 pM) but above the post-prandial physiological range (>100 pM) exhibited no significant changes in abundance. Within gastric biopsies however, increased circulating gastrin (>30 pM) was associated with significantly lower abundance of CgA compared to controls. Abundance of CCK2R mRNA exhibited a similar change in biopsies of both BO and gastric preneoplastic conditions – namely with circulating gastrin there was significantly lower CCK2R mRNA abundance.

The modulation in CCK2R mRNA abundance observed relative to changes in circulating gastrin in both biopsies of BO and gastric biopsies, if confirmed to also effect mature protein production and abundance of receptor at the cell surface, may hinder further gastrin-regulated biomarker discovery in BO. It may be the case that decreases in receptor abundance at the cell surface compensate for increases in

circulating gastrin concentration, altering the sensitivity of BE to gastrin relative to gastrin concentration, in turn having an effect on the abundance of gastrin-regulated biomarkers. This highlights a crucial area where further research is needed to complete our understanding of how CCK2R expression is controlled and how changes in receptor expression relate to changes in other gastrin-regulated proteins, particularly in BO. In other words, it may simply be futile to try to discover and implement biomarkers of gastrin-responsiveness in BO if CCK2R expression itself is gastrin-dependent.

Chapter 6

Putative biomarker expression in gastric adenocarcinoma cell lines

6.1 Introduction

Whilst qPCR provides information regarding the abundance of mRNA of putative biomarker proteins, it cannot provide information on protein expression *per se*. In the present context, direct study of protein expression at the tissue level *in vivo* was limited by the amount of extra biopsy material that could be safely removed from patients alongside the biopsies required to be taken for routine Barrett's surveillance. To maximise the number of putative biomarkers that could be analysed with this limited biopsy material, a decision was made to use the tissue for qPCR, rather than using larger masses of biopsy material for protein analysis at the cost of only being able to examine a few putative biomarker targets.

However, it is possible to use established cell lines derived from human gastric adenocarcinoma, transfected with a copy of the human CCK2R[72], to measure changes in both intra- and extra-cellular protein abundance in response to gastrin. If treatment of these cells with gastrin produces similar changes in mature protein abundance to those predicted from qPCR studies, performed in the previous chapter, it may identify which putative biomarkers are more closely associated with the development of adenocarcinoma than simply benign BO. Extra-cellular proteins in particular could be highly informative because they may contribute to the tumour microenvironment by modifying the extracellular matrix to allow tumour cells to invade as well as providing a source of circulating putative biomarkers, the "gold-standard" for biomarker identification in patients.

6.1.1 Objectives

- Analyse the expression of putative biomarkers of gastrin responsiveness in gastric adenocarcinoma cells with and without expression of the CCK2R.
- Compare the expression of candidate proteins based on changes seen in mRNA abundance in biopsies.

6.2 Methods

Cell lines were maintained as detailed in section 2.6.2 and were plated at a density of 5 million cells per 75 cm^3 treatment flask. To each flask excluding controls, an appropriate volume of unsulphated human G17 diluted in 0.05 M ammonium bicarbonate was added from a stock solution of either 1 or 10 µM to produce the required final concentration. Cells were then returned to the incubator. Cells were exposed to G17 (where applicable) for a treatment period of 24 hours before cell media and extracts were recovered and processed (as outlined in section 2.6.3). Key findings were replicated, but where results were uninformative the experiments were only performed once.

Cell extracts and media were prepared using the previously defined methods (sections 2.6.4, 2.6.5) and frozen at -80°C until being defrosted, on ice, when required for SDS-PAGE and western blotting. SDS-PAGE and western blotting experiments were performed as detailed in section 2.7.

Densitometry was used to quantify the intensity of protein bands revealed via western blot using BioRad™ image lab software version 5.2.1. The latter converts HRP-fluorescence into a numerical value suitable for comparison between samples and, in the case of cell extracts, was also used for normalisation to the housekeeping protein GAPDH.

6.3 Results

6.3.1-4 Biomarker secretion and cellular expression in AGS cells

Treatment of AGS cells with G17 at concentrations of 1 and 10 nM induced no notable, dose-related changes in abundance in media of either pro- or active MMP-1, however there was a slight increase in cellular abundance. Treatment with PMA induced a sizeable increase in both pro- and active MMP-1 abundance in cell extracts and cell media (fig 6.3.1A,B,C,D).

MMP-2 abundance did not change appreciably with gastrin treatment in cells or media but treatment with PMA slightly reduced the abundance of MMP-2 in both cells and media when compared to untreated controls (fig 6.3.2A,B,C,D).

Pro- and active MMP-3 abundance was below the limit of detection in control and gastrin-stimulated medium, but were detectable in cells. Interestingly, the cellular abundance of proMMP-3 remained below the limit of detection at all gastrin concentrations. Whilst active MMP-3 was detectable, abundance exhibited no apparent trend in response to a rising concentration of gastrin. Treatment of cells with PMA however stimulated a profound increase in pro- and active MMP-3 abundance in media, with a modest increase in proMMP-3 accompanied by a modest decrease in active MMP-3 abundance in cell extracts (fig 6.3.3A,B,C,D).

Pro- and active TIMP-1 abundance in media and cells exhibited no discernible changes with gastrin treatment. However, PMA treatment induced a profound increase in abundance of active TIMP-1 in media, and of both pro- and active TIMP-1 in cell extracts (fig 6.3.4A,B,C,D).

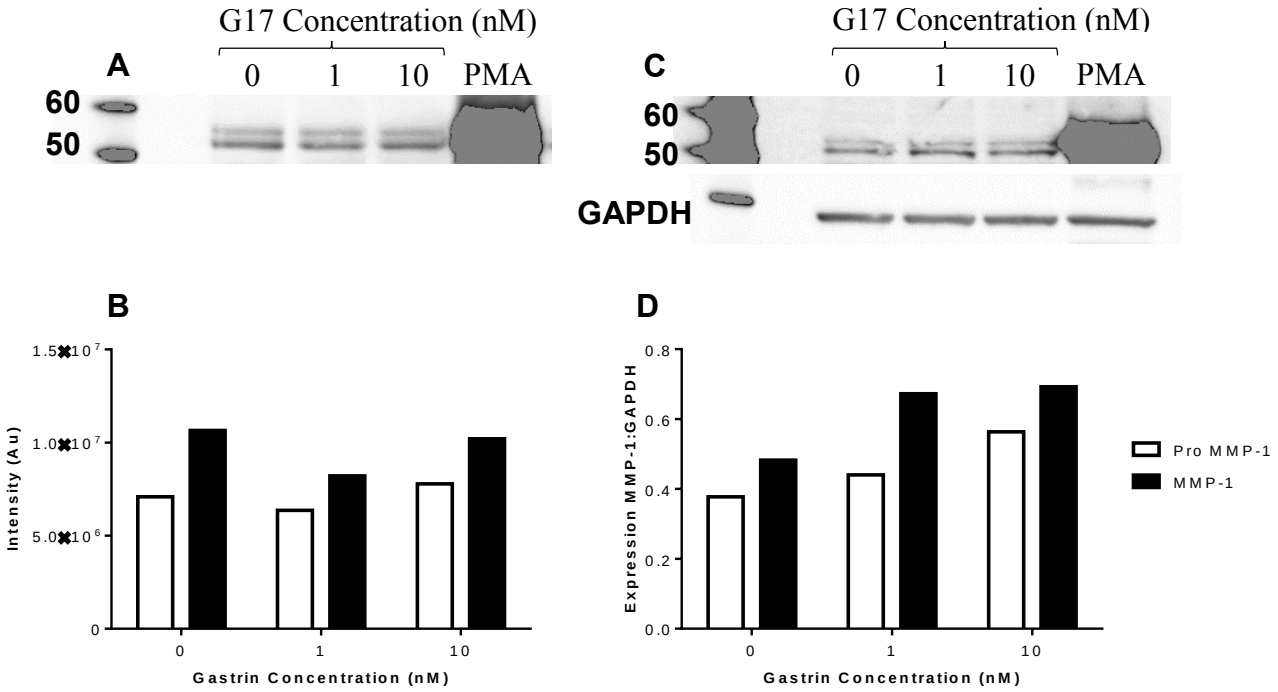

Figure 6.3.1 – **MMP-1 abundance in media is gastrin insensitive, in cell extracts is gastrin sensitive in AGS cells.** Abundance of pro- (54 kDa) and active MMP-1 (52 kDa) in media (A - western blot, B - densitometry) exhibited no notable, dose-related response to gastrin. In cell extracts, pro- and active MMP-1 abundance, relative to GAPDH (C - western blot, D - densitometry), exhibited a slight increase in response to G17. PMA strongly stimulated an increase in pro- and active MMP-1 abundance in media and cell extracts that saturated the system. N=1.

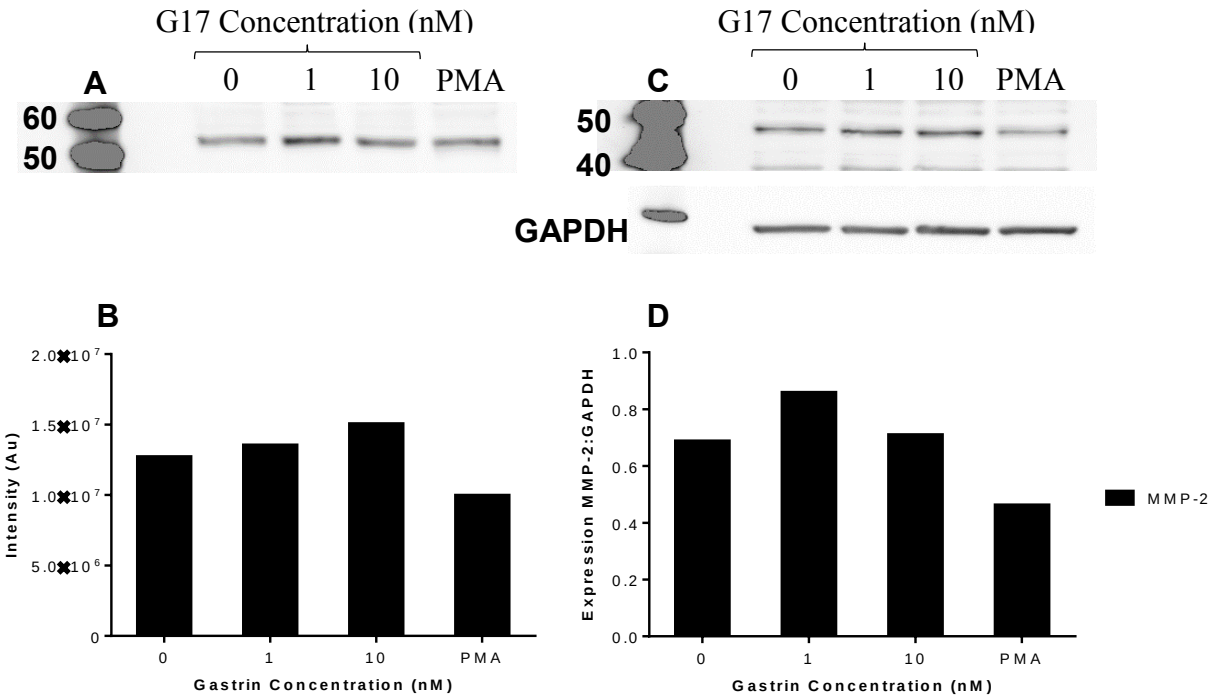

Figure 6.3.2 – **MMP-2 abundance in media and cell extracts is gastrin insensitive in AGS cells.** Abundance of MMP-2 (~54 kDA) in media (A - western blot, B - densitometry) and cell extracts, relative to GAPDH (C - western blot, D - densitometry), exhibited no appreciable dose-dependent response to G17. PMA was associated with a modest reduction of MMP-2 abundance in both media and cell extracts. N=1.

