Investigation of biomarkers associated with hypergastrinaemia and their responses to CCK-2 receptor antagonism

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

Katie Alexandra Lloyd

February 2016

Acknowledgements

Firstly, I would like to thank my supervisors Professor Mark Pritchard, Professor Andrea Varro and (unofficially) Professor Graham Dockray for their extensive knowledge, guidance and academic support that I aim to achieve for myself someday and all my colleagues in Gastro who have helped me through the good and bad times. I would like thank the Institute of Translational Medicine at the University of Liverpool for funding this PhD studentship and Trio Medicines Ltd for partially funding this project.

Secondly, I would like to thank my family: to my loving boyfriend Andy for being my shoulder to cry on when things didn't go as planned (most of the time) and giving me self-assurance when things work out (hardly ever). I thank you for just being there and knowing….you have got it all to come!

To my Nanny and Grandads, I would like to thank you for not only supporting me financially but for always believing in me and giving me the confidence to aim high and to my Granny, although you left us the day I began this PhD journey, you have been with me every step of the way.

Finally, I would like to thank my Mum and Dad for always giving me everything I ever needed to succeed in life. There are no words to describe the love and gratitude I have for you both.

I hope I make you proud…

Investigation of biomarkers associated with hypergastrinaemia and their responses to CCK-2 receptor antagonism

Katie Alexandra Lloyd

Gastrin is a hormonal regulator of gastric acid secretion, but also regulates other cellular functions in the stomach such as proliferation, migration and apoptosis. Elevated serum concentrations of gastrin may dysregulate these processes and contribute to the development of gastric neuroendocrine tumours (NETs) and adenocarcinomas. The inhibition of gastrin or its subsequent downstream signalling pathways may therefore have therapeutic value in gastrin-related disorders including gastric NETs. The aims of this PhD were to investigate the role of gastrin in gastric tumour development and to identify potential biomarkers for the detection of gastrin-associated cellular phenotypical changes which may contribute to the development of gastric pathologies. I also investigated whether three novel cholecystokinin type-2 receptor (CCK2R) antagonists inhibited gastrin-associated morphological changes in the stomach, *in vitro*. miRNA PCR arrays showed that gastrin significantly increased the expression of both miR-222 and miR-376c in human gastric cancer cells which express the cholecystokinin type-2 receptor (AGS_{GR}). Of these two miRNAs, only the increase in miR-222 expression was confirmed using quantitative polymerase chain reaction (qPCR). Using chemical molecular pathway inhibitors, gastrin-induced miR-222 overexpression was shown to occur via activation of the CCK2R and subsequent phosphokinase C (PKC) and phosphatidylinositol-3-kinase (PI3K) pathways. miR-222 expression significantly increased with age and further increased with *Helicobacter felis* (*H. felis*) infection in hypergastrinaemic INS-GAS mice. Similarly in patients with hypergastrinaemia and type-1 gastric NETs, miR-222 expression was increased at baseline relative to healthy control subjects and decreased whilst the patients were taking the CCK2R antagonist, netazepide. miR-222 overexpression caused p27 mislocalisation to the cytoplasm and resulted in decreased p27 mRNA and protein expressions, *in vitro*. Functional analyses using small interfering RNAs (siRNAs) identified that this decreased p27 expression resulted in cell migration and structural remodelling in AGS_{GR} cells. Previous cDNA microarray studies identified additional gastrin-regulated genes in gastric biopsies from hypergastrinaemic patients with type-1 gastric NETs being treated with netazepide. Of eight potential gene candidates, the tissue remodelling protein pregnancy-associated plasma protein-A2 (PAPPA2) was selected for further investigated *in vitro* due to its previous association with tissue remodelling in the placenta. Gastrin treatment increased PAPPA2 mRNA and protein expressions in AGS_{GR} cells and this resulted in increased cell migration and cellular remodelling. Several CCK2R antagonists have previously been developed for the treatment of acid-peptic disorders and hypergastrinaemic conditions including gastric NETs. However oral bioavailability, receptor selectivity and/or drug potency still remain an issue. The inhibition of gastrin-induced cellular phenomena by the novel CCK2R antagonists TR1, TR2 and TR3 was therefore assessed using cell-based assays and their potencies were compared against the previously established CCK2R antagonists, YM022 and netazepide. All compounds caused significant reductions in gastrin-induced cellular processes such as cell growth, migration, structural remodelling and clonogenic survival. TR2 showed the highest potency, but was equally or less potent than both YM022 and netazepide in all assays. These data have therefore identified two potential novel gastrin-regulated biomarkers and a potential novel CCK2R antagonist which may be further developed for the detection and treatment of gastrin-related conditions.

Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
7B2	Prohormone convertase-2 chaperone protein
AA	Amino acid
ACF	Aberrant crypt foci
ACTH	Adrenocorticotrophic hormones
AGCH	Antral gastrin cell hyperplasia
Ago2	Argonaute-2
AID	Activation induced cytidine deaminase
AIDS	Acquired immune deficiency syndrome
AOM	Azoxymethane
AP2	Activator protein-2
AQP4	Aquaporin-4
AREs	AU-rich 3'UTR regulatory elements
babA	Blood-group antigen binding adhesin
BCL	B-cell lymphoma
B-CLL	B-cell chronic lymphocytic leukaemia
BN/GRP	Bombesin/gastrin releasing peptide
BSA	Bovine serum albumin

CAG	Chronic atrophic gastritis
cagA	Cytotoxin-associated gene-A
*cag*PAI	cag pathogenicity island
cAMP	Cyclic adenosine monophosphate
CAT-1	Cationic amino acid transporter-1
CCK	Cholecystokinin
CCK1R	Cholecystokinin type-1 receptor
CCK2R	Cholecystokinin type-2 receptor or gastrin receptor
CDK	Cyclin-dependent kinase
CgA	Chromogranin-A
CLL	Chronic lymphocytic leukaemia
Cox-2	Cyclooxygenase-2
CPE	Carboxypeptidase-E
CRC	Colorectal cancer
CTFP	Carboxy-terminal flanking peptide
D cells	Delta cells
DAB	3,3′-Diaminobenzidine
Dcp	Decapping enzymes

DDC	Diethyldithiocarbamate
DGCR8	DiGeorge syndrome chromosomal region-8
DNA	Deoxyribonucleic acid
ECL	Enterochromaffin-like cell
ED_{50}	Effective dose (50%)
EDTA	Ethylene-diamine tetra-acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EZH2	Enhancer of zeste homologue-2
FAP	Familial adenomatous polyposis
FFPE	Formalin fixed paraffin embedded
FITC	Fluorescein isothiocyanate
FOS	v-fos FBJ murine osteosarcoma viral oncogene homologue
FXR1	Fragile-X mental retardation protein-1
G cells	Gastrin cells
gERE	Gastrin epidermal growth factor response element
Gly-G	Glycine extended gastrin

GORD	Gastro-oesophageal reflux disease
GPCR	G-protein coupled receptor
GRP	Gastrin releasing peptide
H. felis	*Helicobacter felis*
H. pylori	*Helicobacter pylori*
H^+/K^+ ATPase	Proton pump
H_2R	Histamine type-2 receptor
H_2RA	Histamine type-2 receptor antagonists
HB-EGF	Heparin-binding epithelial growth factor
HCC	Hepatocellular carcinoma
HDC	Histidine decarboxylase
HELLP	Haemolytic anaemia, elevated liver enzymes and low platelet count
HIV	Human immunodeficiency virus
Hop	*Helicobacter* outer membrane protein
IC_{50}	Inhibitory concentration (50%)
IGF	Insulin-like growth factor
IgG	Immunoglobulin-G antibodies
IgV_H	Immunoglobulin heavy-chain variable-region gene

IHC	Immunohistochemistry
IL-8	Interleukin-8
IMX	3-isobutyl-1-methylxanthine
JNJ-26070109	[(*R*)4-Bromo-*N*-[1-(2,4-difluoro-phenyl)-ethyl]-2-(quinoxaline-5-sulfonylamino)-benzamide
KCNQ1	Voltage-gated potassium channel
kDa	Kilodaltons
LOH	Loss of heterozygosity
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinases
MBC	Minimum bactericidal concentration
Mcl-1	Myeloid cell leukaemia-1
MEF	Mouse embryonic fibroblasts
MEN	Multiple endocrine neoplasia
MIC	Minimum inhibitory concentration
miRNA	Micro-ribonucleic acid
miRNP	miRNA ribonucleoprotein complex
MMP	Matrix metalloproteinase
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine

MnSOD/SOD2	Manganese sodium dismutase
MNU	N-methyl-N-nitrosourea
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaF	Sodium fluoride
NETs	Neuroendocrine tumours
NIH	National Institute of Health
NLS	Nuclear localisation signal
Non-SPF	Non-specific pathogen free
NPAP60	Nuclear pore-associated protein-60
NSCC	Non-selective cation channel
NSCLC	Non-squamous cell lung carcinoma
OMP	Outer membrane protein
PA	Pernicious anaemia
PACT	Protein activator of the interferon-induced protein kinase
PAL	Peptidyl-α-hydroxyglycine α-amidating lyase
PAM	Peptidyl-α-amidating monooxygenase
PAPPA2	Pregnancy-associated plasma protein-A2 or Pappalysin

PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.1% tween-20
PC	Prohormone convertase
PCA	Parietal cell antibodies
PHM	Peptidylglycine α-hydroxylating monooxygenase
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase-A
PKC	Protein kinase-C
PLC	Phospholipase-C
PNPT1	Polyribonucleotide nucleotidyltransferase-1
PPI	Proton pump inhibitor
Pro-G	Progastrin
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RECK	Reversion inducing cysteine rich protein with kazal motifs
Reg	Regenerating protein
RhoA	Rhodopsin-A
RIA	Radio-immunoassay

RIPA	Radio-immunoprecipitation buffer
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROCK	Rhodopsin-associated protein kinase
ROS	Reactive oxygen species
sabA	Sialic acid-binding adhesin
SCID	Severe chronic immunodeficiency
SFKs	Src family of tyrosine kinases
SHH	Sonic hedgehog protein
siRNA	Short interfering ribonucleic acid
SMAD	Mothers against decapentaplegic homolog
SNPs	Single nucleotide polymorphisms
Sp1	Specificity protein-1
SPEM	Spasmolytic-polypeptide expressing metaplasia
SRP	Signal recognition peptide
T4SS	Bacterial type IV secretion system
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.1% tween-20

TFF	Trefoil factor family peptide
TGF-β	Transforming growth factor-β
TGN	Trans-Golgi network
TNF-α	Tumour necrosis factor-α
TPST	Tyrosylprotein-sulphotransferase
TRBP	HIV-1 TAR RNA binding protein
tRNA	Transfer-ribonucleic acid
TTX	Tetrodotoxin
uPA	Urokinase plasminogen activator
UTR	Untranslated region
vacA	Vacuolating cytotoxin-A gene
VIP	Vasoactive intestinal polypeptide
VMAT	Vesicular monoamine transporter
XPO1	Exportin-1
Xrn1p	Exoribonuclease
YF476	Netazepide or 1-[(3R)-1-(3,3-dimethyl-2-oxobutyl)-2-oxo-5-pyridin-2-yl-3H-1,4-benzodiazepin-3-yl]-3-[3-(methylamino)phenyl]urea
YM022	[(R)-1-[2, 3-dihydro-1-(2'-methylphenacyl)-2-oxo-5-phenyl-1H-1, 4- benzodiazepin-3-yl]-3-(3-methylphenyl) urea]

Z-360	calcium bis[(*R*)-(—)-3-[3-(5-cyclohexyl-1-(3,3-dimethyl-2-oxo-butyl)-2-oxo-2,3,4,5-tetrahydro-1*H*-benzo[*b*]-[1,4]diazepin-3-yl)ureido] benzoate]
ZAP-70	70kDa zeta-associated protein
ZES	Zollinger-Ellison syndrome

Table of Contents

1 Introduction

1.1 Gastrin

1.1.1 History

Primary studies led by Pavlov in 1888 [17] provided fundamental knowledge of pancreatic physiology; prompting the first proposal of hormonal regulation of gut function [18]. Since then, the role of gastrin [19-21] prompted the greatest debate due to the involvement of histamine [22] as some argued that pancreatic secretion could be a direct result of histamine release [23, 24]. Some investigations concluded that only histamine-free (secretin) preparations significantly induced pancreatic secretions [25], however histamine-free gastrin was difficult to isolate. It wasn't until R.A. Gregory and H.J. Tracy published a refined methodology of extracting gastrin from hog antral tissue [26], that adequate testing could be achieved with good reproducibility. It is now universally recognised that gastrin and histamine are two distinct gastrointestinal regulators involved in regulating the same physiological process of gastric acid secretion [27].

1.1.2 Molecular structure of gastrin

1.1.2.1 Transcription and translation

Gastrins are a large family of peptide hormones that are predominantly secreted from gastrin (G) cells in the antrum of the stomach, and are less abundantly secreted in the duodenum, jejunum, colon and pancreas [28]. The human gastrin gene (~4.2 kilobases in length) [29] is located on the long arm of chromosome 17 (q21.2) [30] and when transcribed into messenger RNA (mRNA), consists of 3 exons and 2 introns which encode the gastrin precursor, pre-progastrin [11] (Figure 1). All gastrins are derived from this 101 amino acid (AA) pre-propeptide which contains a signal peptide (21 AA), N-terminal spacer sequence (37 AA), an active gastrin component (34 AA) and an extension peptide, carboxy-terminal flanking peptide (CTFP, 9 AA). Post-translational modifications and differential cleavages of the pre-propeptide can produce 6 gastrin gene products of different lengths (Figure 2).

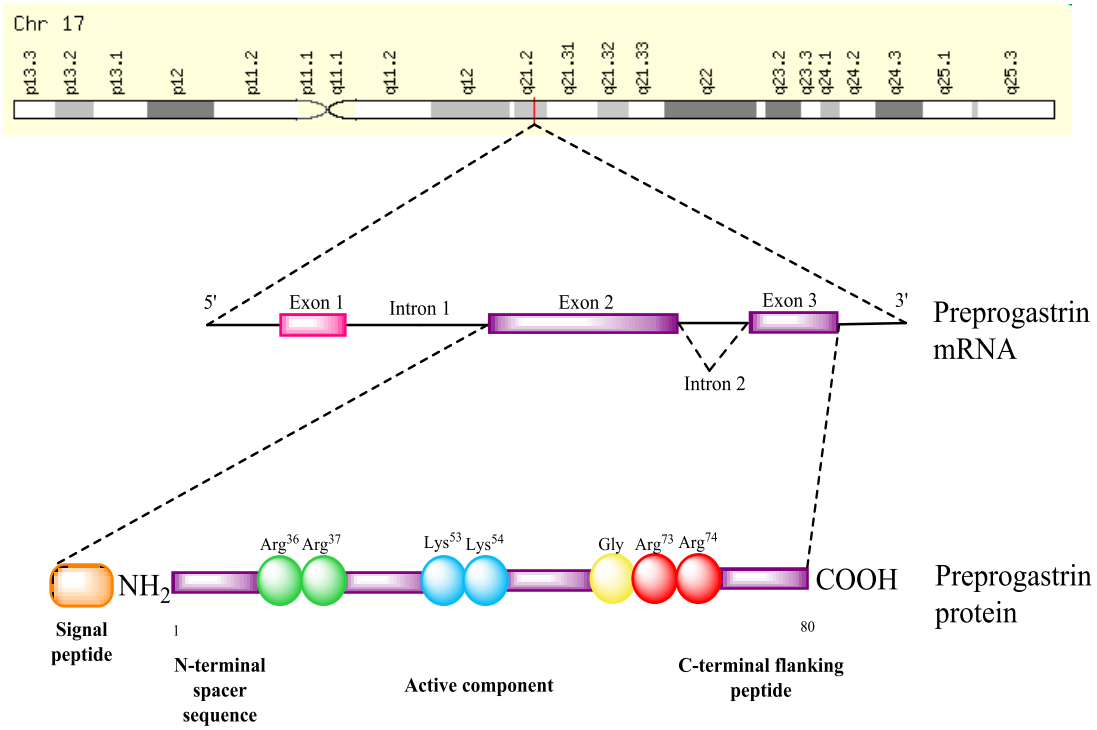

Figure 1. A schematic diagram representing the gene locus at chromosome 17 q21.2 from which the gastrin gene is transcribed. Preprogastrin mRNA contains 3 exons and 2 introns of which only exons 2 and 3 are spliced together and translated into the preprogastrin protein. The preprogastrin protein contains 4 domains: a signal peptide (21AA), an N-terminal spacer sequence (37AA), an active gastrin component (34AA) from which gastrins 17 and 34 are derived and a C-terminal flanking peptide (9AA). Figure adapted from Chao & Hellmich (2011) [3] and Wiborg *et al.* (1984) [11].

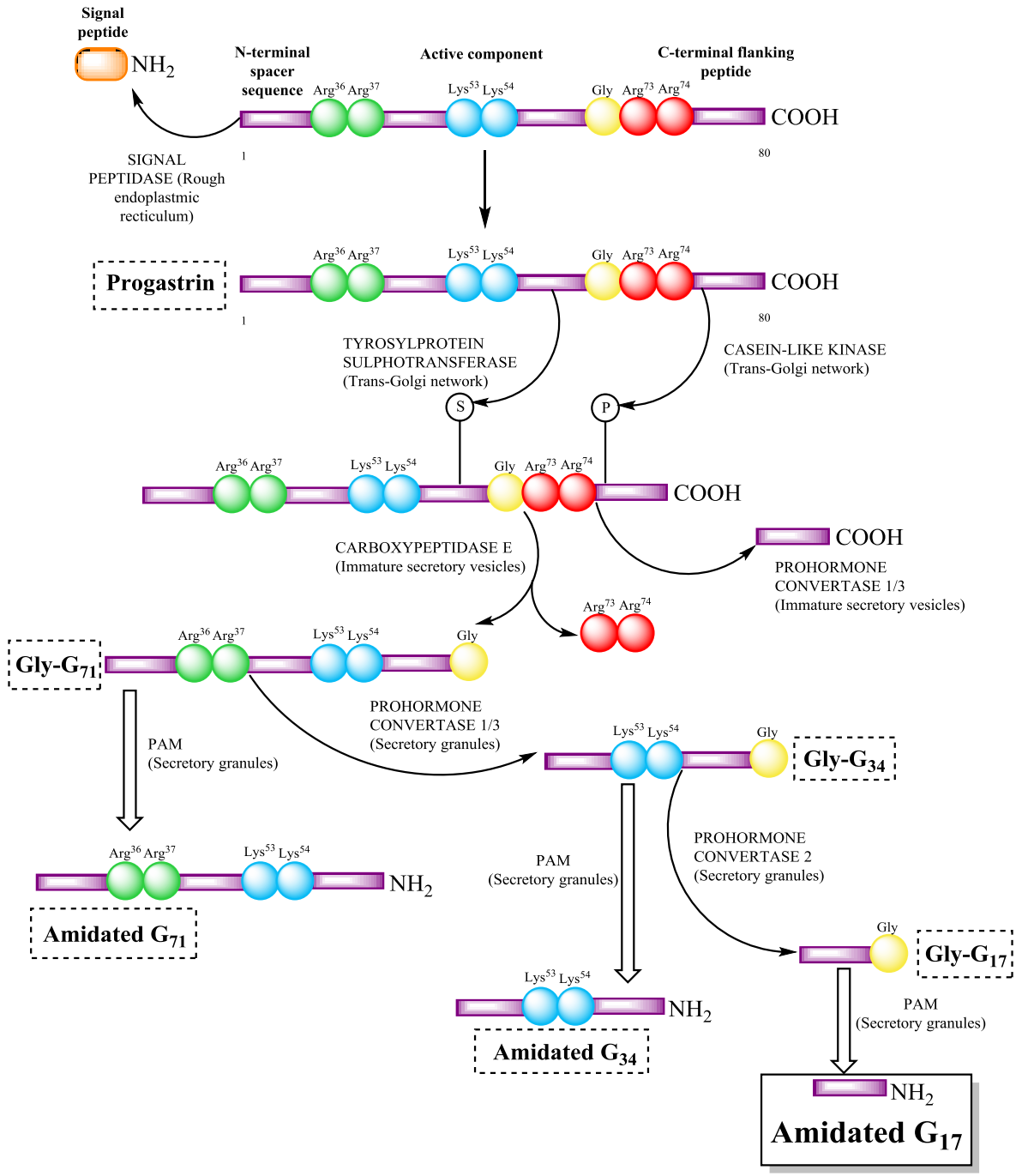

Figure 2. A proposed pathway for the processing of progastrin (Pro-G). Membrane bound signal peptidases remove the signal peptide in the rough ER whilst sulphation and phosphorylation by tyrosylprotein sulphotransferases (TPST) and casein-like kinases respectively, regulate the endoproteolytic cleavage of Pro-G at Arg^{73}-Arg^{74} by prohormone convertase 1/3 (PC1/3), followed by removal of the residual Arg^{73}-Arg^{74} by carboxypeptidase E (CPE). Subsequent cleavage at the second di-arginine site (Arg^{73}-Arg^{74}) by PC1/3 produces Gly-G_{34} which is may be further cleaved at Lys^{53}-$Lys5^{4}$ residues by prohormone convertase 2 (PC2) to form Gly-G_{17}. Peptide carboxy-amidation by peptidyl-α-amidating monooxygenase (PAM) then generates various forms of biologically active gastrins (G_{17}, G_{34} and G_{71}) which may be secreted. Figure adapted from Bundgaard & Rehfeld (2010) [6] and Dockray (1999) [15].

1.1.2.2 Post-translational modifications

Post-translational modifications begin simultaneously with the translation of pre-progastrin in the rough endoplasmic reticulum (ER) [31]. The amino-terminal signal peptide interacts with a signal recognition particle (SRP) to temporarily pause translation of the mRNA by the ribosomes, whilst the SRP binds to a specific receptor on the cytoplasmic side of the ER. Pre-progastrin is directed through the translocon Ser^{61} channel on the cytoplasmic side of the ER by the signal peptide where translation is then resumed. Upon continuation, the signal peptide is cleaved by membrane-bound signal peptidases (between amino acids Ala^{21} and Ser^{22}); generating the 80 AA progastrin (Pro-G) upon completion [32]. Pro-G then progresses through the Golgi apparatus where phosphorylation followed by sulphation may occur in the trans-Golgi network (TGN). The sulphation and/or phosphorylation of Pro-G have been shown to increase protein-protein interactions and regulate cleavage that occurs later in the secretory pathway [33].