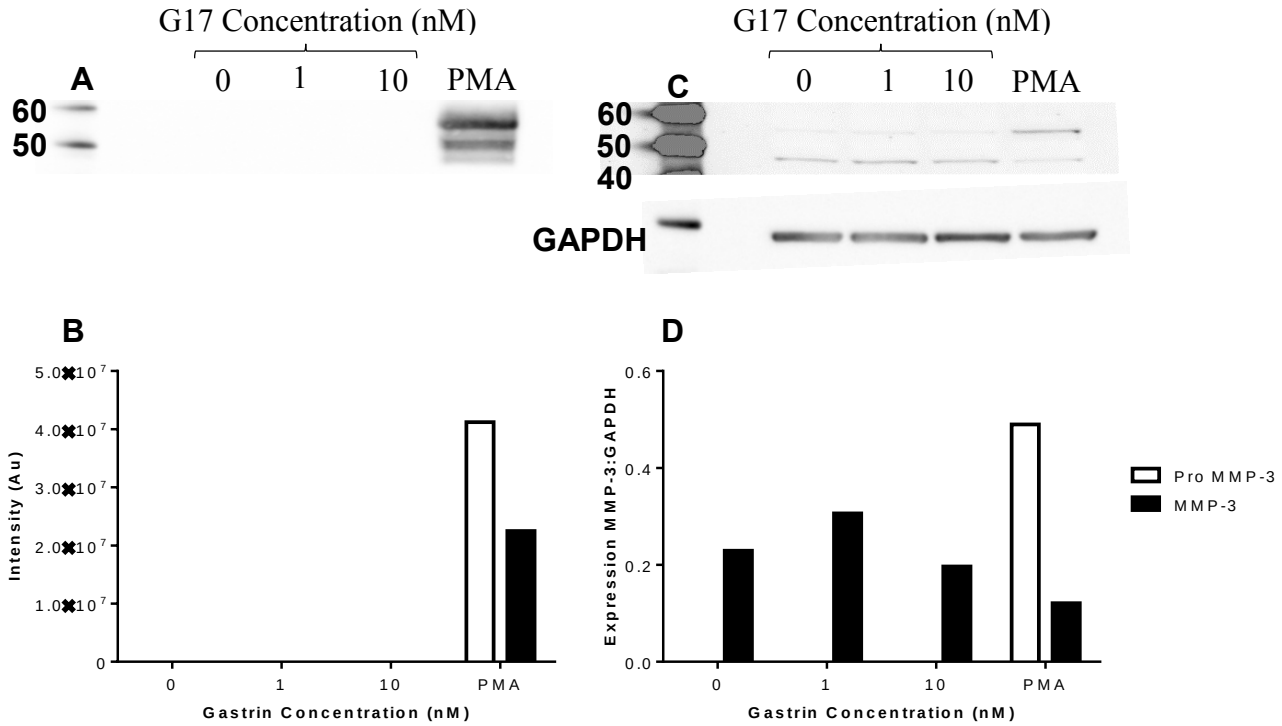

Figure 6.3.3 – **MMP-3 abundance in media and cell extracts is gastrin insensitive in AGS cells.** Abundance of pro-MMP-3 (57 kDA) and MMP-3 (54 kDA) in media (A - western blot, B - densitometry) and cell extracts, relative to GAPDH (C - western blot, D - densitometry), exhibited no appreciable dose-dependent response to G17. PMA induced an increase in abundance of both pro- and active MMP-3 in media and cell extracts. N=1.

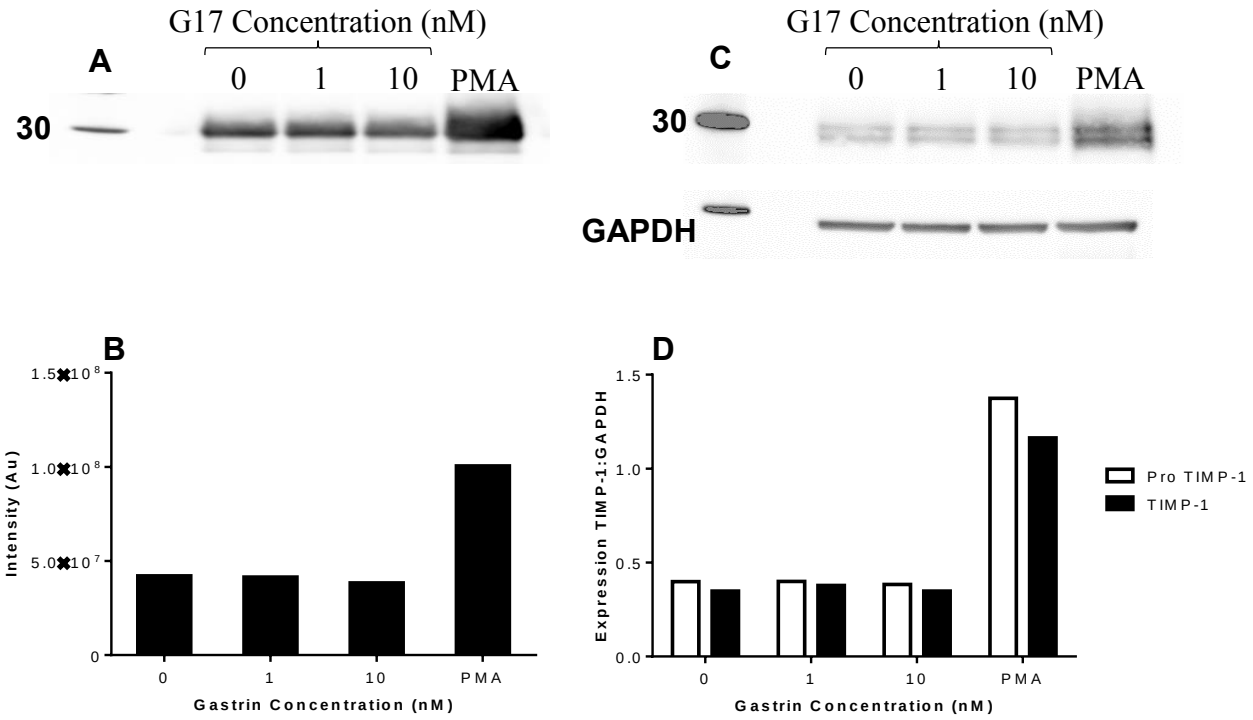

Figure 6.3.4 – **TIMP-1 abundance in media and cell extracts is gastrin insensitive in AGS cells.** Abundance of proTIMP-1 (30 kDa) and TIMP-1 (28 kDa) in media (A - western blot, B - densitometry) and cell extracts, relative to GAPDH (C - western blot, D - densitometry), exhibited no appreciable dose-dependent response to G17. PMA induced an increase in abundance of TIMP-1 in media and both proTIMP-1 and TIMP-1 in cell extracts. N=1.

6.3.5 MMP-1 abundance in media is gastrin-sensitive in AGS-GR cells

In contrast to the results in AGS cells above, treatment of AGS-GR cells with increasing concentrations of G17 (0.1-10 nM) stimulated a dose-dependent increase in pro-MMP-1 abundance in media at concentrations >0.3 nM and dose-dependent increases in media of active MMP-1 at concentrations of 1 nM and above (fig 6.3.5).

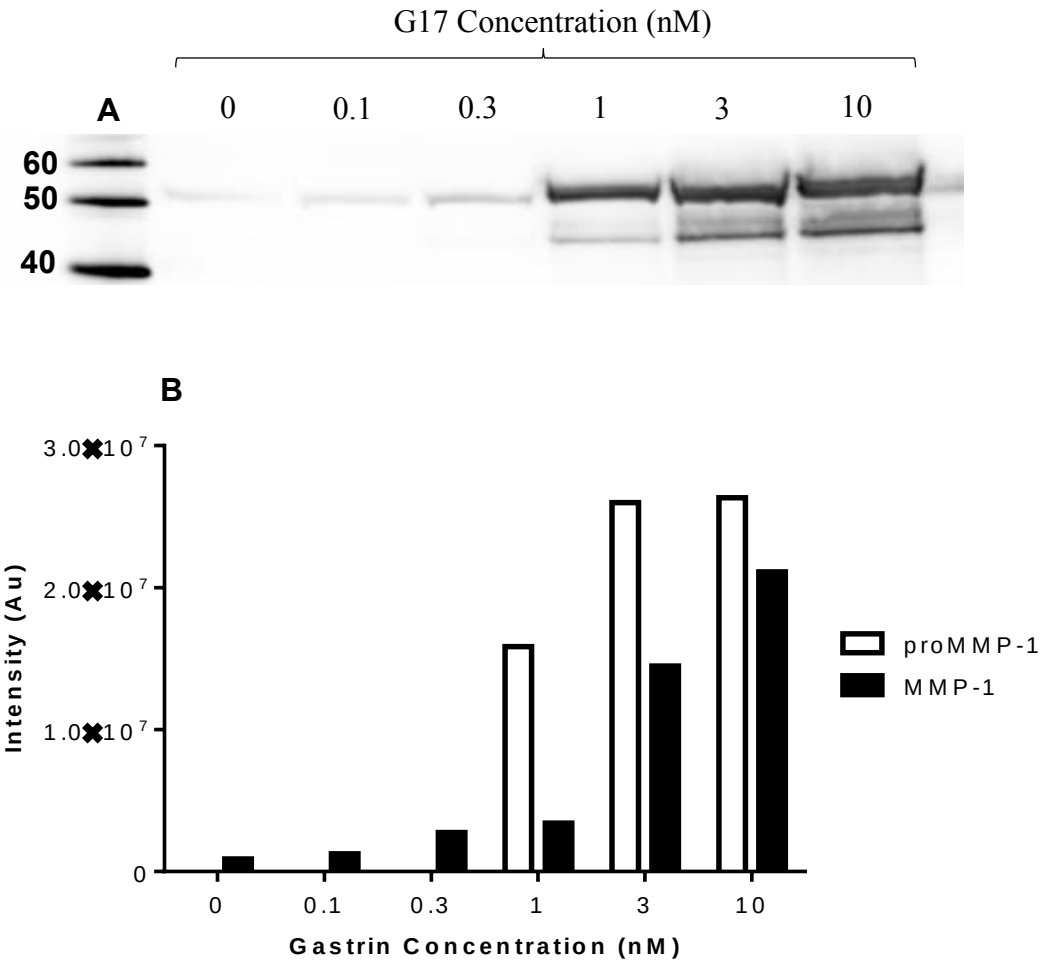

Figure 6.3.5 – **MMP-1 abundance in media is gastrin-sensitive in AGS-GR cells.** Abundance of pro- (52 kDa) and active MMP-1 (54 kDa) in media (A - western blot, B -densitometry) exhibited no appreciable response to G17 at <0.3 nM. However at concentrations >0.3 nM there was a dose-dependent increase in media of proMMP-1; concentrations >1 nM also stimulated an increase in media of active MMP-1. N=1.