1.1.2.3 Cleavage

Following the trans-Golgi network, Pro-G is packaged into immature secretory vesicles where peptide activation and secretory granule maturation occur simultaneously in order to secrete the resulting products. The alternate splicing of Pro-G occurs in these immature secretory vesicles by exo- and endopeptidases typically at three di-basic residues: Arg^{36}-Arg^{37}, Lys^{53}-Lys^{54} and Arg^{73}-Arg^{74} [34] and is dependent upon the sulphation and phosphorylation states of the protein.

In maturing secretory vesicles the first cleavage occurs after the Arg^{73}-Arg^{74} residues specifically by prohormone convertase 1/3 (PC1/3) and this liberates the CTFP [35, 36], which has been shown to exert some independent biological functions [37]. Post-cleavage exopeptidases (carboxypeptidase E) remove the residual C-terminal di-arginine residues leading to glycine extended gastrin-71 (Gly-G_{71}) [38, 39]. PC1/3 also specifically targets the

second di-arginine site (Arg^{73}-Arg^{74}) to cleave glycine extended gastrin-34 (Gly-G_{34}). Gel chromatography of antral extracts from PC1/3-null mouse models indicated that PC1/3, but not PC5/6, is exclusively responsible for gastrin cleavage at both of the di-arginine sites which occur earlier in the processing pathway, as higher concentrations of progastrin (and early processing intermediates) were found in null-mice compared with wild type littermates [40].

Glycine-extended gastrin-34 (Gly-G_{34}) is then further processed into glycine-extended gastrin-17 (Gly-G_{17}) by prohormone convertase 2 (PC2) via partial cleavage after the di-lysine residues (Lys^{53}-Lys^{54}). PC2 has been shown to only be partially responsible for the Gly-G_{34} to Gly-G_{17} conversion as chromatography studies in mouse models with PC2 deletion only showed 50% cleavage of di-lysine residues, whilst mice with a deletion of 7B2, a PC2 chaperone protein, only showed 23% cleavage of the same residues indicating that both PC2 and its chaperone are required for the synthesis of Gly-G_{17} [41]. The cleavage of the two pairs of arginine residues occurs relatively quickly after exiting the TGN into the secretory vesicles (t½~10mins), whereas cleavage of the di-lysine residues (Lys^{53}-Lys^{54}) has been shown to occur at a slower rate (t½~60mins). Using pulse-chase labelling experiments Varro *et al.* found that only ~50% of Gly-G_{34} is further processed to form Gly-G_{17} which may explain the slower cleavage rate of PC2 and its expression later in the secretory pathway [42].

1.1.2.4 Amidation

The final addition of a C-terminal amide functional group has been shown to be a prerequisite for the full biological activity of more than half of all peptide hormones [43, 44] including gastrins, with tissue extracts suggesting that the main amidated forms of gastrin are G_{71}, G_{34} and G_{17} [45]. Peptide amidation has not been shown to be generated through transamidation and instead these peptides are transformed from glycine-extended intermediates by the bifunctional protein peptidyl-α-amidating monooxygenase (PAM)

which contains two catalytic domains peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL), during a two-step process. The first rate limiting step is catalysed by PHM which initiates the redox cycling of enzyme bound copper ions by utilising ascorbate and molecular oxygen to produce peptidyl-α-hydroxyglycine and semidehydroascorbate by-products [46-48]. Following the production of peptidyl-α-hydroxyglycine, which is relatively stable within the secretory granules (pH ~5) [49-51], the second step is catalysed by the lyase domain PAL, which presumably contains a tightly bound zinc cation and occurs spontaneously at alkaline pH to convert the stable intermediate into the α-amidated product and glyoxylate [52-54].

1.1.3 Tissue expression of the gastrin gene

Gastrin gene expression is very tissue specific and differs significantly between human adults and neonates. During foetal development, gastrin is predominantly expressed in pancreatic β cells and, to a lesser extent, the colon. Following the first 72hrs after birth this expression converts to adult expression patterns; becoming primarily located in the G cells of the antral mucosa with low-level expression in the small intestine, pituitary gland, reproductive organs, brain and lungs [55]. This expression shift may be associated with the physiological role of gastrin in cell growth throughout the gastrointestinal tract that is essential for foetal development, in addition to stimulating gastric acid secretion [56].

1.1.4 Regulation of gastrin expression

1.1.4.1 Transcriptional

Many physiological stimuli have been shown to be involved in the regulation of gastrin gene expression and subsequent peptide release, by binding to luminal receptors on the apical surface of antral G cells [57] resulting in the upregulation of transcription factors. Early studies identified high concentrations of biologically active epidermal growth factor (EGF) in gastric secretions [58, 59], and this has been shown to be a highly potent regulator of

gastrin transcription [60]. The upregulation of the gastrin gene by EGF has been shown to depend upon a 40 base pair *cis*-regulatory element of DNA between located upstream of the gastrin promoter region between -82 and -40 base pairs. This fragment of DNA contains the GC-rich gastrin EGF response element (gERE) motif located at 68 base pairs, which is the binding sites of two transcription factors: specificity protein-1 (Sp1) and activator protein-2 (AP2) [61, 62] and mediates the level of RNA polymerase-II transcription and subsequent gastrin gene expression in response to EGF receptor ligands [63].

Gastrin transcription is also negatively regulated by other physiological conditions such as a reduced gastric luminal pH [64]. During feeding, there is increased gastric acid secretion and a reduced intragastric luminal pH and as a consequence, somatostatin release is activated [65]. Somatostatin is a cyclic tetradecapeptide that is released by various tissues such as the antral stomach to inhibit the release of peptide hormones in a paracrine manner. Somatostatin binds to a cis-regulatory element within the human gastrin promoter sequence between -82 and -69 base pairs, which is thought to be adjacent to the gERE, and inhibits EGF-stimulated gastrin transcription [66].

1.1.4.2 Translational

In addition to inhibiting gastrin mRNA transcription, somatostatin has been shown to translationally regulate gastrin by reducing gastrin mRNA stability [67-69]. Studies by Bate *et al*. observed a 2-fold increase in gastrin mRNA translation in rats treated with the acid suppressing drug omeprazole for 5 days. In rats that had fasted for 24hrs there was a 40% decreased in gastrin mRNA translation, whilst gastrin mRNA abundance was unaffected [70]. These data indicate that physiological factors such as luminal pH may control the rate of gastrin mRNA translation irrespective of mRNA abundance.

1.1.4.3 Post-translational

Cellular processing hormones involved in sulphation and phosphorylation have been shown to be important regulators of subsequent endoproteolytic cleavage of Pro-G. Serine phosphorylation has been shown to determine the rate of cleavage and has been shown to occur by *trans*-Golgi membrane bound kinases before sulphation in the secretory vesicles [33, 71]. Like many peptides, progastrin is phosphorylated upon progression through this secretory pathway [72] at a specific Arg-Arg-Ser-Ala-Glu-Glu/Asp domain, which contains a conserved recognition site (Ser-X-acidic residue) for calcium-dependent casein-like kinases. Site-directed mutagenicity studies have indicated that phosphorylation delays the further processing of progastrin [73-75] which subsequently influences the ratio of secreted peptide products.

The sulphation of tyrosine residues has been shown to improve protein-protein interactions with specific sequences for sulphation proposed [76, 77]. In mammals only 50% of progastrin is sulphated [78] in comparison to the structurally similar cholecystokinin (CCK) which exhibits complete sulphation [79]. Studies by Bundgaard *et al.* indicated that the degree of sulphation determines the degree of proteolytic processing [80]. Further examination revealed that glycine-extended gastrins contained a higher degree of sulphation in comparison to their respective amidated forms [81]. Similar patterns of sulphation were observed in the rat [33] with identical sulphation patterns being observed in hog antral tissues [82] indicating that tyrosine sulphation regulates the subsequent post-translational cleavage of gastrins at Lys^{53}-Lys^{54} by PC2.

In endocrine cells such as G cells a secretory vesicle pH of ~5.5 must be maintained, by vacuolar proton pumps (vH^+/K^+ ATPase) [83, 84], in order for adequate generation of gastrin products. The acidic intravesicular environment provides an optimal pH for processing hormones such as PCs and upholds the large electrochemical proton gradient (10^4-10^5 [85, 86])

for amine-proton exchangers. The expression of the two closely related human vesicular monoamine transporters (VMAT) -1 and -2 are primarily limited to endocrine and neuronal cells, respectively and also differ in substrate identification and drug inhibition. VMAT2 has a higher affinity for monoamines compared with VMAT1 and only VMAT2 transports histamine [87, 88]. However, VMAT2 can also be found in the enterochromaffin-like (ECL) cells of the gastric mucosa, which is consistent with the ability for VMAT2, but not VMAT1, to transport histamine. It has previously been established that biogenic amines, particularly serotonin (5-HT) and dopamine, are decarboxylated in G cells and accumulate in the secretory vesicles [89], where they are utilised for peptide amidation. The basic properties of monoamines can also increase intravesicular pH which negatively regulates Pro-G cleavage [15].

Peptide amidation influences the ratio of secreted gastrins and the subsequent degree of biological activity, which can alter further post-translational processing of Pro-G. The concentration of amidating enzyme mRNAs have been shown to be regulated simultaneously with the control of its peptide substrate mRNA levels [90]. The first step in peptide amidation is rate limiting due to the requirement of copper ions, ascorbic acid and molecular oxygen. In rats treated with a copper chelator diethyldithiocarbamate (DDC) there was a decrease in peptide amidation *in situ*, however there was a selective increase in amidating enzyme synthesis and increased gastric acid output [91]. Similarly, the depletion of ascorbic acid has been shown to reduce the amidation of adrenocorticotrophic hormones (ACTH) 1-14 in the pituitary of the rat [92] and guinea pig [93].

Changes in the gastric luminal pH have been shown to affect many physiological processes including the post-translational regulation of gastrin. In starved rats antral and serum gastrin, but not Pro-G, concentrations significantly decreased (~25%) and somatostatin mRNA concentrations significantly increased (36%) when compared with non-starved

counterparts. Refeeding with a nutritious diet caused a significant increase in gastrin mRNA levels which still remained 30% lower than freely fed rats, whereas somatostatin mRNA levels returned to baseline. These studies indicate that both gastrin gene expression and gastrin processing are regulated by luminal pH [94].

The inhibition of gastric acid secretion by the proton pump inhibitor omeprazole and consequential increase in gastric luminal pH has also been shown to increase the transcription of genes encoding PC1/3 and PC2 [95]. In omeprazole treated rats, there were 40% increases in G_{34} conversion to G_{17} through Lys^{53}-Lys^{54} cleavage and in the ratio of glycine extended gastrins (Gly-G_{34}: Gly-G_{17}), indicating that acid induced inhibition of G cells controls the post-translational processing of Pro-G [96]. In other physiological studies it has been established that the cleavage of Pro-G into glycine-extended gastrins at Arg^{73}-Arg^{74}, is temperature-dependent [71, 95] and a 15°C drop in temperature (from 37°C to 22°C) can cause cessation of these secretory processes.

1.1.5 Regulation of gastrin secretion

1.1.5.1 Physiological

Although the *in vivo* mechanisms for the regulation of gastrin secretion by gastric luminal pH, amino acids and calcium are not fully understood, it has been suggested that there are both direct and indirect influences on the recipient G cell. The ingestion of a protein-rich diet, particularly L-isomers of tryptophan and phenylalanine, is a potent stimulator of both acid secretion and gastrin release [97-99] during the gastric phase of digestion. These aromatic L-amino acids are recognised by calcium-sensing receptors (CaR) that are widely distributed throughout the body including the stomach [100], pancreas [101] and brain [102] and their potencies are calcium-dependent, with Ca^{2+} concentrations <0.5mmol/L providing no biological response [103]. CaRs located on antral G cells stimulate gastrin release [100] whereas, CaRs located on parietal cells directly stimulate gastric acid secretion [104]. The amino-acid

sensing mechanisms of CaRs, in combination with increased extracellular dietary calcium ($[Ca^{2+}]_o$), have been shown to activate phospholipase-C (PLC) pathways which evoke the initial mobilisation of stored calcium and open non-selective cation channels (NSCCs) causing an influx of intracellular calcium ($[Ca^{2+}]_i$) followed by a sustained plateaux of $[Ca^{2+}]_i$ concentration, resulting in a concentration-dependent release of gastrin [100, 105].

1.1.5.2 Neural and paracrine

Luminal stimuli have also been shown to exert paracrine and neural signals which can regulate the secretion of gastrin. Intraluminal proteins stimulate cholinergic and two types of non-cholinergic gastric effector neurons, bombesin/gastrin releasing peptide (BN/GRP) and vasoactive intestinal polypeptide (VIP)- expressing neurons [106]. Data have indicated that neurally-induced gastric acid secretion is mediated by the net effect of activated cholinergic neurons which can stimulate acid secretion from parietal cells and BN/GRP neurons that can indirectly inhibit the release of somatostatin from delta (D) cells with a subsequent increase in gastrin secretion [107].

Partially digested proteins (peptones 0.5%) have been shown to potently stimulate the release of gastrin and inhibit somatostatin release [108]. Atropine has been shown to partially inhibit (<50%) the release of gastrin which decreases somatostatin secretion, which in turn stimulates further release of gastrin (above basal levels). The subsequent increase in gastrin secretion due to the inhibition of cholinergic neurons, indicates that both cholinergic and BN/GRP neurons affect the release of gastrin in response to luminal proteins and the reduced somatostatin secretion caused by cholinergic neurons on D cells allows BN/GRP neurons and cholinergic neurons on G cells to stimulate appropriate gastrin secretion [109].

Postprandial distension of the stomach can also activate both cholinergic and non-cholinergic neurons [110]. Data suggest that when the stomach is full there is high-grade

distension causing an increase in cholinergic neuron recruitment, which increases gastrin secretion and decreases somatostatin secretion. As the stomach begins to empty there is a reduction in distension which evokes a mechanical reflex for the inhibition of gastrin secretion by preferentially activating VIP neurons to stimulate somatostatin release. Gastrin and somatostatin responses caused by both high and low grade distension can be reversed with the axonal blocker tetrodotoxin (TTX) indicating neural mediation [109].

1.1.6 Degradation of gastrins

The degradation and clearance of gastrins is essential for the cessation their biological function with excessive biological activity, particularly of G_{17}, contributing to the development of gastric neoplastic conditions such as ECL cell hyperplasia [111] and gastric intestinal metaplasia [112].

Currently the half-lives of G_{17}, G_{34} and G_{71} have been determined in dogs, using a continuous infusion of human gastrin with results of approximately 3, 9 and 90mins respectively [113]. However other studies have identified a longer half-life for G_{17} in humans (5-11mins) [114-116]. Gastrin length has been shown to be the major determining factor for its rate of clearance, with studies in sheep suggesting that the larger the peptide, the longer the half-life as Pro-G had a significantly longer half-life (~42mins) than Gly-G_{17} (~11mins) [117]. The difference between the half-life of Pro-G and its derivatives is thought to reflect physiological application. The 3-fold increase in the half-life of G_{34} compared to G_{17} results in a higher ratio of G_{34}:G_{17} in peripheral plasma than in antral tissue, which may be indicative of maintaining basal gastric acid secretions [118]. Whereas the postprandial response to G_{17} rapidly increases gastric acid secretion to aid digestion and therefore the G_{34}:G_{17} ratio must be maintained via an increased clearance rate of G_{17}.

1.1.6.1 Clearance of G_{17}

Enzymatic metabolism of G_{17} by endopeptidase 24.11 is relatively slow *in vitro* with a half-life of ~67mins, but local concentrations of endopeptidase 24.11 have proven adequate for the rate of degradation observed *in vivo* . Tyrosine sulphation has also been shown to decrease the hydrolysis of G_{17} by endopeptidase 24.11, in contrast to the increased hydrolysis of CCK by the same enzyme [119].

1.1.6.1.1 Renal

The major sites for the metabolism of gastrin are currently unknown. *In vivo s*tudies in dogs have provided evidence suggesting that the kidney extracts ~40% of circulating human gastrin from the renal artery [120] and radiolabelled human gastrin has been shown to accumulate in the renal cortex after intravenous injection [121]. Due to the lack of gastrin present in the urine it has been suggested that the kidneys are a major site of gastrin degradation. However, in humans the role of the kidneys in gastrin clearance is less clear and although hypergastrinaemia has been associated with both acute and chronic renal failure [122, 123], there are few differences between arterial and renal gastrin concentrations [124, 125] which would correspond to a half-life of over 7hrs. However as G_{17} has such a short half-life, renal extraction and metabolism may only provide part of the mechanism and other organs may also be involved.

1.1.6.1.2 Hepatic

Due to its paracrine nature, circulating gastrin must pass through the liver before reaching target tissues. Several studies have investigated whether the liver is a major site for gastrin degradation with conflicting results. Some showed a 40% reduction in gastric acid output in Heidenhain pouches of dogs after a portal injection, compared with caval injection, of human synthetic gastrin [126]. Others found that hepatic inactivation of G_{17} was negligible in the portal and hepatic vein [127], whilst others have proposed that the liver extracts and

metabolises shorter gastrin fragments (<8 AA) with >90% inactivation of the C-terminal tetrapeptide of G_{17} (Trp-Met-Asp-Phe-NH_2) from the portal vein [128], where it is deamidated and secreted in the bile [129].

1.2 Gastrin receptor (CCK2R)

1.2.1 Classification and structure of cholecystokinin receptors (CCKR)

Amidated gastrins induce a biological response via their respective G-protein coupled receptor (GPCR), the cholecystokinin receptor (CCKR) [130] that is located on different target tissues. There are two types of CCK receptor (CCK1R and CCK2R) that have a high sequence homology (~50%) and are distinguished by structural peptide specificity for either CCK or gastrin (Figure 3). CCK1R requires the entire carboxyl-terminal heptapeptide-amide in its tyrosine sulphated form (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-PheNH_2) for high affinity binding, whereas CCK2R recognises the carboxyl-terminal tetrapeptide (Trp-Met-Asp-PheNH_2) present in both CCK and gastrin. Thus, CCK1R binds sulphated CCK with a 500-1000 fold greater affinity than gastrin or non-sulphated CCK, whereas CCK2R binds both peptides with almost equal affinity [131] and therefore, only CCK2R is termed a 'gastrin receptor'.

1.2.2 Tissue distribution of the CCK2R

The tissue distribution of the CCKRs and their ligand affinities have been shown to determine receptor activation and differences in downstream signalling. CCK1Rs are located in specific regions of the brainstem and gastrointestinal tract, as the major function of CCK is to stimulate pancreatic exocrine secretion [132]. However, CCK1R activation has also been associated with increased feeding-induced satiety [133] and intestinal lipids reduce gastric acid secretion and gastric emptying [134]. By contrast, CCK2Rs are widely distributed throughout the central nervous system [12, 135, 136] and particularly on gastric parietal and ECL cells [137] as the main function of gastrin is to increase gastric acid secretion in response to a meal [138]. As CCK and G_{17} bind with equal affinity for the CCK2R, the binding preference is determined by local concentrations of each hormone. G_{17} concentration is higher than CCK in the antral mucosa [139] and in the pituitary [140], whereas CCK predominates in the brain cortex [141].

1.2.3 CCK2R regulation

The gastrin/CCK2R is known to mediate several important biological functions within the stomach including acid secretion. However, less is known about the factors that regulate the receptor itself. The tight regulation of the CCK2R is essential for physiological homeostasis of gastrin/CCK2R signalling and CCK2R upregulation has been observed in many pathological conditions including colon [142] and stomach cancers [143]. The CCK2R has been shown to undergo rapid ligand-induced endocytosis, in response to both CCK and gastrin [144], in which agonist-bound receptors are selectively phosphorylated. Phosphorylation promotes arrestin binding which terminates the downstream signalling cascade and initiates clathrin-dependent and independent internalisation of the receptor [145, 146]. Recent studies have suggested that the C-terminal end of the CCK2R is a critical determinant for β-arrestin recruitment and contains three potential phosphorylation sites [147]. Internalised receptors are then sorted for different routes of trafficking such as recycling to the plasma membrane (both slow and rapid) and lysosomal degradation. Internalisation and trafficking rates differ between receptors as a consequence of different ligand binding or method of internalisation indicating distinct regulatory mechanisms [148, 149].

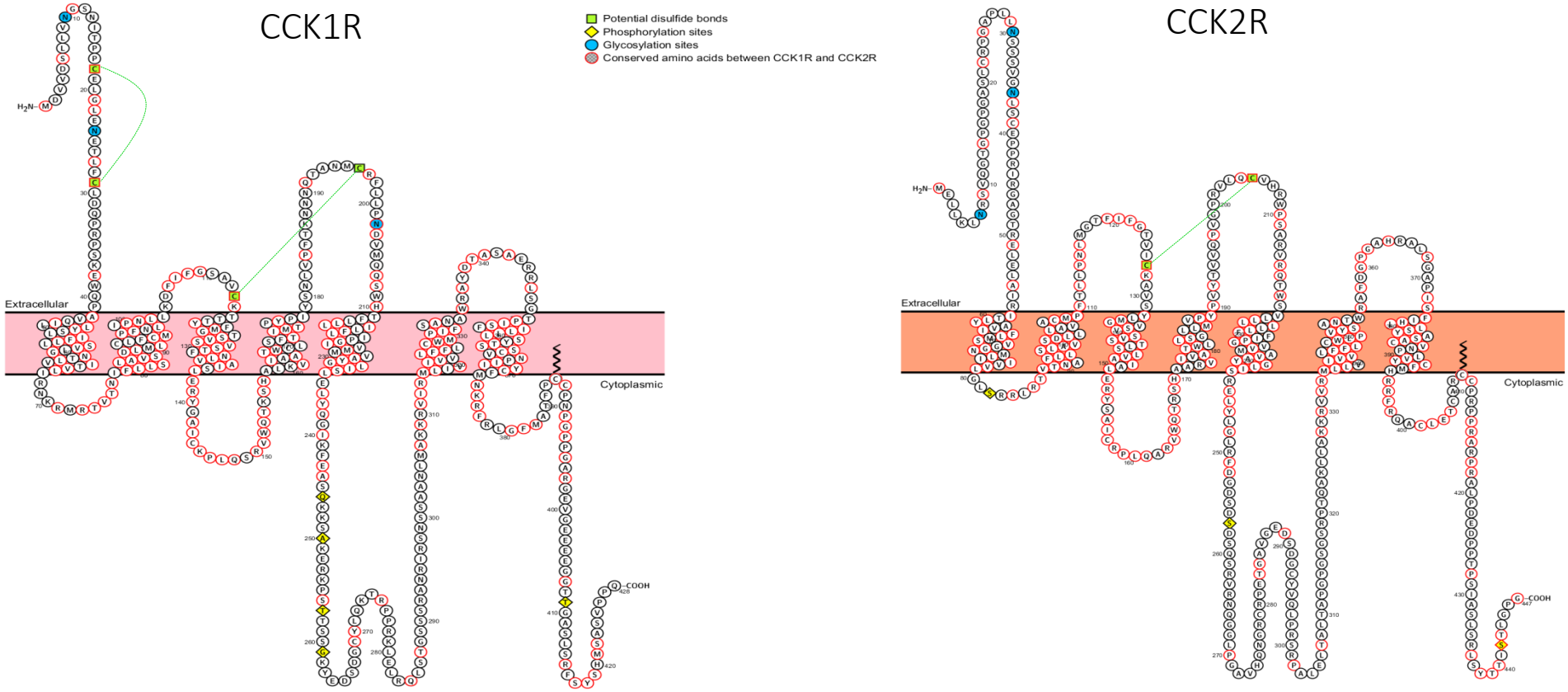

Figure 3. The deduced amino acid sequences of human CCK1 and CCK2 receptors were 428 and 447 amino acids respectively, with approximately 50% sequence homology. These sequences diverge predominantly at the amino-terminus and 3rd intracellular loop, with the greatest homology occurring within the transmembrane spanning domains. Both CCK1R and CCK2R have conserved disulphide bonds between cysteine residues on the 2nd and 3rd extracellular loop that is consistent with tertiary structure stabilisation. Diagram was produced using Protter [5] and adapted from Wank (1995) [12].

1.3 Cellular/physiological effects of amidated gastrin

1.3.1 Architecture of the stomach

The human stomach forms part of the digestive system and is divided into five separate compartments: cardia, fundus, corpus, antrum and pylorus; with occasional functional overlap (Figure 4A). The stomach is responsible for the physical churning of chyme (partially digested food) and secretion of digestive enzymes which catalyse the breakdown of food for nutritional absorption and can act as a barrier for ingested bacteria [150]. Like many organs, the gastric wall consists of multiple layers: the mucosa, submucosa and muscularis externa. The myenteric plexus separates the middle circular and outer longitudinal sections of the muscularis externa and is responsible for the innervation of peristalsis. Blood is supplied to the lesser curvature of the stomach from the aorta via the inferior and superior gastric artery. The greater curvature has a separate blood supply from the right and left branches of the gastroduodenal artery, which runs between the layers of greater omentum and converges with the splenic artery [151]. The blood vessels (and lymph vessels) penetrate through the majority of layers in the stomach, however, they are mostly found in the submucosa due to the close proximity to gastric glands for increased endocrine function (Figure 4B).

1.3.2 Architecture of gastric glands

The corpus mucosa is organised into a glandular structures, with openings to gastric luminal contents (gastric pits or foveolae gastricae). Gland structures differ depending on their location in the stomach and can have one or more branches with distinct cellular compositions (Figure 5). Glands of the corpus mucosa are highly organised structures containing multiple cells types which include parietal cells and endocrine cells; the latter is comprised of many different types. Histamine-secreting ECL cells account for 35% of endocrine cell populations in humans compared with 65% in rats [152, 153]. Progenitor stem cells located in the isthmus [154] migrate towards the surface or the base of the glands and

differentiate into either mucous-secreting cells or parietal, endocrine and chief cells, respectively.

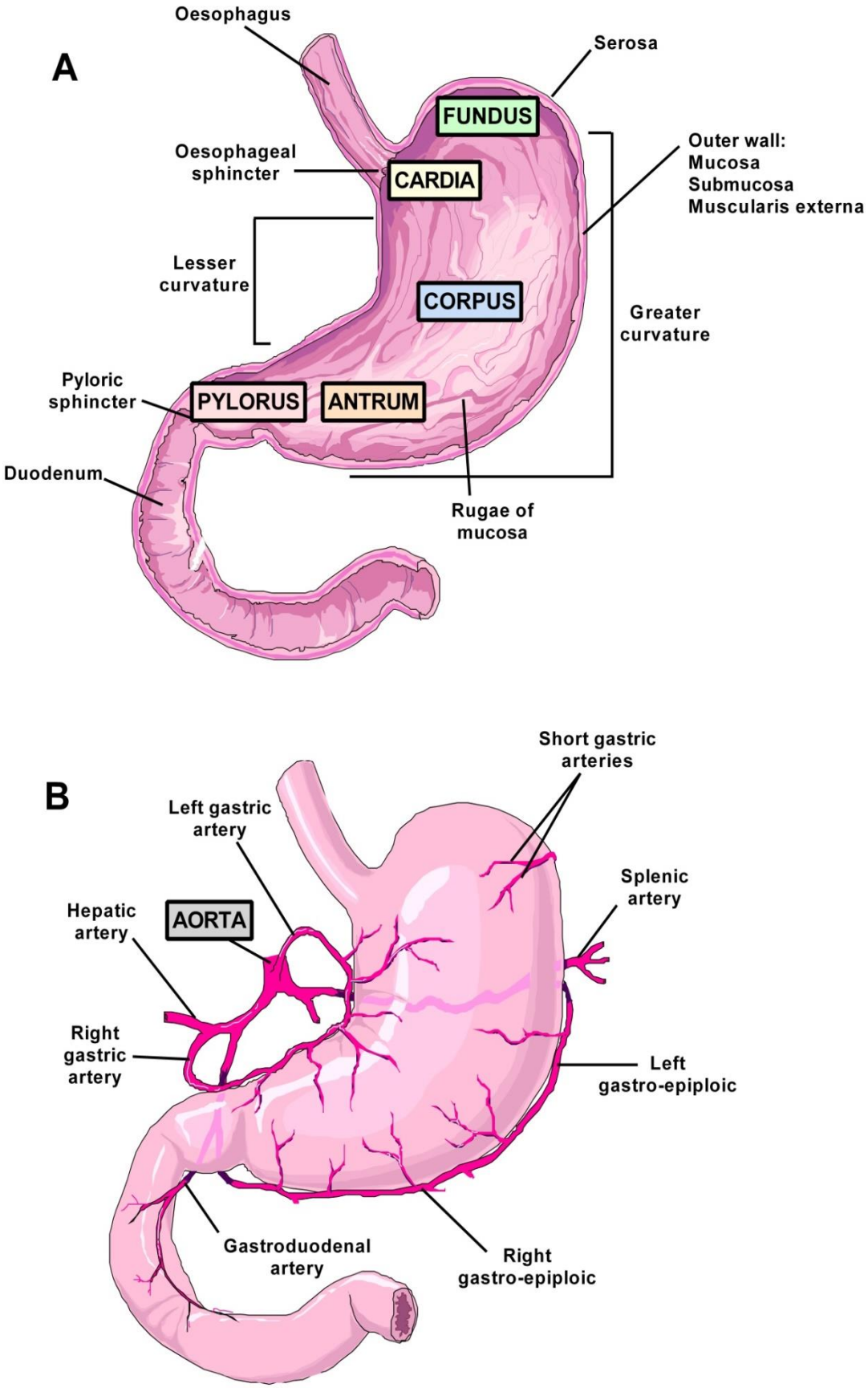

Figure 4. Architecture of the stomach which divides the anatomical regions into the fundus, cardia, corpus, antrum and pylorus (A) and the arterial bloody supply to these regions (B). Figure adapted from Schlossberg & Zuidema (1997) [2].

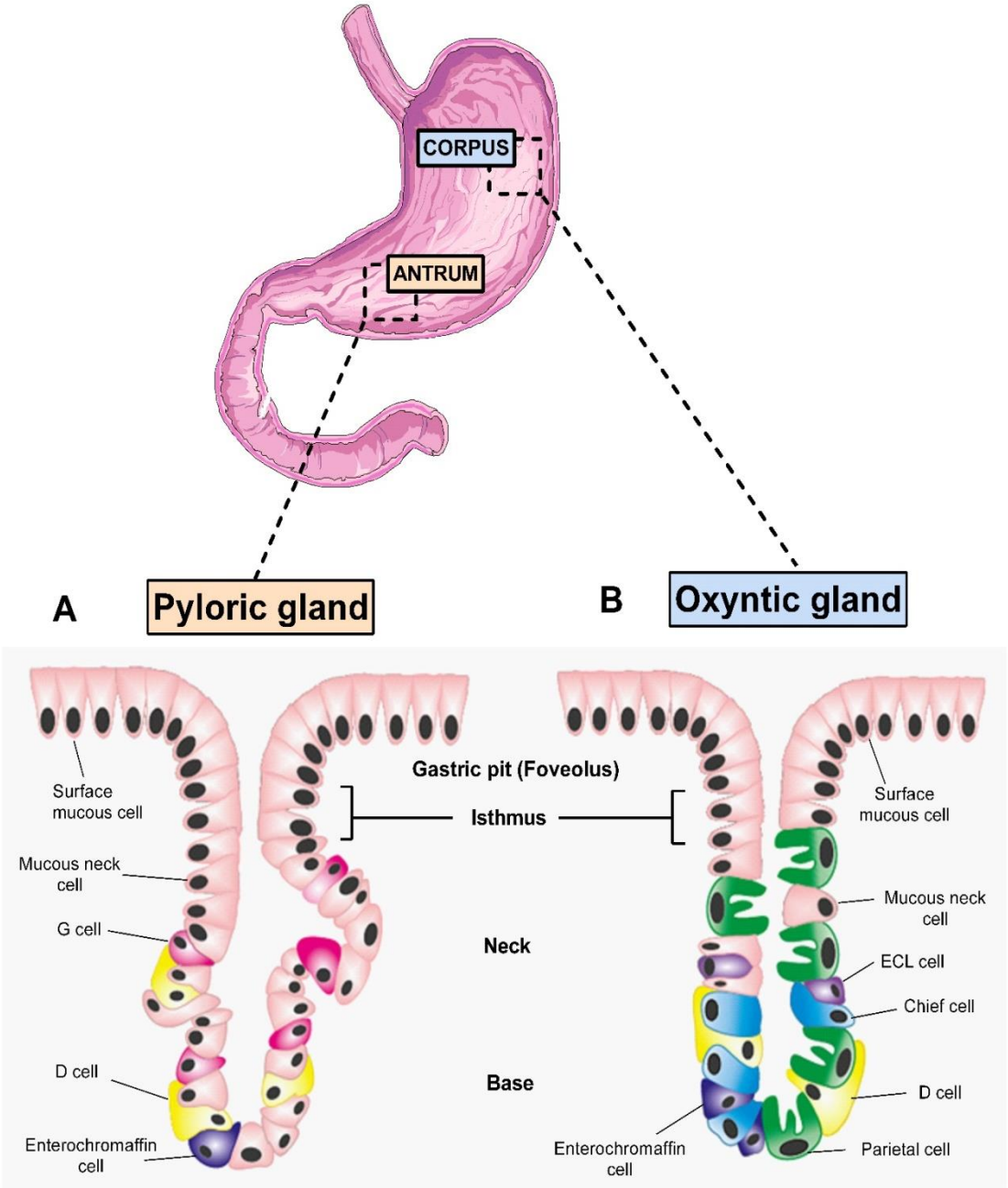

Figure 5. Architecture of a gastric gland located in the pylorus (A) or corpus (B) of the stomach, which contain a pit, progenitor region (isthmus), neck and base that are comprised of different cell types depending upon location. Figure adapted from Schubert (2008) [1].

1.3.3 Gastric acid secretion

Under normal physiological conditions, when digested food enters the stomach via the oesophagus, it distends the stomach walls activating mechanoreceptors (stretch receptors) which synapse on G cells to directly stimulate the release of gastrin releasing peptide (GRP) [109]. In addition to the distension, degraded proteins (particularly L-type amino acids) are potent stimulators of both direct and indirect gastrin and gastric acid secretion [97-99]. Amidated gastrin diffuses into the local capillaries and acts in a paracrine manner on the ECL cells, proliferating cells and chief cells of the corpus via the CCK2R. Once activated, ECL cells synthesise and release histamine which binds to the H_2 receptors on the parietal cells, combining with the signal from the gastrin-CCK2R complex, to secrete protons into the stomach lumen via the H^+/K^+ proton pump [155, 156]. The decrease in gastric luminal pH stimulates the release of somatostatin from D cells which inhibits gastrin and further gastric acid secretion and provides a negative regulatory mechanism [157] (Figure 6).

1.3.4 Cell proliferation

Biosynthetic products of the gastrin precursor, such as amidated gastrin and glycine-extended gastrins can stimulate cell growth within the gastric corpus mucosa [158, 159] and colon epithelium [160, 161] respectively, and several mechanisms of cell hypertrophy induced by amidated gastrin have been investigated. Self-replication has previously been observed in ECL cells obtained from the mouse mucosa, every 2 months [162] which suggests that hypergastrinaemia may directly stimulate MAPK mitogenic signalling via the CCK2R [163], to promote ECL cell hyperplasia and subsequent type-1 gastric NET formation [164]. In addition to direct ECL cell hyperplasia, gastrin-stimulates the release of growth factors such as histamine and transforming growth factor (TGF)-α (known mitogens), and the regenerating (Reg) protein [165] which is a potent regulator of pancreatic β-cell proliferation [166, 167]. Studies have suggested a positive correlation between *Reg* gene expression and gastric mucosal proliferation in rats [168] and gastric mucosal stem cells that do not conclusively express the

CCK2R, incubated with the Reg protein showed a potent and dose-dependent increase in proliferation [169]. These data indicate both a direct and indirect method of gastrin-stimulated proliferation of the corpus mucosa.

1.3.5 Differentiation and maturation of parietal cells

In the gastric mucosa of gastrin-deficient mice (GAS-KO), the development of the major cell types such as parietal cells, chief cells, mucous-secreting cells and endocrine cells, occur independent of gastrin secretion. However, gastrin is responsible for the functional maturation, particularly of parietal and ECL cells. Although immature parietal cells express proton pumps, which are a primary marker for the stage of differentiation of a cell, proton pump α and β subunit expressions are significantly decreased in GAS-KO mice. Other factors involved in acid secretion such as the KCNQ1 potassium channels, AQP4 water channels and creatine kinase B (a source of energy for the ATPase pump) are also significantly reduced in GAS-KO mice [170]. Reduced gastrin production in these transgenic models has been associated with reduced parietal cell mass, but not number [171] possibly due to the reduced histamine concentrations (40%) in ECL cells and subsequent histamine secretion. Basal and stimulated gastric acid secretion [138, 172] can be partially restored by gastrin osmotic infusion (1µl/hr for 1 week). These data suggest that gastrin plays an important role in the differentiation and maturation of the gastric mucosa through regulating parietal and ECL cell physiology.

1.3.6 Apoptosis

Concise programming and regulation of cell death is essential for tissue maintenance and dysregulation of apoptosis is often observed in malignancy. In AGS_{GR} cells treated with gastrin there was a 50-fold increase in plasminogen activator inhibitor-2 (PAI-2) expression which is a major component of the urokinase-type plasminogen activator (uPA) pathway and has been associated with increased apoptosis [173]. *In vivo* hypergastrinaemic mouse

models have also reported an increase in apoptosis in mice with elevated gastrin concentrations compared with normogastrinaemic controls, which is reversed upon treatment with a CCK2R antagonist [174-176]. Similarly, in human biopsies taken from patients with hypergastrinaemia or *Helicobacter pylori* (*H. pylori*) infection, increased apoptosis was observed [175] possibly indirectly via increased circulating PAI-2 concentrations [173, 177]. Alternatively, gastrin has shown to exert anti-apoptotic properties particularly in transformed cell lines such as AR42J, MKN-45 and AGS_{GR} due to CCK2R signalling via the Akt pathway, increased Bax and decreased BCL-2 expression and increased Mcl-1 expression, respectively [178-181]. Together, these data indicate that gastrin regulates apoptosis in gastric epithelial cells dependent upon physiological and pathological conditions via CCK2R activation however the specific mechanisms remain largely unclear.

1.3.7 Angiogenesis

The development and maintenance of new blood vessels (angiogenesis) is an essential physiological function within the stomach and gastrin has been shown to play an important role in the formation of vascular structures. In cell culture models of angiogenesis, human umbilical vein endothelial cells (HUVEC) seeded onto fibroblast monolayers formed tubule structures when exposed to both amidated gastrin-17 (G_{17}) and glycine-extended gastrin-17 (Gly-G_{17}) at optimal concentrations of 10nM. The angiogenic effect was modulated through the CCK2R, as administration of antiserum or CCK2R antagonists abolished the differentiating effects, EGFR signalling pathways that stimulate the release of heparin-binding epithelial growth factor (HB-EGF) which is involved in cell proliferation [182], cyclooxygenase-2 (Cox-2) which is a rate-limiting cofactor in the biosynthesis of prostaglandins [183] and matrix metalloproteinases (MMPs)-2, -3 and -9 from neighbouring fibroblasts which allow structural remodelling. The proangiogenic properties of gastrin have also been observed *in vivo* with increased microvessel density (MVD) in the normal mucosa surrounding colorectal adenocarcinomas in hypergastrinaemic mice [184].

1.3.8 Structural remodelling

The maintenance and structure of gastric epithelia has been shown to be regulated by gastrin through the increased expression of proteases such as uPA and its inhibitors PAI-1 and PAI-2 [185, 186]. Increased expression of MMPs cause a breakdown of the extracellular matrix and reduced structural integrity is frequently observed in gastric neoplasia. The majority of MMPs are commonly expressed in stromal cells, except for MMP7, however there have also been reports of MMP1, -2 and -9 expression within the parietal cells of the gastric mucosa [187]. Gastrin-stimulated AGS_{GR} cells have been shown to have increased MMP1 expression which is mediated by the PKC and MAPK pathways [188]. In patients with MEN-1, usually associated with hypergastrinaemia, and in AGS_{GR} cells there was an increase in MMP9 expression which stimulated epithelial cell invasion [189] which was also mediated by PKC-dependent and independent pathways. Similarly, MMP7 was shown to be upregulated in hypergastrinaemic patients (with and without *H. pylori* infection) via activation of GTPases, RhoA and Rac, which differentially stimulate the NFκB and AP-1 transcription factors to increase cellular migration and epithelial-mesenchymal signalling [190, 191]. Reciprocal interaction between the epithelia and stroma determine gastric mucosal organisation and dysregulation has been associated with the development of gastric tumours due to chronic inflammation or injury [192].

1.3.9 Actions of non-amidated gastrins

Non-amidated gastrins including progastrin, glycine extended gastrins and CTFPs contain the less predominantly expressed forms of gastrin that were originally thought of as being biologically irrelevant. These isoforms are usually expressed in cells lacking the appropriate processing machinery. For example CPE-deficient$^{fat/fat}$ mice showed a slightly reduced expression of amidated gastrin and increased accumulation of progastrin intermediates such as glycine-arginine extended gastrins (86-fold) and glycine-extended gastrins (7-fold) [193]. Progastrin and its intermediates (Gly-G_{17} and CTFP) have been shown to exert trophic

effects on the colonic and gastric epithelia and to be involved in regulating physiological processes. Progastrin, Gly-G_{17} and CTFP are pro-proliferative and anti-apoptotic in both gastric and colonic cancer cell lines. Progastrin and its intermediates Gly-G_{17} and CTFP have been shown to independently promote cell growth in colon-derived cell lines *in vitro* [160, 194] and in the colonic mucosa of mice overexpressing either human progastrin or Gly-G_{17} *in vivo* [37, 161, 195-198], in an autocrine manner. Whilst in AGS cells progastrin, Gly-G_{17} and CTFP have been shown to activate the PI3K pathway to reduce caspase-3 activity and decrease apoptosis [199]; thus increasing the susceptibility for carcinogenesis. However, CTFP has also been shown to promote gastric apoptosis in the normal mucosa of GAS-KO mice [200].