6.3.6 MMP-2 abundance in media and cells is gastrin insensitive in AGS-GR cells

Treatment of AGS-GR cells with increasing concentrations of G17 (0.1-10 nM) provided no clear evidence of a dose-dependent relationship with MMP-2 (fig 6.3.3A,B). Abundance of MMP-2 in cell extracts remained below the limit of detection at all G17 concentrations (fig 6.3.6C).

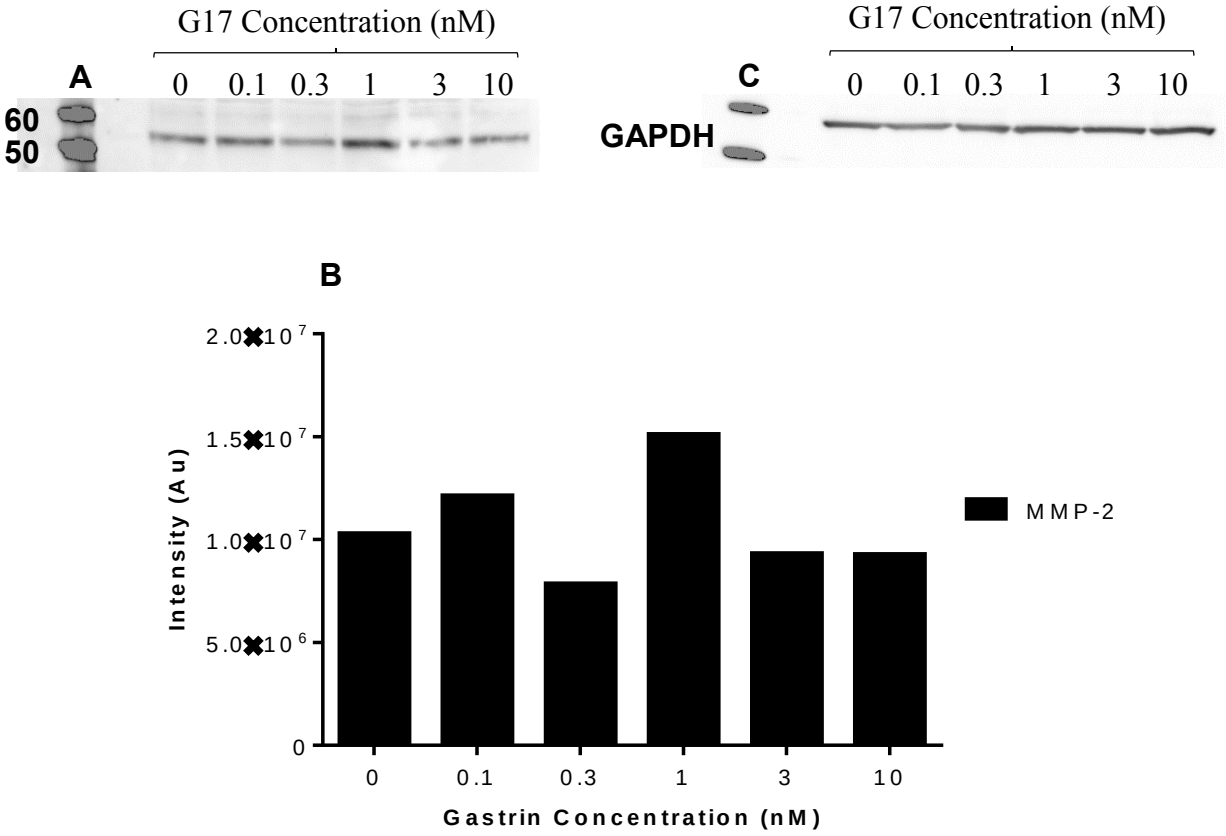

Figure 6.3.6 – **MMP-2 abundance in media and cells is gastrin insensitive in AGS-GR cells.** Abundance of MMP-2 (~54 kDa) in media (A - western blot, B - densitometry) exhibited no appreciable dose-dependent response to G17. Abundance of MMP-2 in cell extracts, relative to GAPDH, remained below the limit of detection at all G17 concentrations (C - GAPDH western blot, blank MMP-2 western blot not shown). N=2.

6.3.7 MMP-3 abundance in media and cell extracts is gastrin sensitive in AGS-GR cells

Treatment of AGS-GR cells with increasing concentrations of G17 (0.1-10 nM) stimulated a dose-dependent increase in media of pro- and active MMP-3 at concentrations of 1 nM and above (fig 6.3.7A,B) and a dose-dependent increase in active MMP-3 abundance in cell extracts (fig 6.3.7C,D).

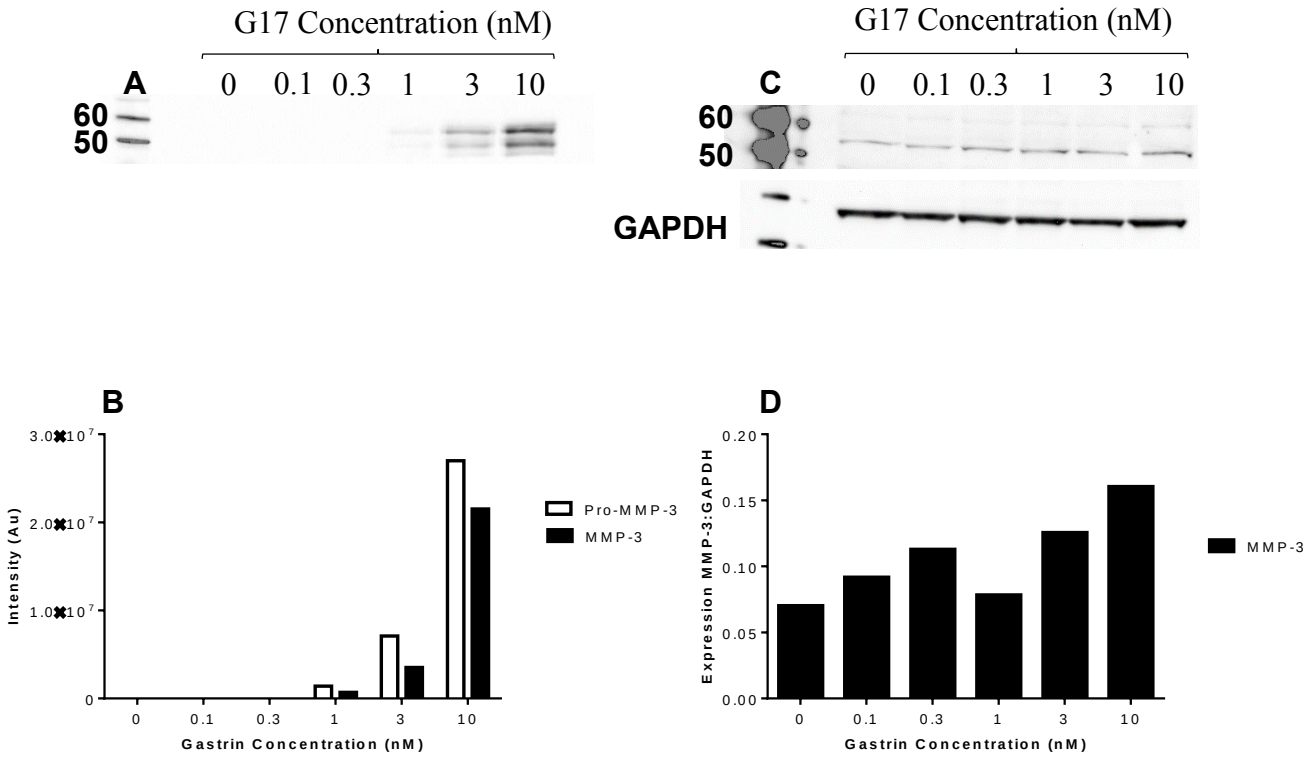

Figure 6.3.7 – **MMP-3 abundance in media and cell extracts is gastrin sensitive in AGS-GR cells.** Abundance of both pro- (57 kDa) and active MMP-3 (54 kDa) in media (A - western blot, B - densitometry) exhibited an increase G17 concentrations ≥1 nM, but MMP-3 abundance in cell extracts exhibited an increase across all G17 concentrations except 1 nM (C - western blot, D - densitometry). N=2.

6.3.8 MMP-7 cellular abundance is gastrin sensitive in AGS-GR cells

MMP-7 abundance in media remained below the limit of detection across all G17 concentrations (fig 6.3.8A). However, treatment with an increasing concentration of G17 (0.1-10 nM) did stimulate a dose-dependent increase in pro- and active MMP-7 abundance in cells at concentrations of 0.1 and 0.3 nM. Concentrations >0.3 nM however appeared to induce a reduction in pro- and active MMP-7 abundance to below the limit of detection at concentrations ≥3 nM (fig 6.3.8B,C).

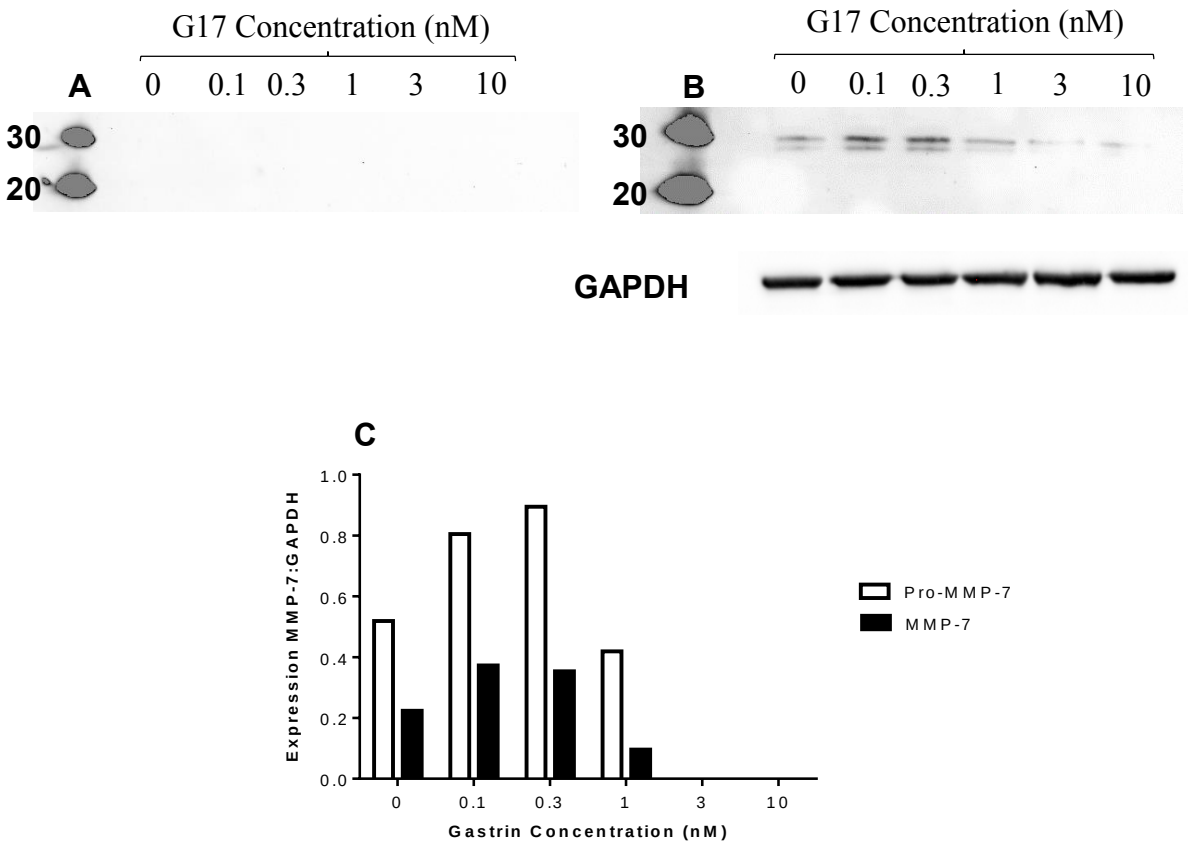

Figure 6.3.8 – **MMP-7 cellular abundance is gastrin sensitive in AGS-GR cells.** Abundance of pro (29 kDa) and active MMP-7 (27 kDa) in media remained below the limit of detection at all G17 concentrations (A - blank MMP-7 western blot not shown). However pro- and active MMP-7 abundance in cell extracts exhibited an increase at G17 concentrations between 0.1-0.3 nM before decreasing at concentrations ≥1 nM (B - western blot, C - densitometry). N=1.