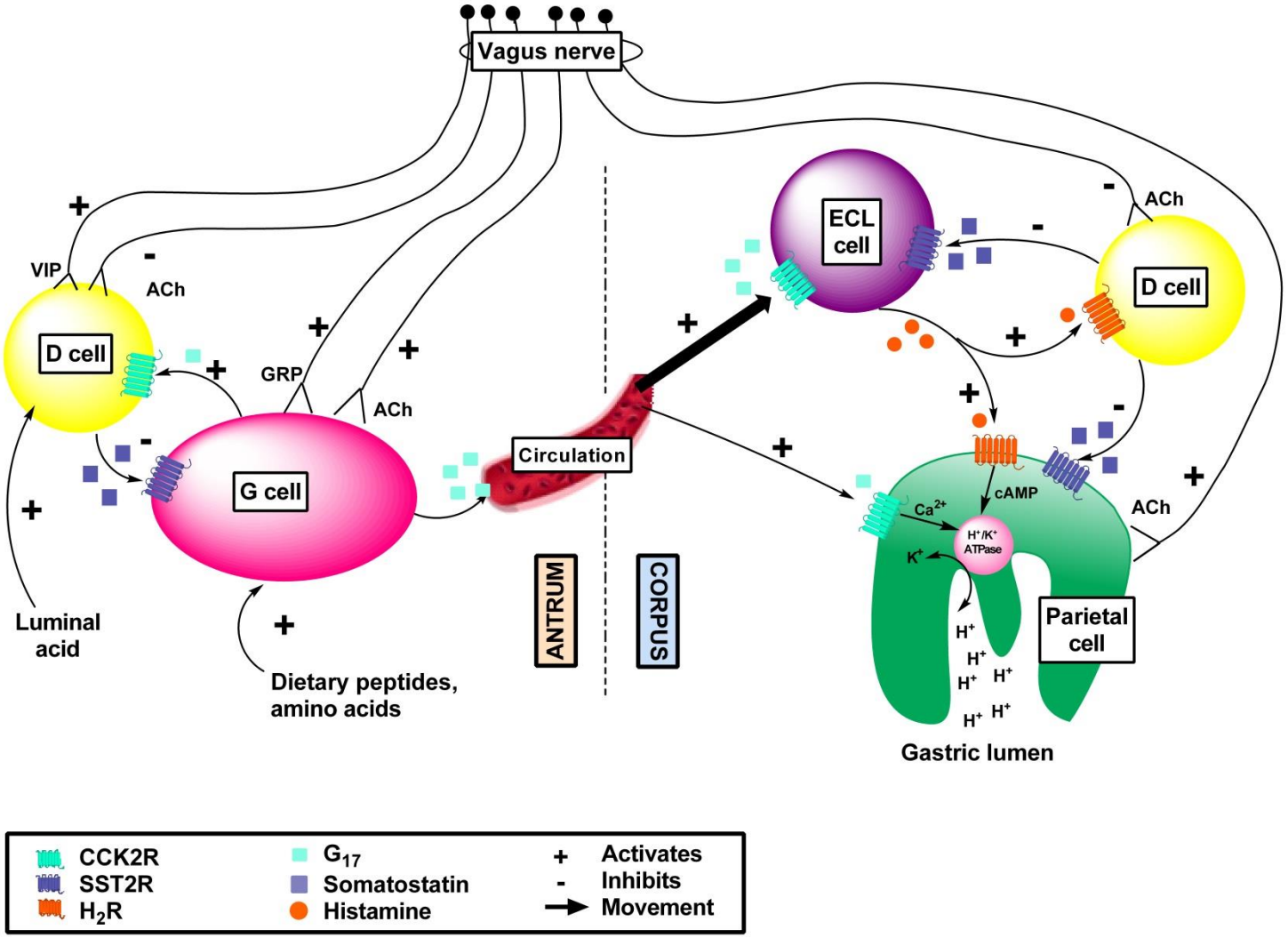

Figure 6. Paracrine, endocrine and neuronal stimulation of gastric acid secretion. Gastrin is secreted from antral G cells in response to dietary peptides and neuronal stimulation which enters the circulation and activates CCK2Rs located in the gastric corpus. Direct CCK2R activation on parietal cells increases intracellular calcium mobilisation and stimulates proton pump (H^+/K^+ ATPase) secretions. CCK2R activation on ECL cells causes the release of histamine and paracrine stimulation of histamine type-2 receptors (H_2Rs) of the parietal cells which also stimulate proton pump secretions. Increased luminal gastric acid and histamine stimulates the release of somatostatin from D cells which binds to somatostatin receptors (SST2R) located on antral G cells and parietal cells which inhibit gastrin and proton pump secretions respectively. Neuronal innervation has been shown to exert both inhibitory and stimulatory effects on D cells, G cells and parietal cells. Figure adapted from Schubert 2008 [1] and Schubert 2015 [9].

1.4 Hypergastrinaemia

Hypergastrinaemia literally means excessive gastrin in the circulation however, most publications classify it as a fasting serum gastrin concentration of >100pg/ml or >48pM. Hypergastrinaemia is associated with the development of many conditions including gastric cancer and has many causes, which are currently characterised depending upon the pH within the gastric lumen.

1.4.1 Causes

1.4.1.1 Hypergastrinaemia associated with normal or hyperacidity

1.4.1.1.1 Zollinger-Ellison syndrome (ZES)

Elevated gastrin concentrations associated with low gastric luminal pH (≤2) are commonly caused by hormone-producing neuroendocrine tumours (gastrinomas) of the pancreas and/or duodenum. The first clinical report of gastrinomas was by Zollinger and Ellison in 1955, who observed recurrent peptic ulceration, gastric acid hypersecretion and less commonly diarrhoea and steatorrhoea in patients with non-specific pancreatic islet cell tumours [201]. This led to the term Zollinger-Ellison syndrome (ZES). However, studies by Roy *et al*. reported only 1 patient out of 1219 (235 National Institute of Health (NIH) patients and 984 cases in the literature) with ZES that exhibited basal hyperacidity [202].

1.4.1.1.2 Antral gastrin cell hyperplasia (AGCH)

A rare cause of hypergastrinaemia is antral gastrin cell hyperplasia (AGCH) that causes an increased serum gastrin response to a standard meal test, which differentiates these cases from gastrinomas [203]. Antral-predominant *H. pylori* infection is present in 50% of AGCH cases and may contribute to increased antral inflammation which overstimulates G cell function, resulting in excessive gastrin production and hyperacidity [204]. AGCH is reversed with surgical antrectomy resulting in a minimal fasting serum gastrin concentration

(<50pg/ml) and 92% reduction in gastric acid secretion [205, 206], however cases due to *H. pylori* infection should now be treated by eradicating this organism.

1.4.1.1.3 Renal failure and uraemia

Although the method of clearance for gastrin is still currently unclear, some studies have suggested that gastrin may be metabolised by the kidneys and liver *in vivo* [120] and by the lung, skeletal muscle and gastric mucosa *in vitro* [207-209]. However, renal excretion of gastrin is thought to be the most efficient method as hypergastrinaemia is frequently observed in patients who have acute or chronic renal failure [122, 123, 210]. In patients with renal failure, gastric ulceration and gastrointestinal bleeding has frequently been reported [211, 212], suggesting that the gastrointestinal bleeding and ulcer formation caused by gastric acid hypersecretion may be secondary to hypergastrinaemia due to the reduced gastrin clearance in renal failure patients [213].

1.4.1.1.4 Post-gastric resection with intact antrum, particularly Billroth II partial gastrectomy

Inadequate removal of all antral tissue during partial gastrectomy or gastrojejunostomy (Billroth II), has been shown to result in recurrent ulcer formation [214]. Although this outcome is usually uncommon (0.5-6%), one possibility could be due to hypergastrinaemia as a result of continued parietal cell activity and a large study conducted in patients with recurrent peptic ulcer disease found that recurrence was caused by a retained gastric antrum in only 4% of patients [215]. However, 2 cases reported by Webster *et al.* support the hypothesis that a retained gastric antrum may be a source of hypergastrinaemia [216].

1.4.1.2 Hypergastrinaemia associated with hypoacidity (elevated gastric luminal pH >2)

1.4.1.2.1 Chronic atrophic gastritis (CAG)

Elevated gastrin concentrations associated with increased gastric luminal pH (>2) are more frequent than hyperacidic conditions, with chronic atrophic gastritis (CAG) being observed in <40% of the worlds' general population [217]. Atrophic gastritis is a premalignant condition that, in some cases, can progress to the development of gastric carcinoma and develops from chronic gastritis, which involves long-term inflammation of the antrum and/or corpus mucosa.

Autoimmune conditions or chronic *H. pylori* infection (discussed in section **1.8 *Helicobacter pylori* (*H. pylori*)**) are the main causes of CAG, types A and B respectively, by initiating the progressive destruction of the gastric mucosa. Absent or reduced parietal cell mass creates an achlorhydric stomach with optimal conditions for prolonged bacterial colonisation and consequential hypergastrinaemia being associated with premalignant disease. Although *H. pylori* usually remain in the gastric lumen, bacterial-cell interactions evoke an inflammatory response which over time can cause CAG. Without successful eradication of this bacterium, prolonged exposure may lead to epithelial cell damage, possibly due to the production of reactive oxygen species, loss of parietal and chief cells and glandular atrophy [218]. Studies by Kuipers *et al*. highlighted the negative impact that *H. pylori* infection has on healthy gastric mucosa as 36% of *H. pylori* positive patients that were initially CAG negative, developed the condition. Of these patients, 38% showed histological evidence of intestinal metaplasia and 6% developed an early onset of gastric cancer [219].

On the other hand, autoimmune CAG is a disorder that selectively attacks acid secreting parietal cells and causes deficiency of intrinsic factor and in the more advanced stages leads to severe vitamin B_{12} deficiency (characteristic of pernicious anaemia). Previous

epidemiological studies have suggested that similar to other autoimmune diseases, autoimmune CAG is more prevalent in the elderly, in females more than males [220], and can be detected by the presence of anti-gastric intrinsic factor and parietal cell cytoplasmic antibodies (PCA) in the serum [221, 222].

1.4.1.2.2 Post-vagotomy

Since peptic ulcer disease can arise as a result of corrosion of the gastric mucosa by acid, several methods were employed to either reduce gastric acid output or intragastric acidity including surgical intervention. Of the patients that received truncal vagotomy and pyloroplasty surgery, the mean basal serum gastrin concentration increased and mean basal gastric acid secretion decreased in both early postoperative studies and longer term studies (<4 years post-surgery) [223]. Our improved understandings of the mechanisms that regulate gastric acid secretion have identified the vagus nerve as a minor contributing factor for gastrin and subsequent gastric acidity. Therefore, the removal or partial removal of the vagus nerve may induce hypergastrinaemia as a result of reduced gastric acid output [224-226].

1.4.1.3 Hypergastrinaemia associated with inhibitors of gastric acid secretion

1.4.1.3.1 Histamine type-2 receptor antagonists (H_2RAs)

Histamine is a biologically active amine that binds to four distinct receptor subtypes (H_{1-4}) located throughout various organs including the gut, lungs, heart and central nervous system, with the resulting agonism/antagonism depending upon tissue location [227-230]. The dual signalling mechanism produced by H_2Rs and their role in cell proliferation and differentiation provide additional targets for therapeutic intervention, particularly for the prevention and treatment of gastric carcinogenesis.

H_2RAs reversibly compete with histamine for binding to H_2Rs on the basolateral surface of parietal cells and suppress both basal and stimulated acid secretion by >70% [231]. Despite

having no observed renal or haematological adverse effects in rats or dogs [232] and healthy subjects [233], H_2RAs have been associated with hypergastrinaemia after both short [234] and long term [235, 236] treatments. Whilst short term studies in humans observed an absence of gastric acid hypersecretion suggesting that parietal cell mass was unaffected [237, 238], histological analysis of experimental animal models receiving short and prolonged cimetidine treatment showed parietal cell hyperplasia [239, 240]. However, these discrepancies may be due to the large doses used in these animal models which would be beyond the therapeutic range in man.

1.4.1.3.2 Proton pump inhibitors (H^+/K^+ ATPase inhibitors)

The development of proton pump inhibitors (PPIs), most notably omeprazole, provided a novel approach to the treatment of acid-relation conditions by blocking the final step in gastric acid secretion. Direct and irreversible inhibition of the H^+/K^+ ATPase pump embedded in the apical membrane of parietal cells blocks the exchange of protons against a high concentration gradient [241] and reduces acidification of the stomach. PPIs quickly surpassed the use of H_2RAs due to an increase in the duration of action, potency, efficacy and low adverse effects. However, achlorhydric conditions of the stomach stimulate further gastrin secretion and the risk of potential hypergastrinaemia remains controversial.

Short term studies in healthy patients and those with duodenal ulcer or ZES taking a single daily dose of 20-30mg omeprazole observed virtually complete elimination of gastric acidity after 6hrs and <70% reduction in stimulated gastric acid secretion after 24hrs which then stabilised. An increase in both resting and stimulated gastrin secretion was also observed secondary to the reduced intragastric acidity, which returned to basal levels approximately 14 days after cessation of treatment [242]. Longer term studies have shown that omeprazole acutely increases serum gastrin concentrations which peak at 2-4 months following the start of treatment and remain relatively stable thereafter (<5 years) [243], with

a greater increase in gastrin concentrations being observed than with H_2RAs due to increased drug efficacy [244]. Although serum gastrin levels were elevated, most patients were not classified as hypergastrinaemic because the concentrations were within the normal range at <100pg/ml. Although omeprazole (and other subsequently developed PPIs) have been shown to increase serum gastrin concentrations [245] there is still conflicting evidence about whether the hypergastrinaemia that is associated with long term PPI treatment has any pathological consequences in man.

1.4.1.4 Other causes of hypergastrinaemia

1.4.1.4.1 Pernicious anaemia (PA)

Destruction of the oxyntic mucosa, in particular parietal cells, reduces chlorhydric acid and intrinsic factor secretion [246]. Intrinsic factor transports dietary vitamin B_{12} (cobalamin) to the terminal ileum for absorption [247] and vitamin B_{12} deficiency has been shown to induce a macrocytic anaemia known as pernicious anaemia (PA) (previously known as Biermer's disease [248] and Addisonian anaemia [249]). PA is currently considered an autoimmune condition due to the presence of intrinsic factor and parietal cell autoantibodies however PA is also the end-stage of autoimmune CAG [250].

Some data have suggested the role of *H. pylori*-induced CAG in the pathogenesis of PA due to pathogen cross-activation of T or B cells [251]. Pathogen-derived antigens have also been shown to evoke the host's immune responses in rheumatoid arthritis which has been strongly associated with CAG [252]. The gastric proton pump has previously been identified as an autoantigen in autoimmune CAG [253-255] and increased numbers of activated gastric $CD4^+$ T cells (which recognise both gastric proton pump and *H. pylori* antigens) have also been observed [256, 257]. However due to the low incidence of *H. pylori* seropositivity in patients with autoimmune CAG and PA [258, 259], this remains unclear.

1.4.1.4.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune condition that is characterised by inflammation of the synovial membranes, synovial hyperplasia and progressive destruction of affected joints. CAG has been previously observed in 62.5% of biopsies taken from patients with RA [252], which accounts for the high number of patients (<30%) that present with hypo- or achlorhydria. However, increased CAG has also been strongly associated with age and is commonly present in Sjögren's syndrome, which is often associated with RA [260] and gastrin concentrations in RA have been variable with some studies recording levels as high as 1000pg/ml [261] whilst others have found no significant differences in comparison to healthy controls [262].

1.4.1.4.3 Human immunodeficiency virus (HIV)/ Acquired immune deficiency syndrome (AIDS)

There have been some reports that infection with the human immunodeficiency virus (HIV) may cause acid hyposecretion and subsequent hypergastrinaemia [263, 264], however the mechanism for acquired immune deficiency syndrome (AIDS)-related gastric disorders is complicated and still remains unclear. Some have suggested that increased parietal cell autoantibodies in immunocompromised patients may facilitate the development of atrophic gastritis [263], a known prerequisite for hypergastrinaemia. However this study provided little information about *H. pylori* or HIV positivity statuses. Other studies have reported conflicting results with only 1.6% of HIV-positive subjects analysed having increased serum parietal cell autoantibodies [265], suggesting a limited role for this mechanism in the development of gastric atrophy.

1.4.2 Diseases associated with hypergastrinaemia

1.4.2.1 Barrett's oesophagus

Barrett's oesophagus is a premalignant disease that is a strong risk factor (125-fold increase) for the development of oesophageal adenocarcinoma [266, 267]. Patients with Barrett's oesophagus show metaplastic columnar epithelial cells in place of normal stratified squamous epithelial cells [268] as a result of chronic gastro-oesophageal reflux disease (GORD) [269, 270]. Therefore, the majority of patients are prescribed acid suppressive therapies such as PPIs or H_2RAs [271] which contribute to increased gastrin secretion [272] and may promote tumour formation [273, 274]. Gastrin binds to CCK2Rs, which are highly abundant in the mucosa of Barrett's oesophagus, to induce the expression of EGF and trefoil factor family (TFF) peptides. The secondary signalling cascades activated can contribute to carcinogenesis via increased Cox-2 expression with further inflammatory responses and reduced apoptosis, and CCK2R activation has been shown to induce hyper-proliferation in oesophageal cancer cell lines [275-277].

1.4.2.2 Cancer

1.4.2.2.1 Gastric adenocarcinoma

Gastric adenocarcinoma development and histological progression follows a specific pathway from normal mucosa to tumour formation through gastric atrophy, metaplasia and dysplasia and although many factors contribute to the predisposition for carcinogenesis it is strongly associated with chronic *H. pylori* infection [278]. During *H. pylori*-induced CAG there is a loss of parietal cell mass which subsequently reduces secretory signals that may control cell growth and differentiation. For example, the sonic hedgehog protein (SHH) is expressed in human and animal parietal cells [279] and physiologically regulates epithelial cell growth and function [280-282] and immunoregulation [283-285]. Patients with CAG have decreased SHH expression and the development of gastric metaplasia has been observed in SSH-deficient animal models [286]. These data indicate that a combination

of chronic inflammation, primarily caused by *H. pylori* infection, increases the production of cytokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS) which damage DNA. These oxidant products in conjunction with the loss of parietal cell regulatory mechanisms are necessary for progression towards gastric tumour formation [287].

Metaplastic cell lineages are progenitors for gastric metaplasia of which there are two types associated with gastric adenocarcinoma: intestinal metaplasia and spasmolytic-polypeptide expressing metaplasia (SPEM). Intestinal metaplasia is the most recognised pre-neoplastic type of metaplasia and is characterised by the conversion of the normal mucosa to intestinal-type goblet cells [288]. However, CAG can also promote the development SPEM which may originate from trans-differentiation of mature chief cells [289] and is characterised by the development of an antral phenotype in the corpus, which expresses TFF-2 and muc-6 as physiological responses to injury [290]. Both types of metaplasia are strongly associated with increased phenotypic and genotypic alterations observed in gastric adenocarcinoma [290-294] and *in vivo* studies have indicated that SPEM may be a precursor for intestinal metaplasia. Mongolian gerbils infected with *H. pylori* have been shown to develop SPEM after 3 weeks in association with CAG, followed by intestinal metaplasia after 24 and 39 weeks from areas of pre-existing SPEM, as both SPEM and intestinal metaplastic lineages were observed in a single gland [295]. Similar histological progression of intestinal metaplasia has also been observed in humans [296].

Unlike metaplasia which may be reversed by *H. pylori* eradication [297, 298], dysplasia is typically irreversible with hyper-proliferative and migratory properties and is strongly associated with the progression to gastric adenocarcinoma [299]. The morphology of dysplastic tissues displays a breakdown of the glandular structure with increased proliferation and evidence of stromal invasion that arise from metaplastic areas of the

gastric mucosa [300]. Malignant transformation is a gradual process with morphological alterations continually occurring throughout the progression to carcinomatous tissue [292].

1.4.2.2.2 Gastric neuroendocrine tumours (NETs) types-1 and -2

Gastric carcinoids (neuroendocrine tumours, NETs) are rare tumours that originate from ECL cells in the oxyntic mucosa of the stomach [301-303] and are classified as three separate types (with an extremely rare fourth type) on the basis of pathological and morphological characteristics [304-306]. Despite being rare, diagnoses of gastric NETs have become more common over the past 50 years as a consequence of increased endoscopic surveillance and improved pathological analysis [307].

1.4.2.2.2.1 Type-1 gastric NETs

Type-1 gastric NETs are the most common form (70-80%) and are associated with elevated serum gastrin concentrations. Previous studies have suggested that the associated pathological changes that occur in the stomach, may be, in part, due to the hypergastrinaemia which develops as a consequence of autoimmune CAG and achlorhydria [302]. Type-1 gastric NETs are typically small (0.5-1cm diameter), well-differentiated, multifocal tumours that are localised within the corpus mucosa and submucosa. In autoimmune CAG there is a loss of parietal cell mass which results in gastric acid hyposecretion with achlorhydria of the gastric lumen and decreased production of intrinsic factor (characteristic of pernicious anaemia). The lack of gastric acid in the stomach continually stimulates the release of gastrin from antral G cells and results in chronic hypergastrinaemia which promotes gastric mucosal ECL cell hyperplasia and contributes to tumourigenesis [308].

1.4.2.2.2.2 Type-2 gastric NETs

Type-2 gastric NETs are relatively rare (5-6%) tumours that are histologically similar to type-1 gastric NETs (small, multiple and polypoid) and can arise in association with gastrin-

secreting tumours in Zollinger Ellison Syndrome and MEN-1 syndrome [309]. MEN-1 syndrome is usually caused by an inherited autosomal dominant menin gene mutation (*MEN1*), although somatic mutations of the *MEN1* gene have frequently been observed in sporadic ZES cases that occur as a single tumour with no symptoms of MEN-1 [310]. Menin is a nuclear protein of dichotomous function as it can both positively and negatively regulate gene expression. In the majority of MEN-1 syndromes, the inherited *MEN1* gene mutation causes a loss of heterozygosity (LOH), thus highlighting menin as a putative tumour suppressor protein within endocrine tissues [311, 312]. In heterozygous *MEN1* mutant and *MEN1* knockout mice, phenotypic alterations mimic the MEN-1 syndrome observed in humans [313, 314] and complete deletion is fatal at E.11.5-13.5 in mouse embryos [315].

Hypergastrinaemia has been shown to increase ECL cell hyperplasia as 30% of patients with autoimmune CAG or PA showed increased ECL cell proliferation, whilst only 7% developed gastric NETs [316, 317]. However, gastric NETs are more frequently observed in patients with ZES associated with MEN-1 syndrome. In cases of ZES associated with MEN-1 but without gastrinoma, there have been reports of reduced gastric NET formation and in patients with ZES without associated MEN-1, gastric NETs were rare. These data suggest that hypergastrinaemia is sufficient for ECL cell hyper-proliferation but requires additional cofactors such as the MEN-1 tumour suppressor gene mutation, for the conversion of hyperplasia to neoplasia [318, 319].

1.4.2.2.3 Gastric mucosa-associated lymphoid tissue (MALT) lymphomas

Gastric mucosa-associated lymphoid tissue (MALT) lymphomas (now known as extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue) are the most common form of gastric lymphoma (70%) and are usually confined to the antrum and distal corpus of the stomach until an advanced stage of disease [320]. Gastric MALT lymphomas are strongly associated with *H. pylori*-induced inflammation (72-98%) [321],

increased gastrin and CCK2R expression [322] and gastric atrophy [323]. Low-grade histological features closely mimic the *Peyer's patch* (B-cell component) observed in MALT and lymphoepithelial lesions have been frequently observed as lymphoma infiltrates spread into the mucosa surrounding the Peyer's patch marginal zone [324, 325]. Low grade tumours have been associated with better prognoses as localised therapy is often curative due to the relatively confined nature of the tumour and significant regression has been observed in many cases after *H. pylori* eradication [326, 327]. In comparison high-grade tumours develop though increased numbers of transformed blast cells that form large sheets or clusters which induce glandular destruction [328, 329] and this has been shown to reduce the 10-year survival rate by 48% [330]. Gene mutations in the NFκB signalling pathway have been implicated in the malignant transformation to high-grade tumours. However there is increasing evidence to suggest that oncogenesis may only occur when genetic aberrations are in combination with immunological stimulation (primarily caused by chronic *H. pylori* infection) [331].