6.3.9 TIMP-1 abundance in media and cell extracts is gastrin sensitive in AGS-GR cells

Treatment of AGS-GR cells with increasing concentrations of G17 (0.1-10 nM) stimulated a small dose-dependent increase in TIMP-1 abundance in media at concentrations >1 nM (fig 6.3.9A,B). Abundance of both pro and active TIMP-1 in cell extracts was stimulated by G17 treatment at all concentrations, with a clear dose-dependent relationship (fig 6.3.8C,D).

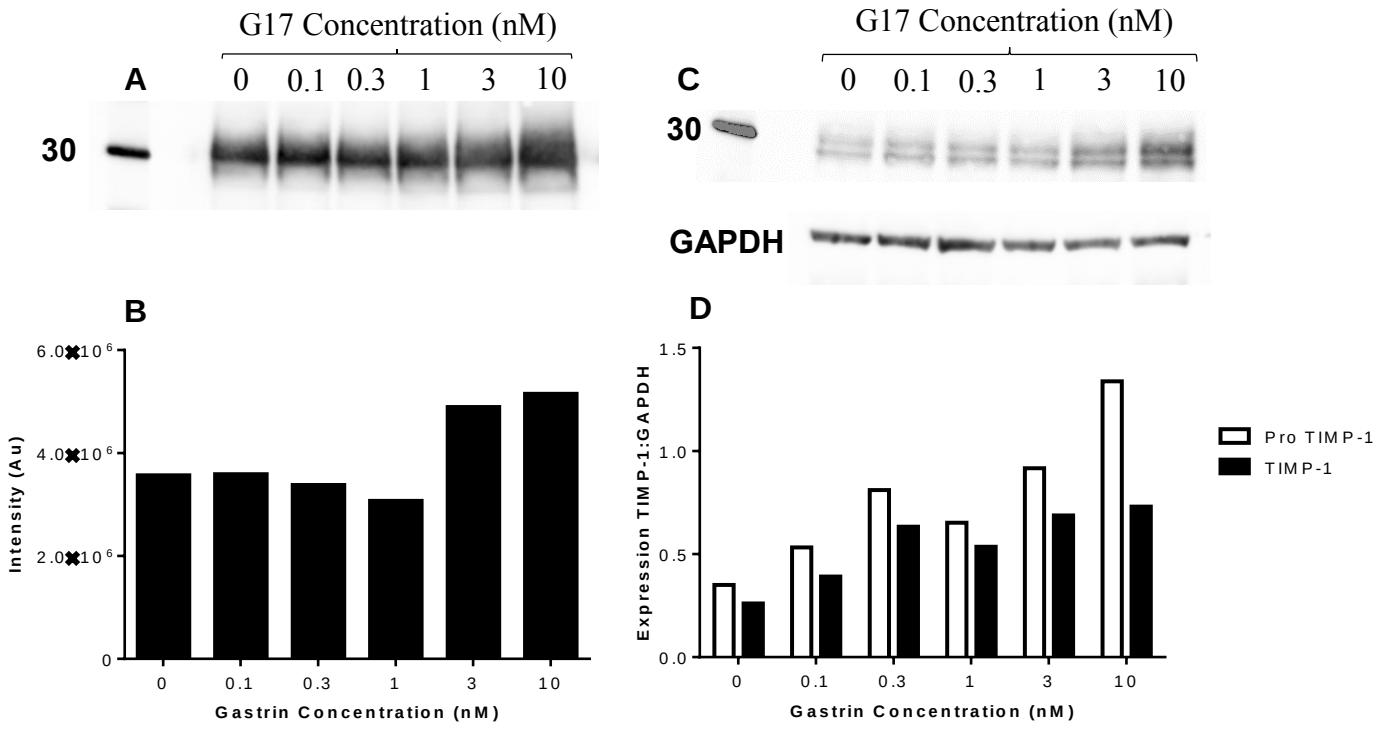

Figure 6.3.9 – **TIMP-1 abundance in media and cell extracts is gastrin sensitive in AGS-GR cells**. Abundance of TIMP-1 (28 kDa) in media exhibited an increase at G17 concentrations >1 nM (A - western blot, B - densitometry). Abundance of both pro (30 kDa) and active TIMP-1 in cell extracts also exhibited an increase at all G17 concentrations (C - western blot, D - densitometry). N=1.

6.3.10 MMP-1 abundance in media is gastrin-sensitive in HGT-GR cells

In order to determine whether the results in AGS-GR cells might be replicated in a second cell line, the experiments were repeated with HGT-GR cells. Treatment of HGT-GR cells with increasing concentrations of G17 (0.1-10 nM) stimulated an increase in abundance of proMMP-1 at concentrations >0.3 nM with an increase in active MMP-1 at a concentration of 1 nM (fig 6.3.10A,B). Cellular abundance of MMP-1 remained below the limit of detection in controls and across all G17 concentrations (fig 6.3.10C).

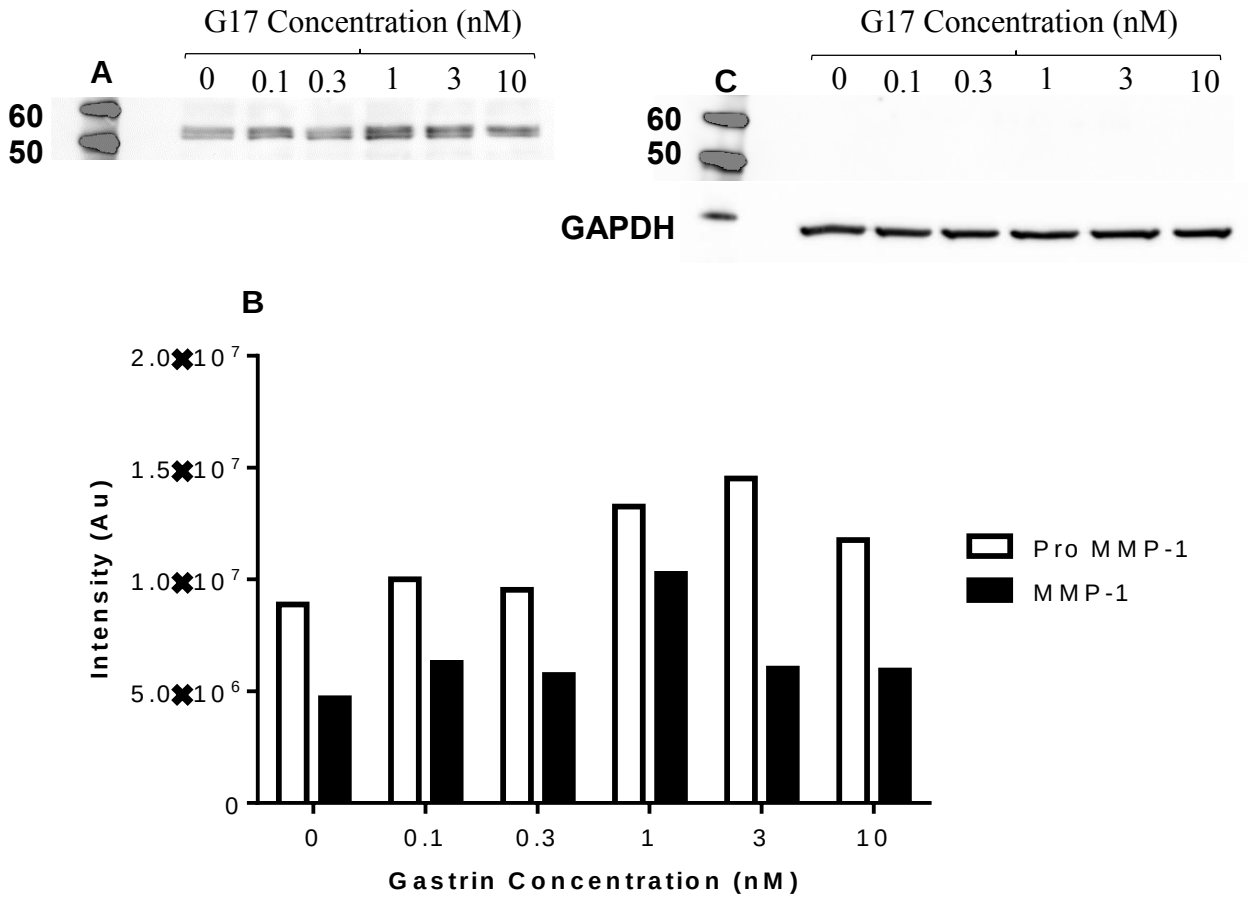

Figure 6.3.10 – **MMP-1 abundance in HGT-GR cells is gastrin sensitive.** Abundance of proMMP-1 (54 kDa) in media exhibited an increase at G17 concentrations >0.3 nM, with an increase in active MMP-1 (52 kDa) at a concentration of 1 nM (A - western blot, B - densitometry). Abundance of MMP-1 in cells remained undetectable at all G17 concentrations (C – GAPDH western blot, blank MMP-1 western blot not shown). N=1.

6.3.11 MMP-2 abundance in media is gastrin-sensitive in HGT-GR cells

Treatment of HGT-GR cells with increasing concentrations of G17 (0.1-10 nM) stimulated a dose-dependent increase in MMP-2 abundance in media at concentrations >0.3 nM (fig 6.3.11A,B). Abundance of MMP-2 in cell extracts exhibited no discernible dose-dependent response to G17 treatment (fig 6.3.11C).