1.4.2.2.4 Pancreatic cancer

Pancreatic cancer is one of the leading causes of cancer-related deaths with less than 1% survival rate (>10 years)[332]. The poor prognosis may be attributed to high levels of proliferation, metastasis and invasion resulting in advanced stage diagnoses. Exogenous gastrin has been shown to increase pancreatic cell growth in some studies [333, 334], but not in others [335] and the mechanism of the trophic effects of gastrin is currently unclear as the CCK2R is only abundantly expressed in drug-induced pancreatic cancer in rodents [336, 337]. However, gastrin has been shown to increase proliferation in pancreatic cancer cells *in vitro* [338, 339] and in xenografted tumours *in vivo* [340]. Studies have indicated that gastrin-induced cell growth may occur in an autocrine manner as primary pancreatic cancer cells have been shown to express the CCK2R and synthesise gastrin [341-343]. *H. pylori* seropositivity, particularly with cytotoxin-associated gene A (*cagA*) positive strains, is a

major cause of hypergastrinaemia and has also been shown to double the risk of pancreatic cancer in humans [344, 345], indicating the potential role of gastrin in pancreatic carcinogenesis.

1.4.2.2.5 Colon cancer

Normal colonic epithelial cells have previously been shown to generally lack the CCK2R and the processing machinery required for the generation of amidated gastrins [346]. However, studies have confirmed that non-amidated gastrins induce local epithelial cell proliferation in colon cancer [347, 348], in an autocrine and paracrine manner [349, 350]. Transgenic hGAS mice (which overexpress human Pro-G) have been shown to have increased colonic proliferation and an increased susceptibility to colon carcinogenesis following DNA damage [198, 351, 352] or *p53* gene mutation [195]. Previous studies have also reported an increase in Pro-G, but not G_{17} or Gly-G, in colorectal cancer patients [353, 354].

Non-amidated gastrins (Pro-G and CTFP) have been shown to possess anti-apoptotic properties due to the activation of the NFκB and Wnt signalling pathways [355, 356]. High levels of Pro-G in primary colorectal cancer cells have also been strongly associated with increased tumour cell growth as larger tumours have been observed in $CD133^{high}/CD44^{high}/Pro\text{-}G^{high}$ cells compared with $CD133^{low}/CD44^{low}/Pro\text{-}G^{low}$ cells in severe chronic immunodeficiency (SCID) mice [357]. In $APC^{Min/+}$ mice, a model of human familial adenomatous polyposis (FAP) which is a rare autosomal-dominant colorectal cancer condition [358, 359], hypergastrinaemia alone has been shown to increase colonic proliferation and malignant potential but was not sufficient to cause carcinogenesis [360]. In addition, hypergastrinaemic patients with ZES, PA or taking acid-suppressive drugs such as PPIs have not previously been shown to have an increased susceptibility to colon carcinogenesis [361, 362], suggesting a limited role for hypergastrinaemia during colon tumourigenesis.

1.5 Mouse models used for the study of gastrin and gastric carcinogenesis

The development of translational animal models is important in order to fully understand the molecular mechanisms involved in human gastric carcinogenesis, for preventative and therapeutic potential. Currently, limitations have occurred due to interspecies variability as anatomies differ between animals and humans. The human and mouse genome both contain ~30,000 protein coding genes [363] and therefore morphological and physiological differences are possibly due to alterations in both coding and non-coding sequences [364], duplicated sequences or gene deletions [365]. Some morphological differences include the gastric fundus lining which contains squamous cells in mice rather than oxyntic mucosa like in humans and rodents rarely develop spontaneous gastric carcinogenesis, with the exception of cotton rats (*Sigmodon hispidus*) [366-368] and some strains of the common African rat (*Mastomys natalensis*) [369, 370]. Therefore the induction of gastric cancer through chemical, bacterial or genetic methods has been explored.

1.5.1 Chemical models

Early studies suggested that aetiological factors such as high dietary salt and nitrate/nitrite intake may contribute to gastric carcinogenesis and therefore, early animal models utilised nitrosamines such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in rats [371, 372] or N-methyl-N-nitrosourea (MNU) in mice [373] in order to induce gastric tumours.

1.5.2 Bacterial infection models

Since the strong relationship between *H. pylori* and gastric cancer was discovered [374] and the bacterium was classified as a type-1 carcinogen in 1994 [375], many different animal models have used *H. pylori*-associated bacterial infection to induce gastric tumours. Due to the successful manipulation of the mouse genome and relatively inexpensive housing costs, mouse models have been the species of choice for many studies. However, there are

a limited number of *Helicobacter* bacterial strains that can successfully colonise the mouse stomach and although successful colonisation of *H. pylori* has been reported in mice [376-378], infection with *Helicobacter felis* (*H. felis*) has been more extensively characterised [379-382].

The isolation and culture of *H. felis*, a natural inhabitant of the gastric mucosa of adult cats [383], allowed the first detailed investigations into bacterial colonisation of the gastric mucosa in small animal models. Although *H. felis* lacks *cagA* and vacuolating cytotoxin A (*vacA*) (the virulence factors associated with increased pathology in humans), chronic infection has shown to induce the progression from severe chronic gastritis to gastric cancer in germ-free mice [384-386], similar to the gastric trophism observed in *H. pylori* infected humans. Lee *et al.* also successfully adapted a strain of *H. pylori* (Sydney strain SS1) that is *cagA* and *vacA* positive with an increased ability for successful colonisation in mice, particularly in C57BL/6 mice but also at lower rates in BALB/c, DBA/2 and C3H/He mice, to provide a standardised model for *H. pylori*-associated disease [387].

1.5.3 Genetic models of gastric cancer

Transgenic animal models have significantly enhanced the understanding of many gastric conditions and the role that gastrin plays during carcinogenesis. Genetic manipulation of the mouse genome in early studies involved oncogenes such as the simian virus 40 large tumour antigens (SV40 Tag) [388], which controls viral infection and alters many biological processes [389]. Stomach-specific SV40 Tag transgenic models using the H^+/K^+ ATPase β-subunit promoter at nucleotides -1035 to +24 showed decreased maturation of parietal cells with subsequent hypochlorhydria [390]. However, these models did not follow the classical pathway of healthy mucosa to gastric cancer via atrophy, metaplasia and dysplasia, and were not associated with CAG or *H. pylori* infection as seen in hypergastrinaemic conditions. Therefore, various transgenic mouse models have been developed to assess the role of gastrin in gastric carcinogenesis.

1.5.3.1 Transgenic mice overexpressing amidated gastrin (INS-GAS)

Pancreatic islet cells produce enzymes such as PCs which cleave progastrin into biologically active forms including amidated gastrins. Therefore, transgenic mice on the genetic background FVB/N which are homozygous for the INS1-GAS transgene, coding for the rat insulin-1 promoter (a gastrin regulator), express the human gastrin gene in pancreatic islet cells and produce an excess of human gastrin [391]. This overexpression of gastrin causes mild hypergastrinaemia, increased parietal cell number and subsequent elevated gastric acid secretion by 12 weeks of age. After 12 weeks, morphological changes are seen within the gastric epithelium including atrophic gastritis and intestinal metaplasia. End-stage disease was found to occur after 20 months in this colony, characterised by invasive gastric adenocarcinoma formation. In comparison, wild-type FVB/N mice did not develop atrophic gastritis, metaplasia or gastric adenocarcinoma under similar conditions. Infection with *H. felis* has been shown to accelerate the development of gastric adenocarcinoma (≤8 months) in 85% of infected INS-GAS mice, with submucosal and intravascular invasion observed in 54% and 46% of animal respectively [112]. Similar pathology has also been shown in INS-GAS mice infected with *H. pylori* after 28 weeks of infection, with early antibiotic eradication of the bacteria (8 weeks post-infection) preventing the development of gastric pathology [392].

1.5.4 Genetic models for the study of gastrin related peptides

1.5.4.1 Gastrin deficient transgenic mice (GAS-KO)

As previously reported, the majority of *H. pylori* infected patients do not exhibit significant hypergastrinaemia, however chronic atrophy may still develop which suggests a susceptibility to gastric cancer independent of circulating gastrin concentration. Several strains of gastrin-deficient mice (GAS-KO) have been developed to further examine the physiological role of gastrin in the stomach. Initial GAS-KO mice developed by Koh *et al.* showed only small architectural changes with slightly reduced parietal and ECL cell

numbers [393]. Since then, independently developed 129/Sv GAS-KO strains have shown reduced gastric acid secretion and increased inflammation due to bacterial colonisation[171]. These mice developed gastric antral tumours when housed in non-specific pathogen free (non-SPF) environments[394]. In transgenic GAS-KO mice developed by Friis-Hansen *et al.* basal and stimulated gastric acid secretion were abolished, however this strain did not develop gastric antral tumours. Histological analysis confirmed gastric mucosal atrophy with an increase in parietal and ECL cell defects, which were partially restored with 6 day gastrin replacement therapy [138]. These data indicate a differential role of gastrin in the physiological functioning and development of both the corpus and antrum.

1.5.4.2 Transgenic mice overexpressing gastrin precursors

1.5.4.2.1 Overexpression of progastrin (hGAS)

Transgenic mice that overexpress the prohormone Pro-G (hGAS) have been developed using a 5.5kb complete human gastrin minigene construct that lacks 1kb of the first intron of gastrin. The lack of hepatic processing machinery results in elevated levels of Pro-G and G_{17} mRNA with normal concentrations of G_{17} and an accumulation of Pro-G peptides. Increased Pro-G in hGAS mice has been shown to increase colonic epithelial proliferation and increases the susceptibility to azoxymethane (AOM)-induced carcinogenesis [351, 352, 395]. Histological examination of hGAS mice observed a significant increase in colon tumour size and number compared with wild-type mice on the same genetic background (FVB/N), with no obvious gastric phenotype as previously observed with INS-GAS mice [396].

1.5.4.2.2 Overexpression of glycine- extended gastrin (MTI/G-Gly)

Similar to hGAS mice, no obvious gastric phenotype is observed in mice that constitutively overexpress glycine-extended gastrins (MTI/G-Gly). PCR mutagenesis of human gastrin cDNA integrates two stop codons after Gly_{72} which is spliced into a metallothionein promoter construct to generate transgene expressing pups (MTI/G-Gly) which overexpress

glycine-extended gastrins in both serum and the colonic mucosa. Gly-G have been shown to exert trophic effects, particularly proliferative, on both normal and malignant colonic cells with increased colonic mucosal hyperplasia observed *in vivo* [161].

In both hGAS and MTI/G-Gly mouse models there was an overexpression and activation of the Src family of tyrosine kinases (SFKs), particularly p60-Src, and subsequent secondary signalling pathways [397] and a significant upregulation of TGF-α which may provide a mechanism(s) for colonic carcinogenesis [398].

1.5.4.3 Gastrin receptor (CCK2R) deficient mice

The CCK2R has previously been shown to be an important regulator of cellular responses to G_{17}, both physiologically and pathologically (see section **1.2 Gastrin receptor**). CCK2R deficient mice exhibit gastric acid hyposecretion with subsequent hypergastrinaemia, abnormal organisation of oxyntic glands and increased gastric atrophy [399-401]. As the CCK2R in these mice is non-functional, gastrin is prevented from stimulating gastric acid secretion and therefore the luminal pH remains elevated. Analysis of cell populations identified an increase in gastrin secreting G cells and a significant decrease in acid-secreting cells (parietal) and differentiation and function of ECL cells [402]. These morphological changes are similar to those seen in INS-GAS mice which exhibit decreased parietal cell mass during atrophy [112] and are in contrast to other hypergastrinaemic models such as *Praomys* (*Mastomys*) *natalensis* or cotton rats (*Sigmodon hispidus*) which exhibit increased ECL cell number and density [370, 403]. However, there is no supporting evidence of gastric cancer development in CCK2R deficient mice, suggesting that the CCK2R is important for mitogenic signalling associated with gastric carcinogenesis.

1.6 Cholecystokinin-2 receptor (CCK2R) antagonists

1.6.1 Non-peptide gastrin receptor antagonists

Over the past twenty years, various attempts have been made to identify compounds that inhibit the CCK receptor in order to selectively block pathological downstream signalling. Many classes of non-peptide receptor antagonists have been designed as novel therapeutic agents including amino acids, dipeptoids, pyrazolidinones, anthranilic sulphonamides, benzodiazepines and ureidobenzodiazepines, with varying degrees of success.

1.6.1.1 Amino acids

4-benzamido-5-(dipropylamino)-5-oxopentanoic acid (Proglumide) was the first clinically available CCKR antagonist [404], however it has since been demonstrated that proglumide does not clearly discriminate between the two CCK receptor subtypes [405]. Chemical alterations of proglumide produced several glutamic acid derivatives, all of which had higher potencies than the parent drug (lorglumide > loxiglumide > proglumide) [406]. Spiroglumide (CR-2194) was initially proposed as an anti-gastrin drug which was more potent (micromolar range) than proglumide *in vitro* and *in vivo* it dose-dependently inhibited pentagastrin-stimulated gastric acid secretion in rats [407] and humans [408]. However due to a relatively low affinity for the CCK2R, new and improved analogues were pursued.

CR-2622, a napth-1-ylamino containing spiroglumide derivative, showed nanomolar affinity for the CCK2R. Although CR-2622 was highly selective and showed good affinity for the CCK2R, the bulky nature of R-group modifications reduced its oral bioavailability [409]. Simultaneously the Rotta group synthesised a basic compound (CR-2345) to investigate whether acidic properties were necessary for stereoselectivity. The substitution of the hydroxyl group on the C-terminus for an *N*-methylpiperazinyl group did not affect

antagonistic activity and CR-2345 also inhibited acid secretion in the perfused rat stomach and in dogs with a Heidenhain pouch or gastric fistula [410]. In addition, CR-2345 exhibited bacteriostatic activity *in vitro* for several *H. pylori* strains that had been isolated from patients with CAG [411]. Although the minimum inhibitory concentrations (MICs) are much higher than current bactericidal antibiotics such as amoxicillin or clarithromycin [412], this is worthy of further investigation due to our current understanding about the role of *H. pylori* during gastric carcinogenesis.

1.6.1.2 Dipeptoids

Several dipeptoid derivatives have been previously developed based on two important amino acid residues (tryptophan and phenylalanine) of CCKR ligands that were potent and selective for the CCK2R [413]. Of these compounds, CI-988 (previously known as PD-134,308) was the most recognised, with nanomolar affinity for the CCK2R [414]. CI-988 has a 1,600-fold higher affinity for the CCK2R and inhibited pentagastrin-induced gastrin secretion in rats with no significant effect on basal gastric acid secretion [415]. It also inhibited gastrin-CCK2R induced cellular proliferation [416]. C-terminal modifications were attempted in the dipeptoid series of drugs in order to increase potency, selectivity and the pharmacokinetic profile such as oral bioavailability which led to the development of a highly potent (IC50: 0.08nM) and highly selective (940-fold) CCK2R antagonist 4-fluorophenyl dipeptoid [417]. However, further studies in rats suggested that CI-988 and related compounds stimulate gastric (partially) and pancreatic (fully) secretions [418]. This in combination with relatively poor oral bioavailability (due to high molecular weight) limited its clinical application for the treatment of gastric disorders.

1.6.1.3 Pyrazolidinones

Utilisation of 'random drug screening' techniques led to the discovery of a class of compounds with moderate CCK2R binding affinity and selectivity, the pyrazolidinones.

Follow-up structure-activity relationship (SAR) studies identified the compound LY262691 with a binding affinity of 31nM for the CCK2R and 11,600nM for the CCK1R [419, 420]. However, the potency of this drug was dependent upon absolute stereochemistry and LY288513 was quickly identified as the biologically active enantiomer of the compound with 19nM and 20,500nM binding affinities for the CCK2R and CCK1R respectively [420]. However, studies examining the effects of LY288513 on gastrin stimulated histidine decarboxylase in rodents identified no inhibitory effects at any of the concentration tested (0.1-30nM/kg/hr) [421].

1.6.1.4 Benzodiazepines

A different approach was adopted by researchers at Merck who identified the first compound to selectively antagonise the CCK2 receptor at nanomolar concentrations (the benzodiazepine analogue L-365,260). Oral bioavailability was increased by the addition of a phenyl ring [422]. Bock *et al.* found that L-365,260 has 140- to 280-fold greater binding affinity for the gastrin CCK2R in the guinea pig compared with the rat pancreas and L-365,260 inhibited ^{125}I-gastrin binding to the gastrin receptor [423]. However, due to poor affinity and the short term duration of action, it was concluded that effective clinical application would be difficult. Therefore, successive attempts at chemical substitutions were made to increase the affinity for the gastrin receptor *in vitro* [424, 425].

1.6.1.5 Ureidobenzodiazepines

Nishida *et al.* suggested another structurally similar 1, 4 substituted benzodiazepine analogue ([(R)-1-[2,3-dihydro-1-(2'-methylphenacyl)-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl) urea] or YM022) that had a 2-fold higher affinity for the CCK2R than the CCK1R. When orally administered, it dose-dependently reduced pentagastrin-stimulated gastric acid secretion in pylorus-ligated rats [426]. Yuki *et al*. confirmed that YM022 was a more potent and highly selective CCK2R antagonist in dogs with IC_{50} values of

0.73nM and 136nM, for CCK2 and CCK1 receptors respectively [427]. However, despite the significantly longer lasting effects on gastric acid secretion *in vivo* [428], insufficient oral bioavailability still remained a problem for this compound. To address this issue, Semple *et al.* substituted various functional groups (Figure 7), to produce a YM022 derivative called netazepide (YF476) [8]. The substitution of the methylbenzene ring at position R_1 for a 3-methylgroup, the aromaticity of the pyridine ring at position R_2, and the presence of a 3-methylamino group at position R_3 enhanced both aqueous solubility and potency, thus increasing oral bioavailability (ED_{50} 21nmol/kg) in dogs [429].

Additionally, the 1,5 substituted benzodiazepine derived calcium bis[(*R*)-(—)-3-[3-(5-cyclohexyl-1-(3,3-dimethyl-2-oxo-butyl)-2-oxo-2,3,4,5-tetrahydro-1*H*-benzo[*b*]-[1,4]diazepin-3-yl)ureido] benzoate] (Z-360) that has been developed by Zeria Pharmaceuticals Co. Ltd, also surpasses the potency for the CCK2R and oral bioavailability compared with YM022 and L-356,260 [430]. Z-360 showed increased displacement of [I^{125}]-G_{17} (0.15nM) binding from the CCK2R with an EC_{50} of 0.1nM [431] and dose-dependently inhibited meal-induced gastric acid secretion in anaesthetised rats and vagus-innervated gastric pouch dogs, with the maximal effect (1mg/kg twice daily) comparable to the PPI omeprazole (30mg/kg once daily) [432].

1.6.1.6 Anthranilic sulphonamides

High throughput screening by Johnson & Johnson identified a novel class of small non-peptide structures based around an anthranilic ring that had good potency and selectivity for the CCK2R, a promising half-life (0.35±0.03hrs) and a low plasma clearance rate (0.42±0.01 L/kg/hr) in rats [433]. Further analogue studies increased the pharmacokinetic profile of the leading compounds in this class. For example, the substitution of the chlorine atom at the 4-position on the anthranilic ring for a bromine atom and similarly the movement of the chlorine atom at the 4-position to the 5-position both increased the

binding affinity (10-fold) for the CCK2R and these became analogues of the initial compound. However, substitution of the sulphonamide moiety for an amide functional group or the sulphonamide moiety at the *meta*-position significantly inhibited biological activity. These data suggested that the sulphonamide group at the *ortho*-position and halogenation of the anthranilic ring were essential for the functionality of these compounds and contributed to the development and success of [(*R*)4-Bromo-*N*-[1-(2,4-difluoro-phenyl)-ethyl]-2-(quinoxaline-5-sulfonylamino)-benzamide (JNJ-26070109) as a potent oral inhibitor of pentagastrin-stimulated acid secretion [433-435].

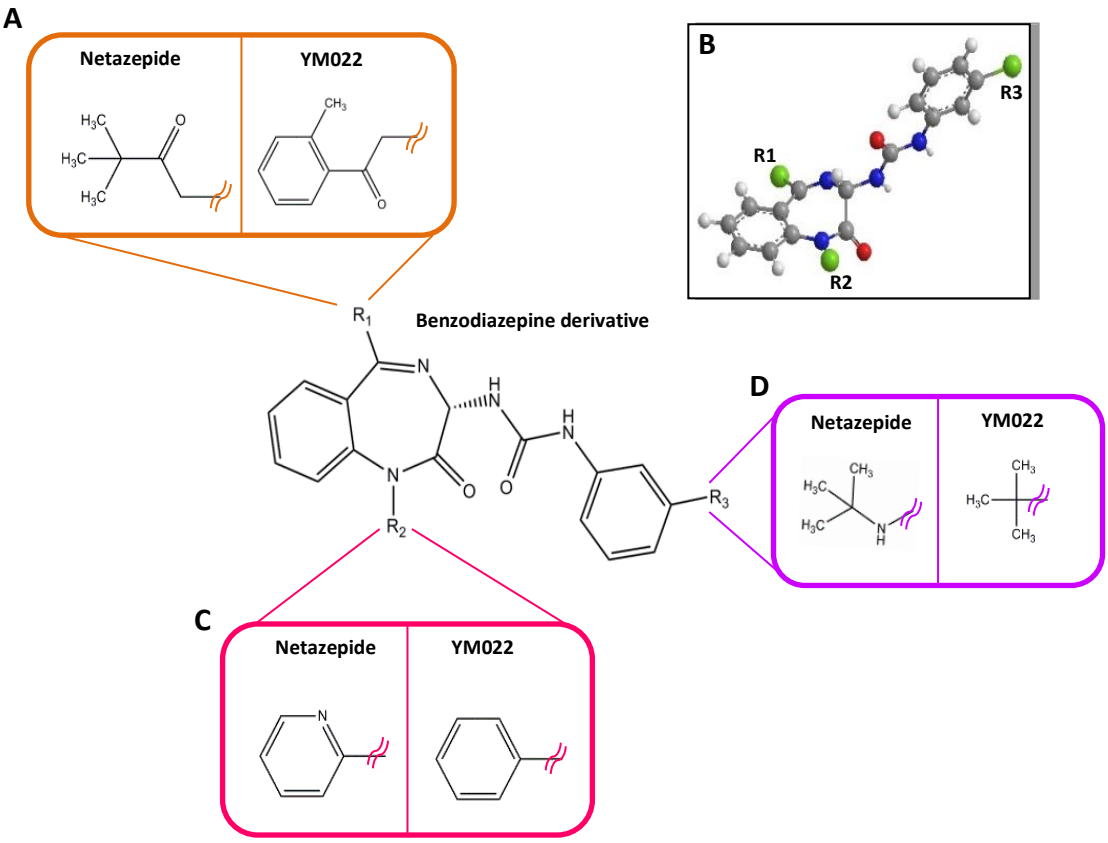

Figure 7. The basic 3D structure of a benzodiazepine derivative (B) and the functional group differences between two benzodiazepine-derived, highly potent and selective CCK2R antagonists YM022 and netazepide (A, C and D). Figure adapted from Semple et al. (1997) [8].

1.6.2 Effects of current CCK2R antagonists

1.6.2.1 In vitro

In humans, the CCK2R is ubiquitously expressed throughout the brain and the gastrointestinal tract and increased expression has been observed in various tumour cell lines including stomach, lung, colon and lymphoma [436-440]. Gastrin has been shown to exert trophic effects on CCK2R bearing tumours both *in vitro* and *in vivo* [441, 442] in a paracrine and potentially in an autocrine manner, as some CCK2R bearing cells additionally synthesise gastrin [350]. YM022 and netazepide have been shown to competitively displace [^{125}I] radiolabelled gastrin-I from the CCK2R with higher potencies than previously developed CCK2R antagonists [426, 429]. Gastrin-induced cellular responses such as DNA synthesis, phosphoinositide hydrolysis, histamine secretion and calcium mobilisation were dose-dependently inhibited following either YM022 or netazepide treatment, indicating that the current CCK2R antagonists YM022 and netazepide may intervene in the activation of the CCK2R to reduce gastrin stimulated trophic effects in human cancer cells [443-447].

1.6.2.2 Animal models

Excessive proliferation of the colonic epithelium has previously been identified as a risk factor for the development of colon cancer [448]. The human gastrin precursor Pro-G is a trophic growth factor for CCK2R bearing colonic epithelial cells located on basal colonic crypts [396]. Previous studies in transgenic hGAS mice have suggested that Pro-G increases CCK2R expression and Pro-G has been strongly associated with an increased susceptibility to drug-induced colon carcinogenesis in both short and long term studies [351, 352, 395]. Inactivation of the CCK2R with YM022 has been shown to inhibit Pro-G stimulated hyperproliferation and increases apoptosis of the colonic epithelium which reduces mucosal thickness and the aberrant crypt foci (ACF) associated with neoplastic progression [449].

Numerous *in vivo* studies have suggested that persistent *H. pylori* infection causes CAG which may progress to gastric adenocarcinoma in susceptible individuals [218, 292, 375]. Transgenic INS-GAS mice develop mild inflammation, moderate hyperplasia and severe mucosal atrophy with intestinal metaplasia and dysplasia after 36 weeks, which is exacerbated by infection with *H. felis* [112]. These preneoplastic changes have been shown to be reduced with administration of YM022 or netazepide, alone or in combination with antimicrobial treatments, in both infected and uninfected INS-GAS mice [450, 451]. Similarly, subcutaneous injection of netazepide (500µmol/kg) has been shown to reduce chronic mucosal inflammation and atrophy in Mongolian gerbils with and without *H. pylori* infection (<18 month treatment), with no significant differences in *H. pylori* colonisation being found between groups [452]. Netazepide at a concentration of 500µmol/kg has also been shown to inhibit ECL cell hyperproliferation and the development of type-1 gastric NETs that spontaneously arise in African cotton rats (*Sigmodon hispidus)* [368, 453] and *Mastomys* rodents (*Praomys natalensis*) [454].

Equally, the recent development of the anthranilic sulphonamide class of CCK2R antagonists identified JNJ-26070109 as one of the most selective human CCK2R antagonists (<1200 fold higher affinity for CCK2R than CCK1R) with high oral bioavailability. Chronic administration of JNJ-26070109 has been shown to reduce pentagastrin-stimulated gastric acid secretion and PPI-induced gastric acid rebound in a dose-dependent manner in rodents and canines [434, 455]. These data indicate that JNJ-26070109 is a promising novel CCK2R antagonist for clinical investigation into the treatment of acid related disorders.

1.6.2.3 Human clinical pharmacology

In support of the animal studies, netazepide has been shown to be a well-tolerated and highly specific CCK2R antagonist in healthy humans and both single and repeated doses of

netazepide cause dose-dependent inhibition of pentagastrin-stimulated or food-stimulated gastric acid secretion [456-458]. In two non-randomised trials, oral administration of netazepide (50mg daily for 12 weeks with a 12 week follow-up) reduced the abundance of gastric biomarkers such as chromogranin A (CgA), histidine decarboxylase (HDC) and MMP-7 which returned to baseline after cessation of treatment and reduced tumour size and number by 30% in patients with autoimmune chronic atrophic gastritis, hypergastrinaemia and type-1 gastric NETs [459, 460].

Due to the expression of the CCK2R on various tissues, CCK2R antagonists also have the potential to inhibit the trophic effects of gastrin associated with the development of other tumours such as pancreatic adenocarcinoma. The benzodiazepine-derivative Z-360 (120mg/day) has been shown to inhibit gastric acid secretion and was well tolerated with minimal adverse effects in phase 1a clinical trials (healthy subjects) at concentrations of <480mg/day for 8 days (quoted as unpublished by Meyer *et al.* [461]). Despite the increased oral bioavailability and tolerability, a phase Ib/IIa clinical trial investigating chemotherapy regimens for patients with advanced pancreatic carcinoma found no significant difference between co-administration of Z-360 and gemcitabine, either drug alone or the placebo. However, increased plasma gastrin concentrations at <240mg Z-360 treatment indicated successful inhibition of the CCK2R [461].

1.6.2.4 Human disease

In vivo studies have previously demonstrated the importance of CCK2R intracellular signalling in hypergastrinaemic conditions such as PA, CAG (both autoimmune and *H. pylori*-induced), acid suppression and gastrinomas [462, 463]. Elevated gastrin concentrations in the circulation stimulate the proliferation of CCK2R bearing gastric ECL cells and contribute to the development of hyperplasia, with subsequent progression to gastric NETs in some cases [464, 465]. Although gastric NETs are usually benign, 2-5% of patients with type-1 gastric

NETs and 10-30% of patients with type-2 gastric NETs develop metastases [466]. Gastrin is an essential regulator of gastric acid secretion via activation of proton pumps on the basolateral membrane of parietal cells [467]. However, gastrin also exerts trophic effects on parietal cells and increased parietal cell mass has been strongly associated with increased gastric acid output which is essential for the development of peptic ulcers [130, 226, 411]. In contrast, corpus colonisation of *H. pylori* has been shown to decrease parietal cell mass and integrity which reduces gastric acid secretion and results in CAG; a known precursor for gastric adenocarcinoma development [219]. Therefore the development of highly potent and selective CCK2R antagonists particularly YM022 and netazepide, have identified areas for therapeutic intervention which may, alone or in combination with other treatments, prevent gastric tumourigenesis or inhibit tumour growth with the potential for tumour regression.

1.7 MicroRNAs (miRNAs)

1.7.1 Significance of miRNAs

MicroRNAs (miRNAs) are a large family of endogenously expressed single stranded non-coding RNAs, 19-25 nucleotides in length, that control post-translational gene expression via binding to the 3' untranslated region (3'UTR) of numerous mRNAs. Since the first miRNA (lin-4) from the *Caenorhabditis elegans lin-14* gene [468] was discovered over 20 years ago, the number of known mature miRNAs has risen into the thousands and is continually increasing. According to previous literature, miRNA genes may contribute to ~1% of the total human genome with a large proportion (<40%) stemming from introns [469], formerly referred to as 'junk' sequences. This highlights the possibility that the number of miRNA sequences and subsequent mature miRNAs may be greater than first thought. As only a partial sequence of complementary target mRNA is required for binding to a miRNA, there is a potential for one miRNA to regulate several different mRNAs and *vice versa* for one mRNA to be regulated by multiple miRNAs. This provides an additional layer of complexity for the functional characterisation of identified miRNAs and their targets [470].

1.7.2 Biogenesis

The biogenesis of mature miRNAs is a complex multistep process (Figure 8). RNA Polymerases-II [471] or -III [472] initially transcribe a long primary sequence pri(mary)-miRNA, which may contain numerous miRNA transcripts. The RNA binding protein DiGeorge syndrome chromosomal region-8 (DGCR8)/Pasha facilitates the cleavage of pri-miRNA to hairpin-like miRNA precursors [473] by the class 2 RNAse-III enzyme Drosha, before being exported through the nuclear pore via the exportin-5/Ran-GTP [474]. The human immunodeficiency trans-activating response RNA binding protein (TRBP) along with the endoribonuclease Dicer, process the ~70 nucleotide lengths of pre-miRNA by removing the stem-loop at the 3' end to generate a small double stranded RNA duplex (miRNA:miRNA*), composed of a single stranded ~22 nucleotide length of mature miRNA and a

complementary strand. Only mature miRNA strands are incorporated into the RNA-induced silencing complex (RISC) and associate with the catalytic subunit Argonaute 2 (Ago2) [475]; which directs the complex to mRNA targets with complementary base pairing sequences, leaving the complementary miRNA* strand to be cleaved.

1.7.3 Physiological functions

MicroRNAs are a class of endogenous non-protein coding short RNAs that post-transcriptionally regulate approximately 30% of the human genome [470, 476], however their regulatory mechanisms are poorly understood. Mature miRNA sequences have been highly conserved throughout evolution in most investigated organisms including humans [477], suggesting a fundamental role during the maintenance of cell physiology.

1.7.4 Target mRNA downregulation

1.7.4.1 Translational repression

It was originally suggested that miRNAs mediate mRNA repression after the initiation of translation for a particular protein, but the point at which this occurred remained unclear. This hypothesis was based on the miRNA lin-4 and its larval mRNA targets (lin-14 and lin-28) that were isolated from *Caenorhabditis elegans* (*C. elegans*) [468]. *Lin-14* and *lin-28* mRNA have been shown to be accumulate in the polysomes (processing bodies) whilst protein concentration is downregulated in late L2 to early L3 larval stages of *C. elegans* development (periods where miRNA mediated repression occurs) which suggest that translation repression occurs downstream of its initiation [478, 479]. However, this hypothesis was challenged by Pillai *et al.* who examined miRNA repression in human cells using luciferase reporters at 3 sites of the 3'UTR of target mRNAs which are mediated by endogenous let-7. After 48hr DNA transfection, luciferase expression was decreased 80-90%, with a 20% reduction in reporter mRNA expression. Moreover sucrose gradient analysis indicated that let-7 inhibited mRNA loading into polysomes, effectively stopping

initiation occurring [480]. Although there are a few discrepancies between experimental methods there is little evidence to contradict either theory, therefore is it possible that inhibition of translation may occur through multiple mechanisms, as the biological outcome of miRNA-mRNA interactions depends on many factors including base pairing, RNA secondary structures, target site accessibility in addition to cellular context [481-484].

1.7.4.2 Target mRNA degradation

Perfect complementary base pairing of miRNAs-mRNAs has been shown to induce endonuclease cleavage of the target mRNAs (also known as 'slicer' activity) [485-487]. Although the majority of perfect base pairing has been shown to direct endonucleolytic cleavage, in some cases this may not be adequate and other factors such as the presence of the Ago2 protein in the RISC complex, are needed for cleavage to occur [488, 489]. Additionally, some imperfect base pairing in plants has also been shown to still induce endonucleolytic cleavage of target mRNAs [490, 491], indicating the importance of specific and catalytically active ribonucleoprotein binding for the cleavage of mRNAs. The cleaved fragments of mRNAs are incorporated into general mRNA degradation pathways which are initiated by deadenylation of the 3' poly(A)-tail [492] followed by either exonuclease activity within the exosome, or processed by decapping enzymes (Dcp) 1/2 followed by exoribonuclease (Xrn1p) degradation [493]. Studies have also suggested that miRNAs may target mRNAs for slicer-independent degradation via AU-rich 3'UTR regulatory elements (AREs) [494] which commonly control mRNA decay [495], however the mechanism for ARE-mediated degradation remains unknown.

1.7.5 Target mRNA upregulation

It is generally assumed that mRNA regulation by miRNAs always results in decreased expression of the target mRNA through degradation or translational repression. However, a study by Vasudevan & Steitz identified that the downregulation of mRNAs is a reversible

process and evidence suggested that some miRNAs may stimulate mRNA upregulation through direct mediation or the inhibition of repression [496].

1.7.5.1 Direct upregulation of target mRNAs

Post-transcriptional upregulation has been shown to occur in response to specific cellular states for instance, different phases of the cell cycle. During the G_0 phase the GW182 protein, which is essential for translational repression, is downregulated allowing Ago2 (which loses 'slicer' activity in this phase) to interact with the Fragile-X mental retardation protein-1 (FXR1) in microRNA ribonucleoprotein (miRNP) complexes, which are recruited for the initiation of ARE-mediated translation to maintain quiescence [497, 498]. In contrast, during the late S/ early G_2 phase of the cell cycle some miRNAs have been shown to additionally repress translation in proliferating cells [499, 500], suggesting that miRNAs may oscillate between activating and inhibiting mRNA translation as a regulatory function of the cell cycle.

1.7.5.2 Indirect upregulation of target mRNAs

The indirect upregulation of target mRNAs involves the removal of inhibitory miRNA/miRNP complexes [501] which have frequently been observed in response to cell stress such as increased hypoxia, radiation and salt concentrations [502-504]. For example, the cationic amino acid transporter-1 (CAT-1) mRNA has been shown to be upregulated in response to amino acid deprivation by the binding of the human antigen R (HuR) protein to AREs, which delays mRNA decay and increases miR-122 dissociation. The removal of repressive miRNPs facilitates CAT-1 incorporation into stress granules [502] which causes polysome recruitment to increase CAT-1 expression and maintains hepatocellular protein synthesis [505].

Alternatively, endogenous miRNA 'sponge' RNAs have been associated with the indirect upregulation of mRNAs as they competitively bind to a common 3'UTR seeding site of

miRNAs and prevent the binding of specific miRNPs to the target mRNA [506, 507]. In prostate cancer cells (DU145), retroviral overexpression of the miRNA sponge *PTENP1* has been shown to derepress the tumour suppressive *PTEN* gene in a Dicer-dependent manner from the oncogenic miR-17~92 cluster, as miR-17~92 has been shown repress *PTENP1* and *PTEN* with equal magnitude. Copy number losses at the *PTENP1* genomic locus have also been frequently observed in sporadic human colon cancers compared with normal colon tissues [508], indicating a growth suppressive function of the miRNA sponges particularly *PTENP1*.

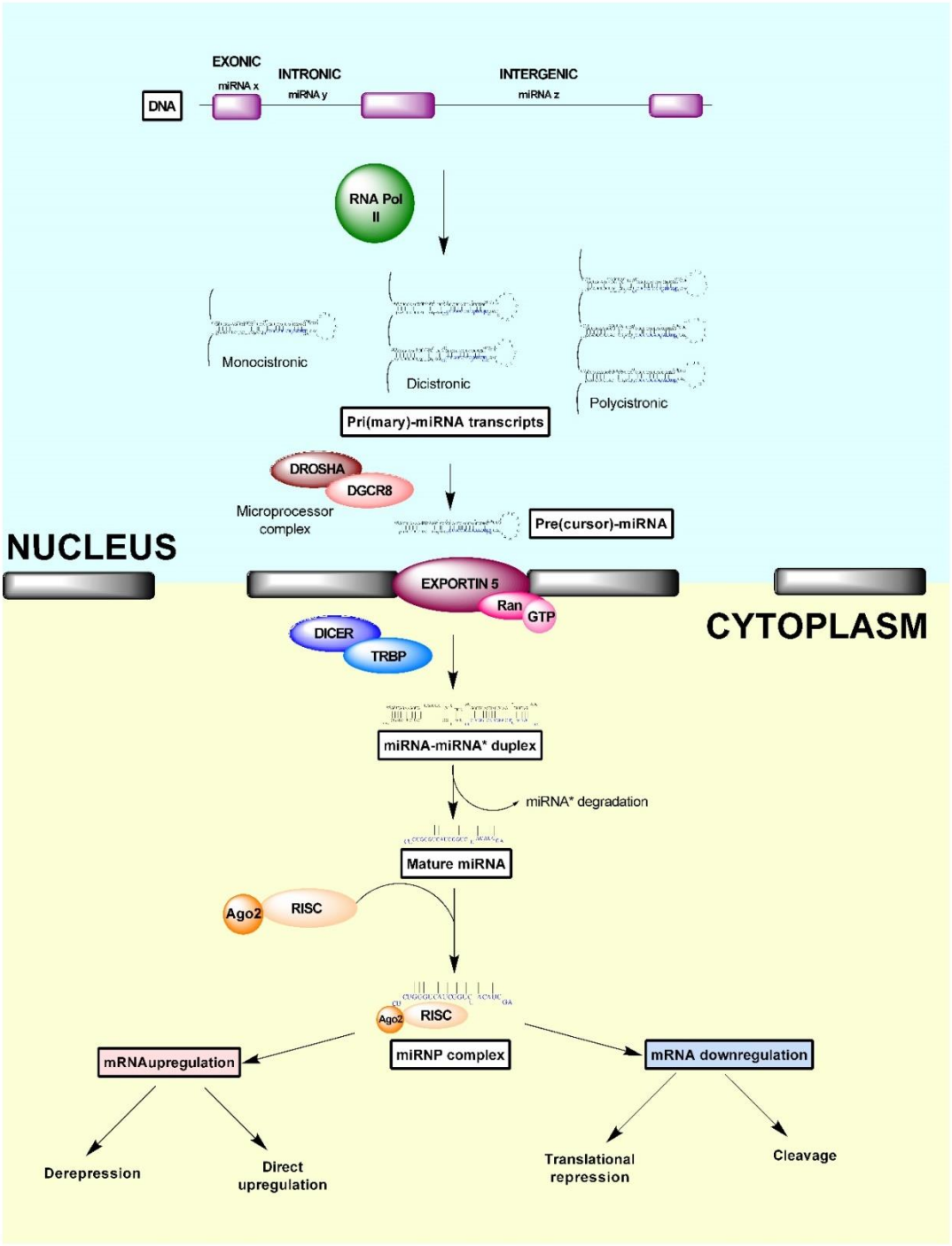

Figure 8. The canonical pathway for miRNA biogenesis in human cells. RNA polymerase-II transcribes the primary miRNA transcript(s) (pri-miRNA) located within exons or introns, or in intergenic regions of DNA. Multiple miRNAs located on the same gene are transcribed as clusters. The microprocessor complex (Drosha/DCGR8) crops the pri-miRNA into precursor miRNA (pre-miRNA) hairpin loops which are exported into the cytoplasm via exportin-5 (EXP5). The majority of pre-miRNAs are further cleaved by Dicer/TRBP which generates mature miRNA strands that can be incorporated into the miRNP complex and directed to target mRNAs. Figure adapted from Lodish *et al.* (2008)[7] and Winter *et al.* (2009)[13].

1.7.6 Regulation of miRNAs

1.7.6.1 Transcription

In humans, miRNA loci are located within multiple genomic regions including coding and non-coding introns and exons. Several miRNAs have been shown to be located in close proximity and are transcribed together as cluster miRNAs (polycistrons) such as the miR-17~92 and miR-221~222 clusters [509]. However, polycistronic miRNAs can also be individually post-transcriptionally regulated. miRNA transcription is regulated by multiple RNA polymerase-II associated transcription factors such as p53 and Myc [510, 511] and epigenetic factors such as DNA methylation and histone modification [512], which determine positive or negative miRNA expression.

1.7.6.2 miRNA processing

In the nucleus, the microprocessor (Drosha and DGCR8 complex) is essential for the cropping of pri-miRNAs (~11bp of the lower stem) into pre-miRNA hairpin loops. The microprocessor can be auto-regulated as DGCR8 stabilises Drosha whilst Drosha destabilises DCGR8 mRNA [513, 514] or regulated by post-translational modifications which increase protein stability and nuclear localisation or either enhance or inhibit processing activity [515-517]. Post-translational modifications such as protein phosphorylation are controlled by RNA-binding proteins such as p53, p72, p68, mothers against decapentaplegic homologs (SMAD) 1-3 or -5 [518-520]. miRNAs are exported into the cytoplasm via exportin-5 (EXP5) (a RAN-GTP-dependent binding protein) that is ubiquitously expressed within the cell. However, it has been suggested that EXP5 can be post-transcriptionally induced by PI3K pathway activation upon entry into the cell cycle (G_1 phase) [521]. Increased nuclear export of miRNAs has also been associated with increased DNA damage due to Akt pathway activation [522] which is a common feature in many cancers and increased nuclear accumulation of miRNAs has been associated with *XPO5* mutation [523]. Once in the cytoplasm, the majority of pre-miRNAs undergo Dicer-dependent cleavage

into mature miRNA:miRNA* duplexes, of which the mature miRNA can then be incorporated into RISC complexes and directed to target mRNA [524]. Human Dicer has been shown to interact with TRBP and the double-stranded RNA-binding protein (PACT) and it has been suggested that TRBP regulates the processing of certain pre-miRNAs as MAPK-mediated phosphorylation of TRBP has been shown to increase growth-promoting miRNAs and decrease tumour suppressive miRNAs such as let-7 [525]. In contrast, the expression of the *TARBP2* gene (which encodes for the TRBP protein) has been shown to be mutated in cancer and it has been suggested that reduced TRBP expression may destabilise Dicer and alter miRNA processing [526]. Therefore the role of these cofactors in the regulation of miRNAs remains unclear as purified Dicer has also been shown to retain a similar processing activity to the RNA binding protein-Dicer complexes [527].