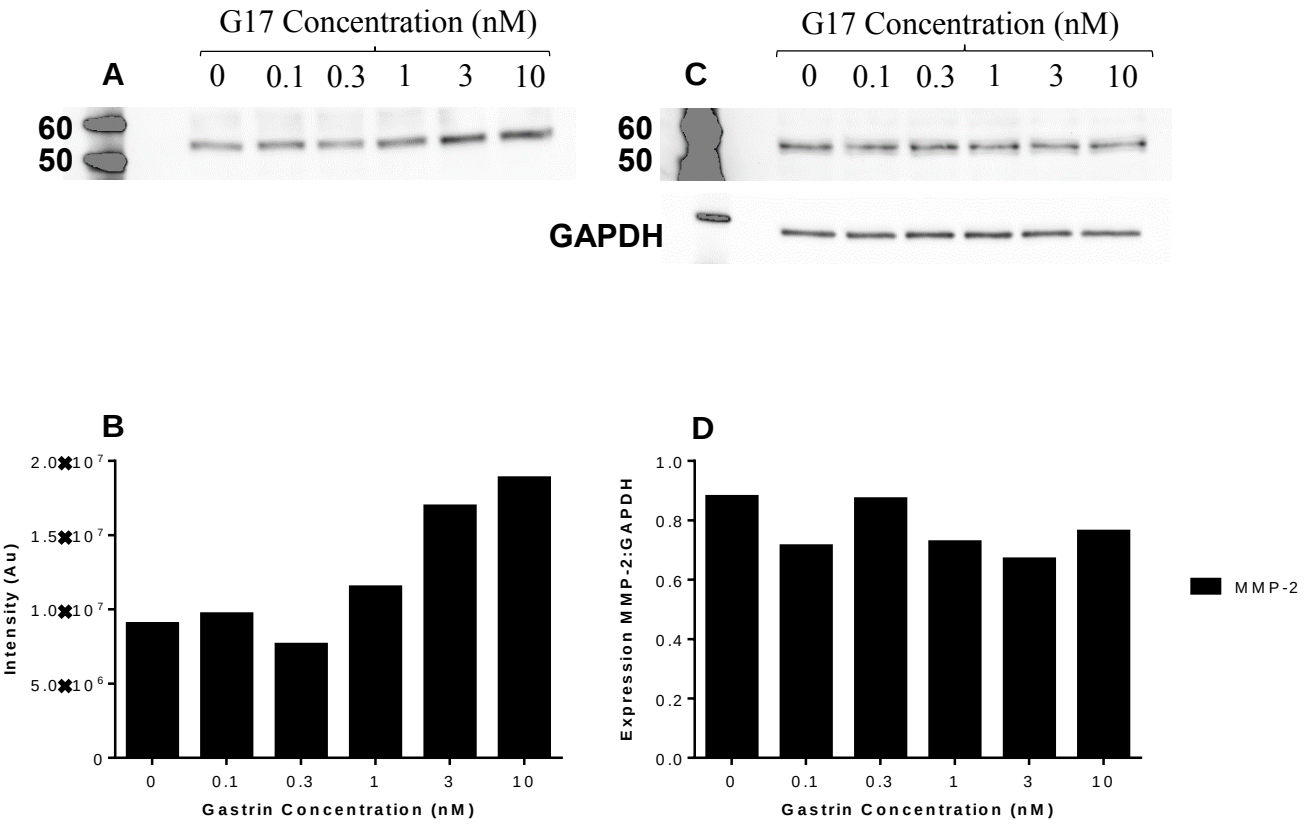

Figure 6.3.11 – **MMP-2 abundance in media is gastrin-sensitive in HGT-GR cells.** Abundance of MMP-2 (~54 kDa) in media exhibited a dose-dependent increase at G17 concentrations >0.3 nM (A - western blot, B - densitometry), MMP-2 abundance in cell extracts however remained gastrin independent (C - western blot, D - densitometry). N=2.

6.3.12 MMP-3 abundance in HGT-GR media and cells is undetectable

Abundance in both media (fig 6.3.12A) and cell extracts (fig 6.3.12B) of MMP-3 remained below the limit of detection in controls and with G17 treatment in HGT-GR cells.

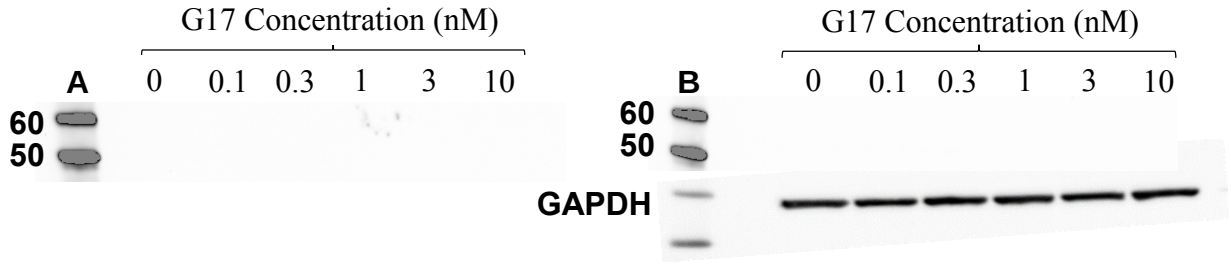

Figure 6.3.12 – **MMP-3 abundance in HGT-GR cells is undetectable.** Abundance of MMP-3 (54 kDa) in media (A) and cell extracts (B – GAPDH western blot, blank MMP-3 western blot not shown) of MMP-3 remained below the limit of detection irrespective of G17 concentration. N=2.

6.3.13 MMP-7 abundance in HGT-GR Cells is undetectable

Abundance in both media (fig 6.3.13A) and cell extracts (fig 6.3.13B) of MMP-7 remained below the limit of detection in controls and with G17 treatment in HGT-GR cells.

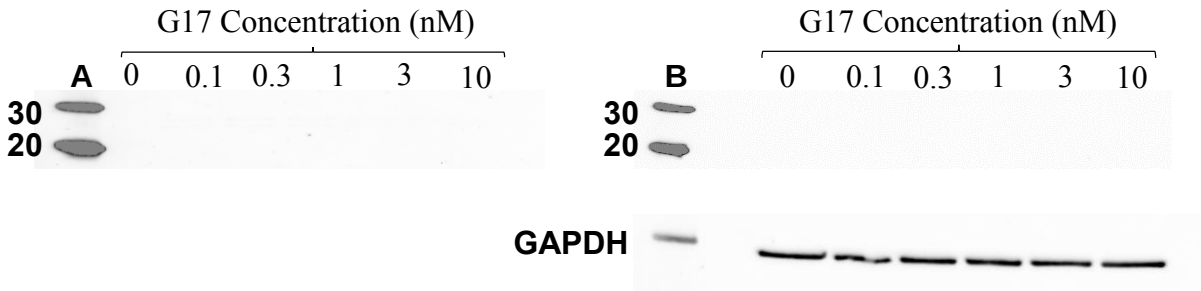

Figure 6.3.13 – **MMP-7 abundance in HGT-GR Cells is undetectable.** Abundance of MMP-7 (27 kDa) in media (A) and cell extracts (B - GAPDH western blot, blank MMP-7 western blot not shown) of MMP-7 remained below the limit of detection irrespective of G17 concentration. N=1.

6.3.14 TIMP-1 abundance in media and cell extracts is gastrin sensitive in HGT-GR cells

Treatment of HGT-GR cells with increasing concentrations of G17 (0.1-10 nM) stimulated a dose-dependent increase in TIMP-1 media abundance at concentrations 0.1-1 nM. However at concentrations >1 nM media abundance (whilst remaining greater than controls) exhibited abundance similar to a concentration of 0.3 nM (fig 6.3.14A,B). Abundance of TIMP-1 in cell extracts remained below the limit of detection in controls and all G17 concentrations (fig 6.3.14C).

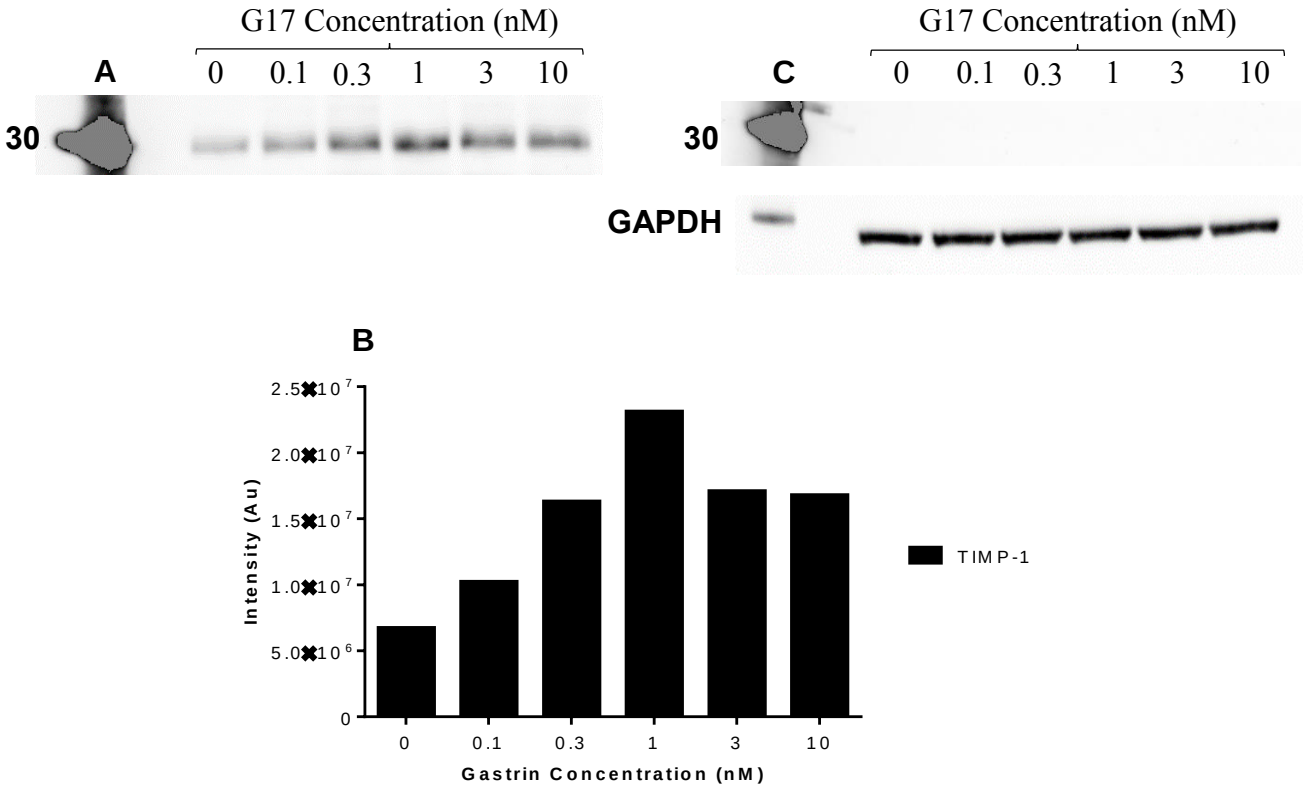

Figure 6.3.14 – **TIMP-1 abundance in media and cell extracts is gastrin sensitive in HGT-GR cells**. Abundance of TIMP-1 (28 kDa) in media exhibited a dose-dependent increase at G17 concentrations between 0.1-1 nM, however concentrations >1 nM exhibited TIMP-1 abundance similar to a concentration of 0.3 nM (A - western blot, B - densitometry). Abundance of TIMP-1 in cell extracts remained below the limit of detection at all G17 concentrations (C – GAPDH western blot, blank TIMP-1 western blot not shown). N=2.

6.3.15 TIMP-3 abundance in HGT-GR cells is undetectable

Abundance in both media (fig 6.3.15A) and cell extracts (fig 6.3.15B) of TIMP-3 remained below the limit of detection in controls and with G17 treatment in HGT-GR cells.