1.7.6.3 miRNA sequence/structure

miRNA transcript sequence and/or structure can be altered in numerous ways and this has been shown to interfere with mature miRNA expression and target binding. Single nucleotide polymorphisms (SNPs) are changes in miRNA base sequences which can alter the transcription of a particular miRNA. For example, SNPs have been identified in the tumour suppressive pri-miR-15a~16-1 cluster transcript (**C**NNC to **T**NNC) and have been shown to reduce the processing activity of the microprocessor which subsequently decreases the expression of mature miR-16 [528]. The addition of untemplated nucleotides to the 3' end of the pri-miRNA (RNA tailing) such as adenylation or uridylation has been shown to interfere with Drosha and Dicer processing to either stabilise the miRNA or facilitate miRNA decay [529-532]. In addition to RNA tailing, the post-transcriptional substitution of an adenosine to inosine (RNA editing catalysed by adenosine deaminases (ADARs)) in pri-miRNA sequences and the *O*-methylation of pre-miRNA sequences, such as pri-miR-142 and pre-miR-145 respectively, have been shown to reduce the binding affinity of microprocessor complexes [533, 534]. It has been suggested that the expression of mature

miRNAs can also be regulated by exonucleases and target mRNAs. For example in melanoma cells the human polynucleotide phosphorylase (PNPT1) has been shown to degrade miRNAs, specifically miR-221, miR-222 and miR-106 [535]. Target mRNAs have also been shown to regulate mature miRNAs by either decreasing [536, 537] or increasing stability [538]. However, the physiological relevance of miRNA stabilisation by target mRNAs still needs to be explored.

1.7.7 Dysregulation in disease

As the binding of miRNAs has been shown to inhibit the translation, increase cleavage or induce the degradation of target mRNAs depending upon complementary miRNA-mRNA binding [539, 540], altered gene regulation can result in many consequential disease states including neurodegenerative diseases such as Alzheimer's [541] and cancers [542-544].

1.7.7.1 miRNAs and cancer

As miRNAs control a large proportion of the genome, their expression patterns are tissue-specific and dysregulation has been observed in the majority of malignancies [545], suggesting the potential for miRNAs to be biomarkers of cancer diagnosis, prognosis and response to therapies. One gene can be regulated by many miRNAs and likewise one miRNA can regulate several genes, including tumour suppressor genes and oncogenes. This adds an additional layer of functional complexity as miRNAs can act as both 'oncomiRs' to promote tumour development or 'anti-oncomiRs' to suppress tumour development, depending upon their tissue expression [546]. However, >50% miRNA genes are located within fragile sites and genomic regions associated with deletion, translocation and amplification in cancers, further indicating their significance during carcinogenesis [547].

OncomiRs are usually upregulated during tumour pathogenesis and inhibit tumour suppressor genes or genes associated with cell cycle regulation or apoptosis. There are many suggested oncomiRs however only a few have been well characterised. The first

direct evidence to suggest that miRNA dysregulation can induce carcinogenesis identified the overexpression of miR-155 and the miR-17-92 cluster (miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1) [548] in various B-cell lymphomas [549-552] due to the amplification of the chromosomal region in which the miRNAs are located. However, these miRNAs have also been shown to be overexpressed in solid tumours of the breast, lung, colon, pancreas, prostate and stomach [542, 553-557].

miRNAs of the miR-17-92 polycistron cluster have been shown to be transactivated by c-Myc, a proto-oncogene that is aberrantly expressed in several types of malignancy [558]. Distinct parts of the cluster have been shown to inhibit the expression of tumour suppressor genes such as *PTEN* (miR-19) thus promoting c-Myc-induced B-cell lymphogenesis [559], in part through a c-Myc-E2F1 reciprocal activation cycle [560]. By contrast other parts of the cluster (miR-17-5p and miR-20a) negatively regulate *E2F1* gene translation to potentially control this putative positive feedback loop [561].

In contrast the downregulation of anti-oncomiRs, which inhibit oncogene translation to prevent tumour development, is more frequently observed in cancers. miR-15 and miR-16 are involved in the physiological regulation of cell growth and apoptosis and target the anti-apoptotic B-cell lymphoma 2 (*BCL2*) gene [562]. Translocation of the BCL-2 protein into mitochondria has been shown to inhibit the mitochondrial/intrinsic pathway and release of cytochrome c, which prevents the apoptotic caspase cascade [563]. However in 68% of B-cell chronic lymphocytic leukaemias (B-CLL), the chromosome 13q14 region, where the miR-15 and miR-15 genes are located, is deleted [564] causing a downregulation of these anti-oncomiRs and allowing the initiation of tumour formation.

The let-7 family was one of the first recognised classes of miRNAs to be shown to orchestrate cell proliferation, migration and differentiation in *C. elegans* [565, 566]. Let-7 negatively regulates a known family of oncogenes, the *Ras* guanidine phosphatases [567],

which activate mitogenic MAPK/ERK signalling pathways that are commonly overexpressed in human malignancy [568]. Similar to miR-15 and miR-16, the let-7 gene has been mapped to human genomic locations that are frequently deleted in lung cancer, resulting in reduced tumour suppressive function of let-7 [569]. Although specific members of the let-7 family are downregulated in cancers (e.g. let-7a and -7c in lung cancer [567]), miRNA expression patterns are highly tissue specific and some members of the let-7 family maintain a high level of expression (e.g. let-7f in breast cancer [570] and let-7b and let-7i in lymphoma [571]), indicating the potential for tumour-specific biomarkers.

1.7.7.2 miRNAs and H. pylori

In addition to transcriptional regulation, *H. pylori* infection has been shown to post-transcriptionally regulate host cellular responses such as cell cycle progression, inflammatory responses, the inhibition of apoptosis, cell invasion and cell migration through dysregulated miRNAs [572]. Epigenetic alterations induced by altered miRNA expression have been shown to enhance tumourgenicity of *H*. pylori-associated gastric cancer.

H. pylori-associated dysregulation of miRNAs has been shown to contribute to gastric cancer pathogenesis. Ectopic expression of miR-101 has been shown to have tumour suppressive functions, in part, through the modulation of the cancer epigenome by inhibition of enhancer of zeste homologue-2 (*EZH2*), Cox-2, Mcl-1 and v-fos FBJ murine osteosarcoma viral oncogene homologue (*Fos*) [573-578]. However miR-101 is reported to be downregulated in *H. pylori*-associated gastric cancer and this has been shown to promote tumour cell proliferation, metastasis, angiogenesis and the inhibition of apoptosis [573].

Aberrant DNA methylation or histone modification is induced by *H. pylori*-infection in the gastric mucosa [579] and has been shown to contribute to increased inflammation [580]. *cagA* positive strains of *H. pylori* significantly reduce the expression of the anti-oncomiR family

of let-7 miRNAs through DNA methylation and histone modification in chronic gastritis and gastric cancer tissues [567, 581]. This in turn activates the Ras/MAPK/ERK pathway which contributes to the malignant phenotype [582].

Acute infection with *cagA*$^+$ *H. pylori in vitro* and *in vivo* also significantly increases the expression of oncomiRs such as miR-1289 compared with uninfected or *cagA*$^-$ infected controls. miR-1289 post-transcriptionally inhibits the translation of H^+/K^+ ATPase-α subunit (HKα) mRNA to further control hypochlorhydric conditions [583]. In both *cagA*-transfected and *H. pylori*-infected human gastric adenocarcinoma (AGS) cells, miR-1290 expression was upregulated in an ERK1/2-dependent manner and miR-584 was upregulated in an NFκB-dependent manner and indirectly by mIR-1290. Both miR-584 and miR-1290 were shown to target the *Foxa1* gene which significantly promoted epithelial-mesenchymal transition. In knock-in mice, miR-584 and miR-1290 overexpression induced gastric mucosal remodelling to intestinal metaplasia [584]. The miRNA clusters miR-17~92 and miR-106b~93~25, miR-194, miR-21 and miR196 were all significantly increased in intestinal metaplasia compared with healthy adjacent tissues and miRNA dysregulation was not reversed upon eradication of *H. pylori* during long term colonisation [585].

Zhang *et al*. showed that oncogenic miR-21 was overexpressed *in vitro* in *H. pylori* infected cells and *in vivo* in gastric cancer tissues compared with healthy uninfected controls, suggesting that the increased miR-21 expression may be due to *H. pylori* infection. In AGS cells, an increase in cell survival, proliferation and cell invasion was induced by miR-21 overexpression via the inhibition of reversion inducing cysteine rich protein with kazal motifs (RECK), a known tumour suppressor of gastric cancer [586].

1.7.8 miRNAs as potential biomarkers of cancer

miRNAs are promising candidates as biomarkers of disease, particularly cancer, due to their high stability in bodily fluids, blood and fixed tissue samples [587, 588]. The stability of

miRNAs has been shown to remain intact under severe conditions including freeze-thawing, boiling, low/high pH (1 or 13) or long term storage and ~50% of miRNAs have also been shown to remain intact after 3hr exposure to RNase enzymes [589]. miRNAs have been shown to be expressed in distinct tissue-specific patterns that can determine clinicopathological features.

1.7.8.1 Diagnostic, prognostic and predictive uses of miRNA profiles for cancer

It has been previously observed that cancers derived from different cell lineages express distinct miRNA profiles that can determine cancer origin and subtype. For example, studies by Ferracin *et al*. were able to correctly identify the origin of 100% of primary tumours and 78% of metastatic tumours from 101 formalin fixed paraffin embedded (FFPE) biopsies using a microarray platform. Further verification in 16 independent tumour samples of unknown origin initially indicated >90% prediction accuracy for most cases [590]. Some specific miRNA profiles have also been shown to possess reproducible results for the early detection of cancers, for example increased expression of miR-21 is indicative of pancreatic ductal neoplasia [591]. Earlier diagnosis of cancer and the identification of the primary tumour site would significantly enhance the specificity and effectiveness of cancer treatment and could significantly increase overall patient survival for many cancers.

In addition to earlier diagnosis and the identification of tumour origin and type, miRNAs have been shown to accurately predict prognosis of disease-free survival and may enhance cancer therapy management. A unique thirteen miRNA signature has been identified in chronic lymphocytic leukaemia (CLL) patients and has been shown to accurately determine abnormal levels of prognostic factors, such as increased 70kDa zeta-associated protein (ZAP-70) and a reduced number of mutations in the immunoglobulin heavy-chain variable-region gene (IgV_H) [592] which have been associated with a more aggressive form of cancer and poor survival rates [593], regardless of disease progression. A five miRNA signature has

been identified for lung cancer prognosis as high expression of miR-221 and let-7a has been associated with increased tumour suppressive function with a good prognosis, whilst increased miR-137, miR-372 and miR-182 has been associated with enhanced metastasis [594]. Similarly, a seven miRNA signature (miR-21, miR-10b, miR-223, miR-338, let-7a, miR-30a and miR-126) has been shown to predict overall survival and disease-free relapse in gastric cancer patients [595]. Notably, single miRNAs have also been shown to accurately predict disease outcome. For example, an increase in miR-210 expression has been associated with an increased risk of relapse and poor clinical outcome in breast cancer, with similar accuracy to a 76 gene mRNA profile (GENE76) [596]. The surveillance of specific miRNA profiles may also allow accurate prediction of patient response to cancer therapies, which is of great importance due to the development of chemoresistance; a major problem in current cancer management [597-599].

1.8 *Helicobacter pylori (H. pylori)*

1.8.1 Potential routes of transmission

Due to the high incidence of *H. pylori* infection, it is possible that there is more than one route for transmission of this bacterium which may include zoonotic, environmental and interpersonal. *H. pylori*-infected gnotobiotic pigs exhibited gastric pathological features similar to human infection [600] and higher incidences of *H. pylori* seropositivity have been observed in adults after occupational exposure to sheep [601, 602]. Four independent studies have also suggested that the isolation of *H. pylori* is possible from both drinking and wastewater [603-606] and recent animal models found that contaminated drinking water increased *H. pylori* infection and gastric mucosal inflammation [607]. Whilst some epidemiological studies have suggested that contraction from environmental sources is possible [607] there is more supporting evidence to suggest that interpersonal transmission is more probable [608-610], however the most important mechanism in humans still remains unclear.

1.8.2 Role in pathogenesis

The *H. pylori* bacterium is well adapted for survival and colonisation of the human gastric mucosa for the lifetime of the host [611] and spontaneous eradication is relatively uncommon (0.1-1.1% per year) [612]. There are frequently no visible symptoms in *H. pylori* infected patients, with the majority not progressing to clinical disease [613]. However for some patients, *H. pylori* infection stimulates the development of chronic gastritis which has previously been associated with peptic ulcers and gastric adenocarcinoma in humans [614, 615]. Environmental, genetic and bacterial virulence factors influence the outcome of infection (Figure 9).

1.8.2.1 Environmental

Many environmental factors have previously been shown to actively contribute to *H. pylori*-induced gastric carcinogenesis. Diet plays a major role in the colonisation and persistence of *H. pylori*, as a synergistic relationship was identified between the bacterium and dose-dependent salt intake. High dietary salt intake has also been shown to induce gastric mucosal damage, in addition to independently increasing cell proliferation and DNA damage, which may result in tumourigenesis [616]. In contrast, an increased intake of antioxidants and vitamins such as vitamin C (ascorbic acid) exhibit a cytoprotective function in acute *H. pylori* infection [617].

1.8.2.2 Genetic

As *H. pylori* infection is asymptomatic for the majority of people, the host's genetics have been shown to determine susceptibility and severity of *H. pylori* infection. Inflammation of the gastric mucosa (chronic gastritis) occurs after successful colonisation of *H. pylori* and can be observed in all confirmed cases. *H. pylori*-induced gastritis commonly develops as a consequence of a Th1-polarised immune response with increased secretion of pro-inflammatory cytokines (IL-1β, TNFα and IL-8) and reactive oxygen/nitrogen species [618-620]. Administration of *H. pylori*-infected unfractionated splenocytes has been shown to result in gastric pathologies including gastritis, delayed-onset of hypersensitivity and metaplasia in uninfected mice [621].

Single nucleotide polymorphisms (SNPs) within genes involved in immune and inflammatory responses have been shown to increase the risk of *H. pylori*-related gastric pathogenesis [622-625]. Studies by El-Omar *et al.* identified that SNPs within the loci of the IL-1 gene cluster, which encode the IL-1α, IL-1β and IL-RN genes, were strongly associated with hypochlorhydria and gastric cancer [626]. Similarly, SNPs located within the promoter

sequence of TNF-α (TNF-α-308*A) and IL-8 (IL-8-251*A) have also been shown to promote gastric atrophy and to increase the risk of gastric cancer development [627, 628].

In addition, chronic *H. pylori* infection has been shown to induce the accumulation of latent somatic gene mutations. For example, whole-exome sequencing and deep sequencing of tumour-associated genes in *H. pylori*-infected patients identified numerous mutations (predominantly C:G>T:A transitions) in the tumour suppressive *TP53* gene, in cancerous and non-cancerous gastric biopsies [629]. The progressive accumulation of somatic mutations within the *TP53* gene may cause dysregulation of the cell cycle and initially drive tumour formation.

1.8.2.3 Bacterial virulence factors

In addition to host genetic factors, bacterial virulence genes have also been shown to determine the clinical outcome of chronic *H. pylori* infection. Previous evidence has suggested that *H. pylori* evolved simultaneously with humans as bacterial genetic diversity, which varies due to point mutations, gene insertions and/or deletions [630, 631], has been reported within the same host [632, 633]. Although the majority of *H. pylori* bacteria reside within the gastric lumen, approximately 20% have been shown to interact with the gastric epithelia [634-636] and different bacterial genotypes have been shown to induce differential epithelial responses, some of which have been shown to increase the risk of gastric tumourigenesis [637].

The most extensively characterised virulence factors are *cagA* and *vacA* genes which have been associated with malignant phenotypes [579, 638]. However, some studies have reported cases of gastric cancer with *cagA*-negative strains of *H. pylori* [639], indicating the potential role of other virulence factors during the development of gastric neoplasia. The *cagA* gene is located within the 31 gene cag pathogenicity island (*cag*PAI), which also encodes for the majority of functional components of the bacterial type IV secretion system (T4SS) [640]. The

T4SS facilitates the deployment of bacterial products such as the *cagA* gene product (CagA) and peptidoglycan, into the host epithelial cell cytoplasm [641]. CagA has been shown to subsequently interact with multiple secondary messenger proteins which activate the MAPK, PI3K-Akt and NFκB pathways [642] which contribute to increased mucosal inflammation and susceptibility for gastric cancer [643-646]. *cagA*$^{+}$ strains can also independently increase the transcription of activation-induced cytidine deaminase (AID) in the gastric mucosa which causes mutations in immunoglobulin DNA [647].

The *vacA* gene is not located within the *cag*PAI and encodes for the vacuolating cytotoxin-A protein (VacA). The bacterial toxin VacA has been shown to suppress T-cell activation and disrupt the epithelial cell barrier, primarily through the induction of cytoplasmic vacuolation, *in vitro* [648] and causes epithelial cell damage and ulcer formation *in vivo* [649]. Almost all *H. pylori* strains contain the *vacA* gene, however variations within the 5' terminal (s), middle (m) or intermediate (i) regions, of which the 's' and 'm' regions are further divided into subtypes s1a, s1b, s1c/s2 and m1/m2, have been shown to influence cytotoxicity [650]. *In vitro*, *vacA* s1/m1 strains have been shown to produce higher concentrations of cytotoxin with increased vacuolating activity being observed compared with s1/m2 or s2/m2 strains [651]. Similarly *in vivo*, infection with *H. pylori vacA* s1/m1 strains have been associated with increased risk of peptic ulcer [652-654] or gastric cancer [655-657]. VacA and CagA are functionally associated, as both have been shown to downregulate the effects of the other on the gastric epithelium, to potentially limit mucosal destruction and allow bacterial-cell interaction [658].

Helicobacter outer membrane (Hop) proteins are the largest of the outer membrane protein (OMP) families which encode ~4% of the *H. pylori* genome [635]. Hop proteins include adhesins such as blood-group antigen-A (BabA/ HopS) [659], sialic acid-binding adhesion (SabA/HopP) [636, 660], OipA (HopH) and HopQ [661], which determine the initial colonisation of

the bacteria and success of long-term infection. Bacterial adhesins have also been strongly associated with increased *H. pylori* density, neutrophil infiltration and high levels of chemokines such as interleukin (IL-8) [662], which correlate with a more intense inflammatory response and the development of more severe gastric lesions.

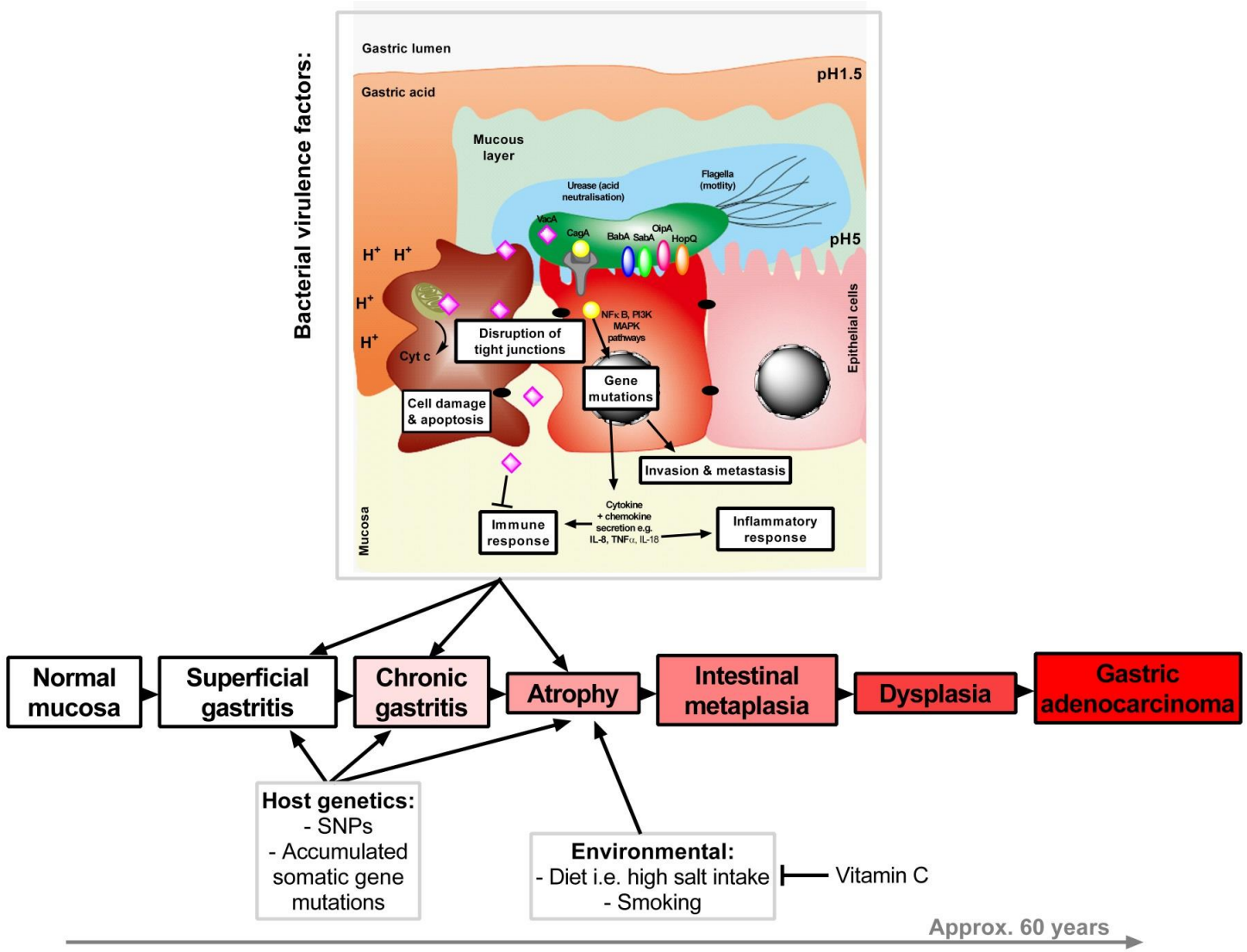

Figure 9. The environmental, genetic and bacterial virulence factors which contribute to *H. pylori* associated pathogenesis in humans. Single nucleotide polymorphisms and accumulated gene mutations have been shown to contribute to the development of gastritis and glandular atrophy. Environmental factors such as poor diet and smoking have also been associated with increased glandular atrophy which has been shown to be reduced with increased vitamin C intake. Bacterial virulence factors also contribute to early stage pathogenesis by the disruption of tight junctions, gene mutations, increased cell damage, invasion and metastasis and increased immune and inflammatory responses. The accumulation of all three risk factors has been shown to severely increase the development and progression of gastric adenocarcinoma. Figure adapted from Conteduca *et al.* (2013) [10], Monack *et al*. (2004) [14] and Wadhwa *et al*.(2013) [16].