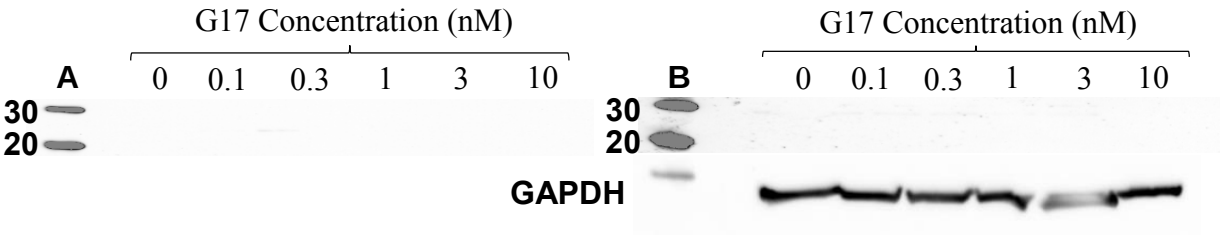

Figure 6.3.15 – **TIMP-3 abundance in HGT-GR Cells is undetectable.** Abundance of TIMP-3 (24 kDa) in media (A) and cell extracts (B – GAPDH western blot, blank TIMP-3 western blot not shown) remained below the limit of detection at all G17 concentrations. N=1.

6.3.16 Overall summary of biomarker sensitivity to gastrin in cell lines tested

In order to compare the data obtained across both cell lines, sensitivity to gastrin stimulation was expressed as the lowest gastrin concentration required for a change in abundance in cell media (Table 6.1). Only MMP-1 emerges as a robust indicator of gastrin sensitivity in both cell lines.

	AGS	AGS-GR	HGT-GR
TIMP-1	-	++	+++++
TIMP-3	N/A	N/A	-
MMP-1	-	+++	+++
MMP-2	-	-	+++
MMP-3	-	+++	-
MMP-7	N/A	-	-

Table 6.1 – **Overall summary of biomarker sensitivity to gastrin in cell lines tested. +++++ =** response at 0.1 nM G17, ++++ = response at 0.3 nM G17, +++ = response at 1 nM G17, ++ = response at 3 nM G17 and + = response at 10 nM G17 and - = no response to gastrin. N/A means that particular biomarker was not analysed in that cell line.

6.4 Discussion

AGS cells proved to be insensitive to gastrin-stimulated putative biomarker expression, as expected, due to the absence of CCK2R expression. However activation of PKC via PMA induced changes in expression of MMP-1, MMP-3 and TIMP-1 indicating the capacity of these cells to mount a response to the appropriate stimulus. In sharp contrast, in cells expressing CCK2R i.e. AGS-GR cells, and in another cell line (HGT-GR cells) there were robust responses to G17. The responses of the two cell lines were, however, different and only MMP-1 and TIMP-1 emerged as robust indicators of gastrin sensitivity. This difference in biomarker expression profile to the same stimulus in two seemingly similar cells lines sharing a common origin, highlights the importance of using multiple cell lines to validate potential biomarker targets even within the same cancer type. The difference also indicates the complexity of the biomarker profile expressed by even similar cancers.

Whilst determining the abundance of circulating or cell specific putative biomarkers can be informative in the discovery of novel biomarkers, it does not offer a complete view relating to the mechanisms involved. Measuring abundance in cell extracts and media, as performed here, reflects the total balance between biomarker synthesis, secretion and degradation. In cases of increased abundance, in cells and media, with gastrin treatment the balance of evidence would suggest gastrin stimulated synthesis and subsequent secretion from cells into media although further research would be required to confirm this.

Working towards the initial aim of identifying a potential circulating biomarker of gastrin-regulated changes in BO, it is arguably more important to consider gastrin-regulated putative biomarker abundance in media compared to cells, especially when considering the function of molecules investigated (modulators of extracellular matrix degradation) are extracellular in function. Hence the consideration of media abundance, rather than cellular, in the assessment of biomarker sensitivity in formation of table 6.1. However, if examining the expression of putative biomarkers in biopsy material was the final goal then consideration of cellular abundance would have been more important.

Utilising cell lines allowed us to overcome some of the inherent limitations we faced examining gastrin-regulated protein expression *in vitro*. These limitations included being unable to examine protein secretion from BO in response to gastrin (as measurements from blood would be non-specific due to its circulatory nature); being able to examine protein abundance in more than one cell line confirmed to be cancer-derived compared to BO cells, the majority of which would never progress to aquiring cancerous mutations, and thus have a different protein expression profile and being able to examine the response in protein abundance across a range of G17 concentrations from the same cells, rather than the fixed, singular gastrin concentration point we had for each BO biopsy patient.

The CCK2 receptor is a G protein-coupled receptor, and activation of the receptor activates intracellular signal transduction pathways that begin with members of the receptor coupled G_q subfamily of GTP-binding proteins, which mediate receptor activation of phospholipase C- β (PLC- β). PLC- β in turn produces secondary messengers 1,4,5-inosital triphosphate (IP_3) and 1,2-diacylglycerol (DAG), with IP_3 inducing Ca^{2+} from the endoplasmic reticulum into the cell cytoplasm. The increased Ca^{2+} in cytoplasm coupled with DAG in turn activate numerous Ca^{2+} and lipid-regulated proteins including members of the protein kinase C (PKC) family.[342] PKC signalling is often dysregulated in gastric cancer and previous work has implicated a role of PKC signalling in altered expression of putative biomarkers such as MMP-1,[343] MMP-2,[344] MMP-3[345] and MMP-7.[346]

Phorbal 12-myristate 13-acetone (PMA), a potent activator of protein kinase C was included as a positive control in the treatment of AGS cells as a method of mimicking the action of CCK2R activated PKC signalling within non-CCK2R expressing cells. PKC activation via PMA induced an increase in MMP-1,-3 and TIMP-1 and a decrease in MMP-2 abundance in media and cells in AGS cells; a similar pattern of response was observed with gastrin treatment in AGS-GR cells. Thus, PMA provides a useful positive control and the data are consistant with the idea that gastrin is acting via the PKC pathway to induce the changes in putative biomarker abundance that we observed in CCK2R expressing cells.

Comparing our results to those in published work, Kumar *et. al.*[211] have previously identified gastrin-stimulated release of MMP-1 in both AGS-GR and HGT-GR cell lines and linked it to cell migration, a crucial function for cancer invasion and development of ACO. This matched with our data as we also saw significant increases in MMP-1 secretion in both CCK2R expressing cell lines when treated with gastrin.

MMP-2 expression has previously been examined within AGS-GR cells and it was also found that gastrin stimulated a small decrease in media abundance of MMP-2 in cells[212] which concurs with our findings of AGS and AGS-GR cells exhibiting lower and variable expression with gastrin treatment. A literature search provided no evidence however of the analysis of MMP-2 secretion or production in HGT-GR cells with which to compare our findings.

Previous work analysing gastric cancer tissue has identified a significant increase in MMP-3 expression in advanced stage gastric cancer when compared to early stage[328] however gastrin concentrations within patients were not considered in this study. Other work detailing a direct link between gastrin stimulation of gastric cancer or either cell line tested and MMP-3 expression has not been previously published.

Previous studies have identified circulating and gastric MMP-7 stimulation by gastrin in human gastric corpus biopsies and patient plasma samples, mouse gastric biopsies and a G17 concentration-dependent increase in MMP-7 promotor activity in AGS-GR cells.[132] Treatment of cultured gastric glands with G17 stimulated MMP-7 in medium which in turn stimulated proliferation in human gastric myofibroblasts. Kumar *et. al.*[211] also noted that there was a significant increase in the relative transcript abundance of pro-MMP-7 in gastric corpus biopsies from human patients treated with PPIs compared to controls with circulating gastrin above the reference range of normal serum gastrin, there was no significant different however between patients treated with PPIs and controls when circulating gastrin concentrations were within the reference range. Our results were broadly in line with those published for AGS-GR cells, despite secreted MMP-7 in media being undetectable, there was gastrin stimulated pro and active MMP-7 production in cells up to a concentration of 1 nM above which pro and active MMP-7 production fell below the limit of detection. This supports the published data[132] relating to increased promotor activity and production

of MMP-7 in response to gastrin within AGS-GR cells however in our hands increased production of MMP-7 did not translate to an increase in secreted MMP-7 to media. A literature search provided no previous studies of MMP-7 production or secretion in HGT-GR cells with which to compare our findings.

Previously, a meta-review analysing the expression of TIMP-1 in tumour tissue or blood as a potential biomarker of gastric cancer showed that elevated TIMP-1 expression correlates with poor patient outcome[326] and has previously been associated with MMP-1 as a biomarker of significant interest in the field.[327] Our data identified gastrin-stimulated secretion of TIMP-1 in both cell lines, with greater sensitivity in HGT-GR compared to AGS-GR cells.

Comparing gene expression experiments to protein expression within my own research, MMP-1 was the only biomarker for which gene expression data from Barrett's patients (gastrin and Barrett's biopsies) and protein expression data from gastric adenocarcinoma cells was available for comparison, due to the cell lines used not expressing CgA or HDC.[347] Gastric biopsies from Barrett's patients exhibited a trend towards increased gene expression of MMP-1 with PPI treatment, likewise in Barrett's oesphagus biopsies MMP-1 gene expression was significantly higher when fasting gastrin concentration was between 30-100 pM and in both cell lines tested MMP-1 exhibited a gastrin-stimulated increase in secretion of similar sensitivity.

The main findings of this chapter is that despite the similarities between cell lines (both being gastric adenocarcinoma derived) there is variability in the expression profile of putative biomarkers in response to gastrin. Not surprisingly, there is also variability in the expression profiles of putative gastrin-regulated biomarkers in cell lines when compared to qPCR in BO derived biopsies. Across all results chapters and all putative biomarker targets analysed, MMP-1 stands out as the best candidate for further study due to its ubiquitous sensitivity to gastrin in all systems examined.

Chapter 7

Discussion

7.1 Overview

The main findings of this thesis are that:

a) patients with BO treated with PPIs are more likely to exhibit elevated circulating gastrin concentration than normal subjects;

b) in gastric biopsies derived from normal and Barrett's patients there is a similar relationship between serum gastrin and abundance of CgA, CCK2R and HDC mRNA, however only control biopsies exhibited a relationship between circulating gastrin and MMP-1 mRNA abundance;

c) patients with preneoplastic conditions of the stomach exhibited significantly increased circulating gastrin concentration relative to progression of the condition along the Correa cascade, reduction in CCK2R mRNA abundance was also linked to degree of cascade progression; CgA mRNA abundance was significantly decreased in biopsies exhibiting intestinal metaplasia;

d) across all BO biopsies, putative biomarker mRNA abundances that exhibited a relationship with serum gastrin concentration where: CgA exhibited no significant abundance change in subjects with gastrin <100 pM and significantly lower abundance >100 pM; MMP-1 exhibited significantly higher abundance with gastrin between 30-100 pM and significantly lower abundance with gastrin >100 pM; MMP-7 exhibited significantly higher abundance in patients with gastrin between 30-100 pM; COX-2 exhibited the same abundance expression profile as MMP-1; SHH exhibited significantly lower abundance with gastrin >100 pM; unexpectedly, CCK2R abundance was significantly lower with gastrin >100 pM;

e) in BO patients, serum abundance of miRs-21, -221 and -222 was significantly higher in patients with gastrin between 30-100 pM;

f) BO patient biopsy miRNA abundance for miR-21 exhibited significant variance in all gastrin concentration groups precluding statistical analysis; miR-221 abundance was significantly lower with circulating gastrin concentration >30 pM while miR-222 abundance was significantly lower with >100 pM gastrin concentration;

g) cell models of gastric adenocarcinoma expressing the CCK2R (ie AGS-GR and HGT-GR cells) treated with gastrin exhibit different putative biomarker expression

profiles and sensitivities; however TIMP-1 and MMP-1 present as common markers of gastrin activity.

7.2 Recent changes in the state of the field

7.2.1 Liquid biopsy for biomarker detection

During the course of this thesis substantial progress was reported by others in using "liquid biopsy" i.e. plasma biomarkers to detect cancer; in particular, impressive success was obtained using multiple biomarkers at both the DNA and protein level.[348] The latter combine detection of ctDNA for the presence of cancer in the body combined with protein tests to determine localisation of the cancer, overcoming some previous limitations of liquid biopsies. These biomarkers were obtained from previous research analysing the mutations common in various localised cancers and applying that library to liquid biopsies; theoretically a similar development pathway could be followed for blood-based biomarkers of preneoplastic progression in which biopsy analysis (as I have performed) would form the first step.

One of the most important attributes of a liquid biopsy based test is the ability to detect cancer as early as possible, preferably in patients with preneoplastic conditions or those in a high risk group before cancer has a chance to develop to improve prognosis. Despite covering a range of 16 genes and 8 proteins median sensitivity for the test overall remained at 70% covering a range of 98-33% for ovarian and breast cancers respectively. Most applicable to my research was the discovery rates for oesophageal and stomach cancer: 70 and 75% respectively. As noted by the authors of the paper however, the median specificity of the test for early stage cancers dropped to only 43%, with oesophageal cancer being the lowest at 20%.

This drop in detection rate highlights the limitations in utilising a test based on ctDNA in blood from dying cancer cells, since a) cancer has to be already established for detection and b) the sensitivity of the test is directly proportional to the number of cancer cells dying and releasing ctDNA. However limitations also exist in protein analysis of liquid biopsies, whilst changes in the abundance of circulating protein biomarkers linked to cancer can be detected with high-sensitivity they're rarely specific enough to identify the location of the cancer.

This study utilised a combination of both types of biomarkers to try and overcome these limitations, to some success, and provide cancer detection and localisation in one test. It highlights the importance of examining multiple biomarkers together as part of a panel and multiple sources of biomarkers (proteins, ctDNAs, miRNAs etc.) to account for limitations of any singular biomarker or biomarker source.

7.2.2 Application of a GI blood based biomarker

At the start of the present thesis, it was considered that MMP-7 might be a particularly promising biomarker. In this context it is therefore interesting that using a combination of metabolomic and proteomic techniques it was found that circulating MMP-7 + ɣ-glutamyltranspeptidase as a result of epithelial injury was specific for the detection of biliary atresia.[349] Again, the use of multiple platforms facilitated the development of a rigorous biomarker panel.

The idea that MMP-7 could be used as a biomarker for epithelial injury in the GIT mirrors one of the potential hypotheses I was investigating in BO. In our work however, it would appear that MMP-7 is not a reliable marker for tracking the effect of gastrin on BO.

7.3 Effect of PPI treatment in patients with Barrett's oesophagus

7.3.1 Gastrin response to PPIs

There has been evidence that patients with BO exhibit significantly lower circulating gastrin concentration than control patients when neither were treated with PPIs[350] which, considering extensive research detailing PPI induced hypergastrinaemia,[74] raises the question of how circulating gastrin concentration would respond to PPI treatment in Barrett's patients. Rather few studies appear to have specifically determined if PPI treatment influences circulating gastrin in Barrett's patients, however a previous study did find that in Barrett's patients, long-term PPI treatment induced a significant increase in circulating gastrin. This increase stimulated increased restitution of BE in the short-term, however long-term treatment did not induce significant changes in the length of BO between low and high dose PPI treatment groups.[351] Thus the concept for the first stage of our research was to identify if in our cohort of BO patients PPI treatment was associated with hypergastrinaemia which may have an effect on BE as previously reported. When controlled for age, sex and *H.*

pylori infection status patients with BO treated with PPIs exhibited significantly higher circulating gastrin concentration compared to controls treated with PPIs.

A possible reason for this difference for this may be that Barrett's patients initially have had a higher circulating gastrin concentration prior to PPI treatment, a possibility supported by the higher mean gastrin concentration in Barrett's patients without PPI treatment compared to controls without treatment. However the change in circulating gastrin concentration in response to PPI treatment within patient groups was still greater in Barrett's patients than controls, implying that BO patients respond disproportionally to PPI treatment. Within our cohort other common causes of hypergastrinaemia such as *H. pylori* infection, atrophic gastritis or ZES were ruled out and no studies have reported changes in the stomach of Barrett's patients that would suggest changes in the stomach associated with hypergastrinaemia to explain this disparate response. Currently no studies have been performed on circulating gastrin concentration in matched control and Barrett's patients which tracked individual patients prior to and post-PPI administration, however this kind of study would be the "gold standard" in providing further evidence to support the idea of Barrett's patients circulating gastrin concentration responding differently to controls when both are treated with PPIs.

It is also possible that Barrett's patients had been treated with PPIs longer than control patients as patient information did not address this directly, which represents another point for further study - comparing length of PPI treatment to changes in circulating gastrin in both groups to identify if gastrin increase is time-dependent or perhaps PPI dose-dependent.

It would also be pertinent to involve fasting and post-prandial gastrin measurements to assess whether or not gastrin response to food was typical, or whether Barrett's patients' response was disproportional. A longitudinal study of this nature would also address the factor of whether PPI treatment length impacts changes in circulating gastrin concentration. An intervention study comparing BO and control patients administered PPIs for the first time (with circulating gastrin monitoring from pre-administration onwards) would be the gold-standard however may prove unfeasible on a large scale. A well-designed, well-powered cohort study as described above

would be the next logical step in investigation of PPIs effect on circulating gastrin in patients with BO.

7.3.2 Stomach response to PPI treatment

Despite BO being an oesophagus focused condition, our research exhibited evidence of changes in gastrin-regulated genes occurring in the stomach of Barrett's patients when treated with PPIs, even when gastrin matched. All samples analysed were histologically examined to ensure that patients with BO did not exhibit altered gastric mucosal morphology. However, it is possible that in the stomach of Barrett's patients there are subtle changes in ECL or parietal cell populations that were not detectable during histology but would be detectable using much more sensitive qPCR analysis.

The increases we observed in CgA and HDC abundance would suggest increased ECL-cell mass in keeping with the known effects of prolonged PPI therapy/hypergastrinaemia.[74] This leaves either parietal cell loss (a nominal contributor to total CCK2R expression) or more likely, reduced expression in either or both of those cell types. Previous work on whether CCK2R expression was regulated by gastrin[174] indicated that gastric CCK2R abundance was increased by hypergastrinaemia in transgenic mouse models and gastrin treatment of mouse gastric pit precursor cell line GSM06.[352] Decreased expression of the CCK2R with increased gastrin concentration was therefore unanticipated and raises important questions as to how changes in CCK2R expression are related to gastrin concentration and sensitivity to gastrin. Currently the mechanisms that control CCK2R expression are not fully understood so further research in that area may provide an understanding of the phenomenon we observed. This finding could have wide-reaching implications in the field of gastrin study, offering a new perspective on older studies and in the search for biomarkers of the activity of gastrin on cells, specifically BO and ACO.

The use of gastric cell lines transfected with the CCK2R may provide us with more answers concerning how CCK2R expression is controlled. By utilising cell models, we can apply a number of inhibitors downstream of CCK2R activated signal transduction pathways and using qPCR determine if relative abundance changes.[174] If a decrease in CCK2R abundance is not replicable in cell lines, it could indicate that CCK2R expression is modulated via a paracrine pathway and animal models will need to be used to examine further.

7.4 Biomarkers of Barrett's Oesophagus

7.4.1 Expression of putative biomarkers of gastrin stimulated progression

None of the potential biomarkers analysed exhibited our predicted trend of a significant change in abundance in the most at-risk patient group. These findings however follow logically when considering our analysis of changes in the expression of the CCK2 receptor within our samples.

7.4.2 CCK2 receptor expression is associated with gastrin concentration

The most novel finding of my work (and indeed that with most wide-reaching impact) was an inverse correlation between CCK2R mRNA abundance and circulating gastrin concentration in BO biopsies. This finding is crucially important because it indicates that in patients with elevated gastrin, for example, due to PPI therapy, there may exist an auto-regulatory compensation mechanism that reduces BE responsiveness to gastrin.

If so, the search for gastrin regulated biomarkers of progression in BO will be much more complex, as one would expect reduced expression of biomarkers in cases of reduced CCK2R expression, which coincides with the most at-risk group – those who would potentially most benefit from discovery of a biomarker for gastrin driven BO progression.

Our data, being qPCR based rather than targeted to mature CCK2Rs at the cell surface, is novel in CCK2R expression studies. In the cell work performed by Dimaline et. al, hypergastrinaemia caused the CCK2R to be internalised and stored in vesicles, this is consistent with the possibility that when receptors are in abundance, transcription of CCK2R mRNA could be downregulated. Likewise comparing to the rat data whereby rises in gastrin concentration stimulate increased gastrin receptor abundance it would follow a rise in transcription of CCK2R mRNA would accompany.

These results outlining the variance in CCK2R population and between different expression models of the receptor emphasize the importance of studying receptor abundance as well as hormone levels to the total understanding of an endocrine response. A lack of elucidated mechanisms that control and influence expression of the CCK2R represents a key missing component in gastrin research that requires further study to fully understand the role gastrin plays in BO progression.

Clinically, the observed variance in CCK2R abundance may also impact the usefulness of CCK2R antagonists as an alternative to PPI therapy for use in treating reflux disorders and potentially BO.

7.4.3 Strengths and limitations of the Barrett's study

The study design outlined in chapter 5 had several advantages compared to previous Barrett's biomarker studies. Principally it included primary human tissue samples of BO rather than animal models or transfected cell lines, so results derived are representative of human physiology. Secondly, we have linked circulating gastrin concentration to its physiological function within the body (stimulation of gastric acid secretion) and expressed our biomarker results along that range. This is unique as large scale meta-analysis of BO and ACO rarely include a section on gastrin concentration (indeed many individual studies do not consider it) even in studies specifically considering PPI usage and Barrett's to ACO progression risk.[353] This represents an oversight considering the predictability that a) many Barrett's patients will be taking PPI medication b) Barrett's patients taking PPIs do not all exhibit the same change in circulating gastrin concentration as a result and c) gastrin has possible proliferative effects on BO.[129, 289] Lastly, circulating gastrin concentration was determined with a rigorously validated assay.

There were however some limitations to our study. At the beginning of the study it was not possible to do formal power calculations on biomarkers of gastrin driven BO progression simply because no previous research had been done on the phenomenon. A correlation between circulating concentration and BO progression risk was only suggested in 2010[289] and 2011[354] meaning research elucidating a possible link is still in its infancy. This made determination of an appropriate cohort size for the discovery of putative biomarkers difficult. Lastly our study was cross-sectional, with the majority of gastrin concentrations falling below the "high risk" group of interest for biomarker discovery. It would be advantageous to design prospective studies to focus solely on high-gastrin patients over an extended period of time and examine how gastrin-regulated gene expression changed relative to degree of dysplasia.

7.5 Consideration of alternative models of BO

Whilst human biopsies represent the gold standard of starting material for potential biomarker analysis, there is potential to use animal models of BO as a platform for putative biomarker discovery and pathophysiology of the condition.[355] Animal models with induced BO such as by surgical intervention could also be used to provide normal biopsy tissue (pre-induction) to provide a better control starting point with which to compare changes in gene expression in BO and progression thereof relative to gastrin.

Although cell culture and organotypic models of BO have been used previously, they cannot represent the genetic diversity, clonal dynamics and stromal and host-immune interactions involved in clinical neoplastic progression.[355] Although they do provide a much quicker method of obtaining proteins suspected to be able to act as biomarkers in response to specific stimuli (in our case, gastrin) and as such play a pivotal role in primary screening for biomarkers. Another exciting possibility is the use of patient derived organoids[356] of both tumour and preneoplastic tissue that would allow detailed in vitro screening for putative biomarkers for the effects of gastrin prior to in vivo studies.

A novel method of obtaining cells from the lower oesophagus without the need for endoscopy– the cytosponge – is currently being trialled for Barrett's diagnosis and biomarker analysis. Whilst use of the cytosponge has boasted a high-degree of accuracy in BO diagnosis (sensitivity 79.5-87%, specificity 92.4%) it has limitations in that it samples oesophageal epithelium cells from the entire length of the oesophagus, not just Barrett's metaplasia, and the research group behind it has yet to identify a suitable panel of biomarkers to accurately identify patients at a high-risk of progression.[291]

Methods of biomarker discovery either focus on the DNA, RNA, protein or epigenetic level, referred to as genomics, transcriptomics, proteomics or epigenomics respectively.[357] A number of biomarkers discovered within these disciplines have already been developed into assays for human cancers. These include prostate-specific antigen test for prostate cancer in blood (proteomics), methylated vimentin as a DNA based biomarker (genomics) and DNA based FISH assays utilised for bladder cancer detection (genomics).[358]

The majority of cancer biomarkers are measured either in the tumour mass or in blood, however in our study there was no existing tumour to investigate, meaning the gold

standard for a biomarker of the effect of gastrin on BO would be a blood based assay. A blood based assay would be the most minimally invasive method of tracking changes in biomarker abundance, would require no extra training or equipment to obtain samples and would not interfere with scheduled surveillance of BO.

7.6 Future experiments

An obvious progression from my work would be to analyse whether changes observed at the transcriptional level relative to gastrin concentration translated to comparable changes in mature protein production.

Immunohistochemistry based experiments could provide data on the location of changes in protein abundance in response to gastrin; for example cells within biopsies that display a more dysplastic phenotype may also be those exhibiting more extreme changes in protein expression. Currently all my qPCR data obtained from biopsies assumes that the cells within the biopsy consist of a homologous population of similarly metaplastic Barrett's epithelium, displaying similar changes in mRNA expression. It would also be possible to study the location of the CCK2R within the cells of biopsies and see if it correlates with changes in protein expression, further strengthening the proposed hypothesis that activation of the CCK2R by gastrin is responsible for changes in gene expression.

Characterisation of an isoform of the CCK2R linked to increased risk of pancreatic cancer[359] and shown to be constitutionally active[172] indicates further experimentation should involve characterisation of the specific variant (or variants) found in BO. There has been success with the use of a monoclonal antibody raised to the unique 69 amino acids included in the variant, so experiments previously used to detect the variant in pancreatic tumours could be applied to ACO biopsies. Currently there is no research investigating whether the variant is expressed in ACO as it is in pancreatic cancers. Very recently attention has focused on how natural genetic variations in GPCRs could impact pharmacological response[360] of GPCR therapies, under the umbrella of the emerging field of personalised medicine. It could be the case that genetic variation in the CCK2R sequence can impact the responsiveness to gastrin in BO and the potential usefulness of emerging CCK2R antagonists as a treatment option.

7.7 Conclusion

To conclude, I set out to identify biomarkers that could be used to detect the effect of gastrin on BO. They could then potentially be used to create an assay identifying progression risk of gastrin-driven BO to ACO to identify patients earlier, before histological changes are noticeable during endoscopy. What I discovered is despite published work identifying significantly increased risk of BO progression at high gastrin concentrations, none of the biomarkers we analysed suggested a robust association with circulating gastrin concentration over the range up 500 pM. We did however identify significant changes in the abundance of the CCK2R within samples, relative to gastrin concentration. This finding could have large biological implications in future research concerning the role of gastrin on tissues expressing the CCK2R, antagonist therapies for reflux disease and beyond.

The approach taken within this thesis also provides a paradigm for further biomarker studies and emphasises the importance of using multiple approaches, techniques and biological sources, for rigorous putative biomarker discovery.

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Appendices

I.I Measurement of serum CgA concentration

Alongside measurement of CgA transcript abundance in gastric biopsies, serum CgA from the samples was also analysed using RIA.

I.I.I Method

Serum chromogranin A (CgA) concentrations were determined by RIA utilising a proprietary kit (Eurodiagnostica, Cat No. RB321) according to the manufacturer's instructions. In brief, serum samples were diluted 1/10 in assay diluent and incubated with ^{125} I-CgA and a CgA specific antibody alongside appropriate standards and quality controls for 24 hours at 4°C.Antibody-bound and free radiolabel were separated by addition of 500µL of a double antibody solid phase solution to each tube. The tubes were then briefly vortexed and incubated at 4°C for 60 minutes before being centrifuged at 3000rpm for 15 minutes still at 4°C.

The supernatant was decanted from the pellet, with the radioactivity of each counted for one minute using a Packard Bell RIAstar gamma counter. The B/F ratio was calculated allowing for non-specific binding of the radiolabel determined from control tubes (no antibody).

I.I.II Serum CgA abundance was significantly higher in patients treated with PPIs

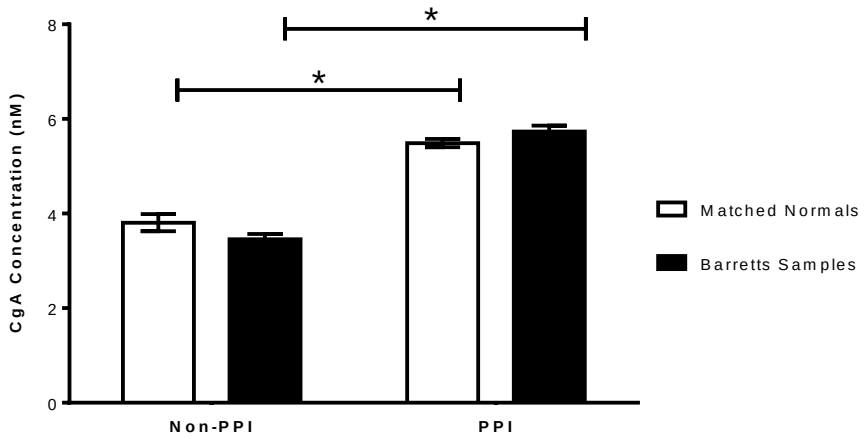

Figure I.II.I - **A comparison of the serum chromogranin A concentration in normal and Barrett's patients as measured by RIA.** Data is presented as mean ± SEM, $p<0.05$ 2-way ANOVA with Tukey's correction.

I.II.III Conclusion

As with transcript abundance in gastric biopsies, CgA concentration in serum also appears to be increased in both control and Barrett's patients when treated with PPIs (fig I.II.I).

I.II Measurement of serum B12 in patients with corpus atrophy

Due to the requirement of intrinsic factor secretion from parietal cells for the absorbance of vitamin B_{12} from the diet, serum B_{12} concentration was investigated in patients with corpus atrophy when treated with PPIs.

I.II.I Method

Serum samples were tested for the concentration of active vitamin B_{12} in serum samples using a commercial active B_{12} ELISA kit (Biohit HealthCare, UK) according to manufacturer's instructions. Serum samples were diluted 1:200 on manufacturer's recommendation using the supplied diluent and vortexed. To the plate 100 µL of diluent buffer (blank), supplied calibrator solutions (for creating a standard curve) and supplied positive and negative control samples were added in duplicate as well as patient samples; samples were then sealed and incubated for 30 min at 37°C. The solution was then removed by inverting the plate and firmly tapping on absorbent tissue, and the plate was washed three times with 350 µL of supplied wash buffer per well, ensuring all wash buffer was removed between washes by firmly tapping the inverted plate onto absorbent tissue. To each well, 100 µL of conjugated antibody solution was added and the plate incubated at 37°C for 30 min before removal, after which the washing step was repeated. Next, 100 µL of supplied substrate solution was added to each well and incubated in the dark at room temperature for 30 min before 100 µL of provided stop solution was added to complete the reaction. The absorbance of the well was then measured at 450 nM on a Tecan GENios plate reader using manufacturer supplied software. Comparison of the absorbance values for the serum samples to the standard curve values allowed extrapolation of patient B_{12} concentration.

I.II.II Results

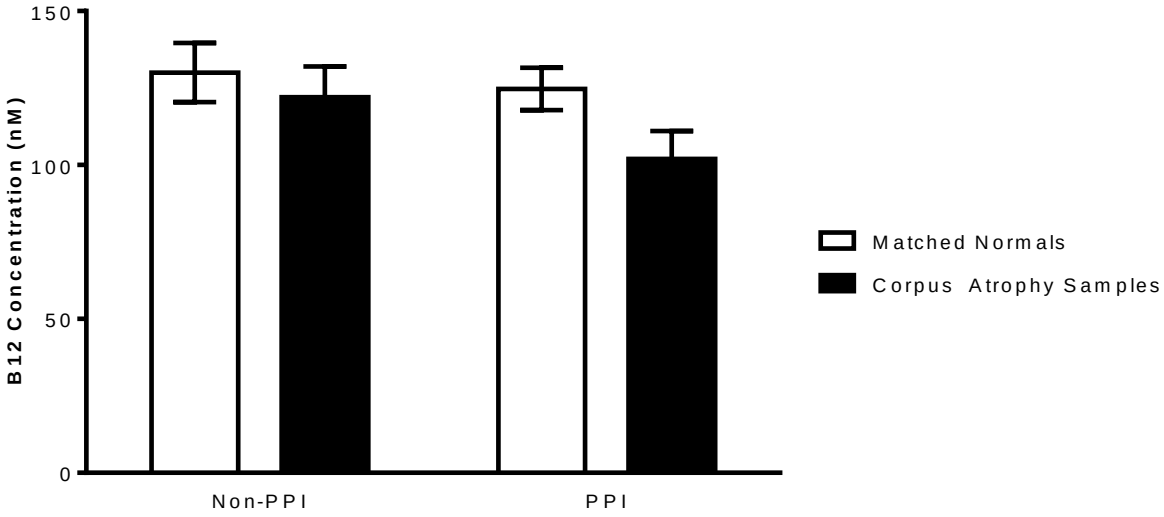

Figure I.II.II - **A comparison of the serum active B_{12} concentration in normal and Barrett's patients as measured by ELISA.** Data is presented as mean ± SEM, $p<0.05$ 2-way ANOVA with Tukey's correction.

I.II.III Conclusion

No significant differences in active B_{12} concentration were identified between normal patients or those exhibiting corpus atrophy. Likewise no significant difference in B_{12} concentration was identified as a result of treating patients with PPIs (fig I.II.II).