1.9 Hypothesis

Gastrin induces differential expression patterns of genes associated with gastric premalignant changes such as cellular proliferation, migration and structural remodelling, which can be reversed with CCK2 receptor antagonism. This could provide mechanistic insight into gastrin-associated carcinogenesis and may provide potential novel biomarkers for the early detection of gastric tumours.

1.10 Aims

1. To assess differentially expressed miRNAs within human gastric adenocarcinoma cell lines that have been transfected with the human CCK2 receptor (AGS_{GR}) with and without gastrin treatment
2. To identify possible activated pathways that may alter the transcription of the chosen miRNA(s)
3. To identify and verify protein targets of the miRNA(s) and assess the functional consequences of their dysregulation *in vitro* and *in vivo,* in both hypergastrinaemic INS-GAS mice and humans with type-1 gastric neuroendocrine tumours
4. To assess whether gastrin affects the expression of PAPPA2 mRNA and protein in AGS_{GR} cells
5. To evaluate the functional consequences of gastrin-induced alterations in PAPPA2 expression in AGS_{GR} cells
6. To assess whether three novel CCK2 receptor antagonists reduce/reverse gastrin-induced phenotypic alterations *in vitro*

2 Materials and methods

2.1 Reagents

Amidated gastrin (G_{17}) was from Bachem (St.Helens, UK), Ro-32-0432, PD-98059 and LY-294002 were from Calbiochem (Nottingham, UK) and YM022 was from Tocris Bioscience (Bristol, UK). Netazepide (YF476) and compounds TR1, TR2 and TR3 were gifts from Trio Medicines Ltd (London, UK). All other routine supplies were from Sigma (Poole, UK) unless otherwise stated.

Human PAPPA2 and GAPDH qPCR primer pair sequences were purchased from Eurogentec (Southampton, UK) (Table 1). Human miR-222 (MS00007609), RNU62 (MS00033740) and CDKN1B (p27) primers (QT00998445) were purchased from Qiagen (Sussex, UK).

Human PAPPA2 siRNA, human CDKN1B (p27) siRNA and non-targeting control siRNA were purchased from GE Dharmacon (Lafayette, USA) as a combination of 4 pooled siRNA sequences (SMARTpool: ON-TARGET*plus*™ PAPPA2 siRNA, SMARTpool: ON-TARGET*plus*™ human CDKN1B (p27) siRNA and SMARTpool: ON-TARGET*plus*™ Non-targeting Pool siRNA) for maximal gene silencing (Table 1). DharmaFECT 1 transfection reagent (GE Dharmacon, Lafayette, USA) was used for effective siRNA transfection in AGS_{GR} cells.

Chemically synthesised miR-222 mimic (MSY0000279), miR-222 inhibitors (MIN0000279), miR-1 mimic positive control (MSY0000416) and miScript negative control (1027271) were all from Qiagen (Sussex, UK). All other routine supplies were from Sigma (Poole, UK) unless otherwise stated.

Table 1. Primer and small interfering RNA (siRNA) sequences.

Target gene	Primer sequences	Purchased from
PAPPA2	Forward: GCATCTCAGCTGTGGCTCTA Reverse: AGTTACTGGGAGCCGAAAGAC	Eurogentec (Southampton, UK)
GAPDH	Forward: CAGCAAGAGCACAAGAGGAA Reverse: GTGGTGGGGACTGAGTGT	Eurogentec (Southampton, UK)
Target gene	**siRNA sequences**	**Purchased from**
CDKN1B ($p27^{kip1}$)	CAAACGUGCGAGUGUCUAA GCAGCUUGCCCGAGUUCUA ACGUAAACAGCUCGAAUUA GCAAUGCGCAGGAAUAAGG	GE Dharmacon (Lafayette, USA)
PAPPA2	CAUCAUCGCAGGUGUGUUU GCCCAAGCAUUCCCUUAAA GGGCUCCGUUCACCAACUA CAAGAGGGCAUACAUGAGU	GE Dharmacon (Lafayette, USA)
Non-targeting	UGGUUUACAUGUCGACUAA UGGUUUACAUGUUGUGUGA UGGUUUACAUGUUUUCUGA UGGUUUACAUGUUUUCCUA	GE Dharmacon (Lafayette, USA)

2.2 Cell lines

A human gastric adenocarcinoma cell line (AGS) and a transfectant stably expressing the human CCK-2 receptor (AGS_{GR}) [173, 663], provided by Prof. A. Varro (Liverpool, UK) were cultivated in nutrient mixture F-12 Ham's medium supplemented with 10% Foetal Bovine Serum (Gibco, Paisley, UK), 2mM L-Glutamine and 1% combined antibiotics streptomycin and penicillin (complete media). Cells were maintained in a humidified atmosphere of 5% CO_2/ 95% O_2 in Galaxy R (Wolf Laboratories, Newton, MA) incubators at 37°C and AGS_{GR} cells sustained antibiotic selection with 2µg/ml puromycin for 7 days before experimentation.

Rat exocrine pancreatic tumour cells (AR42J) that constitutively express the gastrin-CCK2 receptor were purchased from the European collection of cell cultures (ECACC, Salisbury, UK). Cells were routinely cultivated in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum and 1% combined antibiotics streptomycin and penicillin (complete media) and maintained at 5% CO_2/ 95% O_2 in Galaxy R incubators (Wolf Laboratories, Newton, MA) at 37°C.

All cells were harvested with 1× trypsin-EDTA solution (0.05% trypsin (v/v), 0.02% EDTA (w/v) in PBS) at 70-80% confluency and only cells at passage numbers between 1 and 30 were used. All cells were serum starved for 24hrs before treatment.

2.3 Animals

All *in vivo* experiments were carried out under UK Home Office project licence numbers 40/3392 and 70/8457, granted after ethical approval. Animals were housed in specific pathogen free facilities with access to food and water *ad libitum* at the University of Liverpool. Male wild-type FVB/N mice were purchased from Charles River Laboratories (Margate, UK) and male INS-GAS mice on the genetic FVB/N background were generated in-house and were homozygous for the INS1-GAS transgene, coding for the rat insulin-1

promoter (a gastrin regulator), which produced an excess of human gastrin [28]. This overexpression resulted in mild hypergastrinaemia, increased parietal cell number and subsequent elevated gastric acid secretion within 12 weeks of birth. After 12 weeks, morphological changes were seen within the gastric epithelium including atrophic gastritis and intestinal metaplasia. End-stage disease occurred after about 40 weeks in this colony, characterised by invasive gastric adenocarcinoma formation [396]. All mice were pathogen free and maintained under normal housing conditions for a minimum of 1 week prior to experimentation. Whole stomachs were taken and opened via the greater curvature then washed in sterile PBS. Whole stomach mucosal scrapings were obtained using sterile glass slides and preserved in either 500µl RNA later for RNA extraction or 200µl RIPA buffer for protein extraction. All blood samples were collected via cardiac puncture and separated by centrifugation by Dr. Bryony Parsons and *H. felis* bacteria for mouse infection studies was provided by Dr. Bryony Parsons.

2.4 Human samples

Human serum and gastric corpus biopsies were obtained by Dr. Andrew Moore from 8 patients enrolled on studies 1 and 2 of the phase-2 clinical trial for the assessment of netazepide in subjects with autoimmune atrophic gastritis, hypergastrinaemia and multiple type-1 gastric neuroendocrine tumours, as previously described [460]. Control samples were obtained from 10 patients who had a normal upper GI endoscopy, normal gastric antral and corpus biopsies, no evidence of *H. pylori* infection, were not taking proton pump inhibitors and who had fasting serum gastrin concentrations <40pM. All human sample collection had appropriate ethical approval and informed patient consent.

2.5 Sample preparation

$1x10^6$ AR42J, AGS or AGS_{GR} cells were seeded into T75 vented cap cell culture flasks (Corning, Sunderland, UK) in complete media and were left to adhere for 24hrs.

Treatments were applied accordingly in serum free media. Following treatment, cells were detached with trypsin and harvested into 15ml centrifuge tubes (Corning, Sunderland, UK). Cell suspensions were then centrifuged in the Heraeus Multifuge 3 S-R (Thermo Fisher Scientific, Hemel Hempstead, UK) at 1500rpm for 5mins and the precipitate-free liquid removed. The remaining pellet was re-suspended in 10ml phosphate buffered saline (PBS) and centrifuged at 1500rpm for a further 5mins, discarding the supernatant. The pellet was re-suspended again in 1ml PBS, transferred into a 1.5ml Eppendorf tube and spun in a HAWK 15/05 refrigerated microcentrifuge (Sanyo/MSE, London, UK) a final time at 4°C, 5000rpm for 15mins. The upper layer of PBS was aspirated and discarded without disturbing the pellet which was stored at -80°C prior to use.

2.6 RNA isolation and reverse transcription

Total RNA was isolated from cells and tissues using the miRNeasy Mini Kit and from serum using the miRNeasy Serum/Plasma kit (both from Qiagen, Sussex, UK) according to the manufacturer's instructions. The concentration of RNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Hemel Hempstead, UK) and each sample was diluted to a final concentration of 2.5ng per reaction. Using the miScript RT II Kit (Qiagen, Sussex, UK) with the HiFlex buffer enabled cDNA synthesis of mRNA and with the HiSpec buffer enabled cDNA synthesis of mature miRNAs only. Samples were stored as undiluted cDNA at -20°C prior to real-time PCR.

2.7 MicroRNA PCR Array

The synthesised cDNA was prepared using the miScript SYBR Green PCR Kit (Qiagen, Sussex, UK) according to the manufacturer's instructions. The mixture was then loaded into each well of the 96-well miScript miRNA PCR array plate (Qiagen, Sussex, UK) and run in a real-time LightCycler 480 (Roche, Sussex, UK). miScript miRNA PCR arrays contained 84 of the most abundant and best characterised miRNAs that have been arrayed in

biologically relevant pathway-focused panels with 8 endogenous control miRNAs. Data was analysed using the $\Delta\Delta C_T$ method for relative quantification using the miScript miRNA PCR array data analysis software (Qiagen, Sussex, UK). Normalisation was against the average threshold cycle of the entire plate minus Ct values >35 (the maximum threshold value) and 4 control genes (2x miRTC and 2xPPC) that were separately used to assess reverse transcription and PCR performance.

2.8 Quantitative polymerase chain reaction (qPCR) primer assays

Mature miRNAs were assessed using primer assays with SYBR green for miR-222 with endogenous RNU62 or spike-in exogenous *C. elegans* miR-39 used for normalisation according to the miScript miRNA Primer Assay Handbook (Qiagen, Sussex, UK). Messenger RNA was assessed using primer assays for CDKN1B (p27) with GAPDH for normalisation according to the Quantitect Primer Assay Handbook (Qiagen, Sussex, UK) and run in a real-time LightCycler 480 (Roche, Sussex, UK). Each sample was run in quadruplicate and analysis used the $\Delta\Delta C_T$ method for relative quantification.

2.9 Small interfering RNA (siRNA) transfections

AGS_{GR} cells were transfected with SMARTpool: ON-TARGET*plus*™ human CDKN1B (p27) siRNA (L-003472-00-0005) or SMARTpool: ON-TARGET*plus*™ Non-targeting Pool siRNA (D-001810-10) (both from GE Dharmacon) for 48hrs according and using DharmaFECT 1 transfection reagent. AGS_{GR} cells were seeded onto 24 well plates ($3x10^4$/well) in complete media and left to adhere for 24hrs. In separate 1.5ml Eppendorf tubes, the siRNA and transfection reagent (GE Dharmacon, Lafayette, USA) were diluted in serum free media to a final volume of 50µl per reaction and incubated for 5mins at room temperature. The diluted 50µl of siRNA was added to the 50µl transfection reagent, mixed by gentle pipetting and incubated for 20mins at room temperature. The transfection reaction was diluted further in 400µl antibiotic-free complete media for a final volume of 500µl. The

culture media was aspirated from the cells and 500µl of the appropriate transfection mix was added to each well. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2/ 95% O_2 for 24-72hrs, media was replaced with fresh complete media every 24hrs to reduce cytotoxicity. Cell culture medium was then changed to serum-free medium when 10nM G_{17} treatment was applied.

2.10 MicroRNA (miRNA) mimic and inhibitor transfections

AGS_{GR} cells ($3x10^4$/well) were seeded into 24 well plates in 500µl complete media and left to adhere for 24hrs before being transfected. The chemically synthesised miR-222 mimic (MSY0000279) or inhibitor (MIN0000279), miR-1 positive control (MSY0000416) or miScript negative control (1027271) (all from QIAGEN) were diluted in 100µl serum-free media. The HiPerfect transfection reagent (301704) (QIAGEN) was then added (3-4.5µl) and incubated for 10mins at room temperature to allow the formation of transfection complexes. The complexes were then added dropwise onto the cells and incubated for 24-72hrs. Cell culture medium was then changed to serum-free medium when 10nM G_{17} treatment was applied.

2.11 Sulforhodamine-B (SRB) assay

AGS_{GR} cells ($2x10^3$/well) were seeded in 96 well plates and grown for 48hrs in complete media, with fresh media applied every 24hrs. Cells were treated with TR1, TR2 and TR3 in the absence and presence of G_{17} 10nM in complete media at 24hr intervals for a further 48hrs. DMSO (1%) and G_{17} 10nM treatments were used as negative and positive controls respectively, with YM022 and netazepide (100nM) treatments used as positive inhibition controls. After 48hrs, cells were fixed using methanol/acetic acid, stained with 0.4% SRB in 1% glacial acetic acid and absorbance was read at 570nm using a Sunrise plate reader (Tecan, Switzerland) to assess cell proliferation. Treatments were performed n=8.

2.12 Haemocytometer count assays

AGS_{GR} cells ($2x10^5$/well) were seeded in 6 well plates and left to adhere for 48hrs in 2ml complete media; fresh media was applied every 24hrs. Cells were treated with TR1, TR2 and TR3 in the absence and presence of G_{17} 10nM in complete media at 24hr intervals for a further 48hrs. Untreated and G_{17} 10nM alone treatments were used as negative and positive controls respectively, with YM022 and netazepide (100nM) treatments used as positive inhibition controls. After treatment, the media was removed and floating cells were counted. Adherent cells were washed twice with PBS and trypsinised until all cells were in suspension and these were then counted using a haemocytometer.

2.13 Cell migration assays

Monolayers of AGS_{GR} cells were grown on 24 well plates in complete media before a cell-free region was created using a 2µl pipette tip. Cells were washed twice in PBS followed by two washes in serum free media before the treatment was applied. Whole cells that had migrated into the denuded region were counted and scratch wound width was measured using a graticule at 0hrs and 8hrs post-treatment. Representative images were taken at these times using a Zeiss Aviovert 25 microscope (Carl Zeiss Microscopy, New York, USA).

2.14 Cell morphological assays

AGS_{GR} cells ($1x10^4$/well) were seeded in 24 well plates in complete media and left to adhere for 24hrs before treatment. After treatment, cells were fixed using 3:1 methanol: acetic acid and stained with 0.3% crystal violet. The number of cells that presented long processes were counted as a percentage of total cells in 3 reference fields (>100 cells) per treatment and representative images were taken using the Zeiss Aviovert 25 microscope (Carl Zeiss Microscopy, New York, USA).

2.15 Clonogenic survival assays

AGS_{GR} cells were seeded at a low density (500 cells/well in 2ml of complete media) in 6 well plates and left to adhere for 24hrs. Cells were pre-treated for 20mins with or without compounds TR1, TR2 or TR3 at a range of concentrations. Cells were then treated with or without 10nM G_{17} for 6hrs, followed by treatment with or without 8µM etoposide for 1hr. Fresh complete media was applied after etoposide treatment and the cells were left to form colonies. After 10-12 days, cells were washed and fixed using 3:1 methanol/acetic acid and stained with 0.3% crystal violet for 10mins. Colonies containing at least 100 cells were counted.

2.16 Immunofluorescence visualisation

AGS_{GR} cells were seeded onto 13mm diameter coverslips (VWR international Ltd, Leicestershire, UK) in 24 well plates (Costar, High Wycombe, UK) at a density of $1x10^4$/well in complete media and left to adhere for 24hrs before treatment was applied. After treatment, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30mins. Following the removal of the fixative, cells were washed a further two times with PBS and permeabilised with 0.2% PBT (0.03g BSA, 10ml PBS and 20µl Triton-X 100) at room temperature for 30mins.

For immunocytochemical analysis cells were blocked in 10% swine serum (Dako, Cambridgeshire, UK) for PAPPA2 antibody or 10% rabbit serum (Vectorlabs, Peterborough, UK) for p27 antibody for 45mins at room temperature before overnight incubation with either rabbit polyclonal anti-Plac3 (PAPPA2) primary antibody (Thermo Fisher Scientific, Hemel Hempstead, UK) or mouse monoclonal anti-p27 antibody (BD Biosciences, Oxford, UK) diluted 1:500 in PBS in a humidified chamber at 4°C. For enhanced specificity salt washes were applied to cells before incubation with swine anti-rabbit FITC conjugated secondary antibody (Dako, Cambridgeshire, UK) or rabbit anti-mouse FITC conjugated

secondary antibody (Vectorlabs, Peterborough, UK) diluted 1:500 in 1% BSA in PBS for 1hr, protected from light. For phalloidin staining, after fixation and permeabilisation cells were incubated with either Alexa Fluor™ 488 or Alexa Fluor™ 594 phalloidin diluted 1:500 in PBS in a humidified chamber for 1hr at room temperature protected from light.

Cells were washed three times in PBS for 10mins each, still protected from light, before mounting with Vectashield® mounting media with DAPI (Vectorlabs, Peterborough, UK) onto glass slides for visualisation. Images were captured using the Olympus BX51 fluorescence microscope (Olympus, Sussex, UK) at 6 reference fields (>100 cells) per treatment and relative intensities of nuclear and cytoplasmic staining was analysed using AxioVision Rel. 4.8 software. Cells stained with secondary antibody alone were used as non-specific binding controls.

2.17 EdU incorporation assays

AR42J cells ($1x10^4$/well) were seeded onto 13mm diameter coverslips in 24 well plates, in 1ml complete media and left to adhere for 24hrs. Cells were pre-treated in serum free media for 20 mins with or without compounds TR1, TR2, TR3, YM022 or YF476 at the minimum concentration which fully inhibited the effects of 10nM G_{17} in AGS_{GR} cells, and this was followed by treatment with or without 0.1nM G_{17} for 8hrs. Cells were visualised using the Click-iT® EdU Imaging Kit (Life Technologies, Paisley,UK) as follows: after 6hrs of 0.1nM G_{17} treatment, cells were additionally treated with 10µM EdU in DMSO for a further 2hrs. Cells were then fixed using 1ml 3.7% formaldehyde in PBS for 15mins at room temperature. The fixative was then removed and cells were washed twice with 1ml of 3% BSA in PBS. Cells were then permeabilised in 1ml 0.5% Triton X-100 in PBS for 20mins at room temperature, followed by two washes in 1ml 3% BSA in PBS. The Click-iT® reaction cocktail was prepared as per the manufacturer's instructions and 500µl was added to each well, cells were then incubated for 30mins at room temperature, protected from light.

Once the reaction cocktail was removed, cells were washed once with 1ml 3% BSA in PBS and once with 1ml PBS. For nuclear staining, 1ml Hoechst 33342 (component G) 1:2000 in PBS was added to each well and cells were incubated for 30mins at room temperature, protected from light. After nuclear staining, cells were washed twice with 1ml PBS and mounted onto slides using Vectashield® mounting medium (Vectorlabs, Peterborough, UK). Images were obtained using an Olympus BX51 fluorescence microscope and analysed using ImageJ. Cells demonstrating EdU fluorescence were scored as a percentage of total cells in three separate fields (>100 cells) per treatment.

2.18 Protein extraction and western blotting

Cell lysis was achieved by suspending harvested cell pellets on ice in radio immunoprecipitation (RIPA) buffer containing 425mM NaCl, 1% v/v Igepal CA-630, 1mM EDTA, 0.5% w/v sodium deoxycholate, 50mM Tris, 0.1% w/v SDS, 10mM NaF and 0.5mM sodium orthovanadate. Suspensions were centrifuged at 13,000rpm for 15mins at 4°C and the supernatant, which contains the protein, and transferred to fresh Eppendorf tubes which were stored at -80°C prior to use.

Protein concentrations were quantified by Quick start™ Bradford protein assay (Bio-Rad, Hertfordshire, UK) using standard concentrations of bovine serum albumin (BSA). Protein samples were denatured in Laemmli buffer at 95°C for 10mins and electrophoresed on 12% SDS-polyacrylamide gels, followed by transfer onto 0.45µm pore nitrocellulose membrane (mdi; membrane technologies INC, Ambala Cantonment, India). Nonspecific antibody binding was blocked by incubating the membrane in 5% non-fat milk in PBS with 0.1% Tween-20. Membranes were incubated with the following primary antibodies: mouse monoclonal p27 antibody (BD Biosciences, Oxford, UK) at a dilution of 1:5000 or mouse monoclonal anti-actin antibody (Neomarkers, Freemont, CA) at a dilution of 1:2500. The secondary antibody was horseradish peroxidase (HRP)-conjugated rabbit anti-mouse

immunoglobulins from Dako (Cambridge, UK) at a dilution of 1:2000. Membranes were developed using Supersignal (Pierce, Tattenhall, UK) and chemiluminescence was detected using a Bio-Rad ChemiDoc XRS+ (Bio-Rad, Hertfordshire, UK). Densitometry was performed using ImageLab software and results were normalised to the expression of actin.

2.19 Statistics

Data are presented as means or percentage of control ±SEM. All experiments were repeated in at least triplicate and either one-way ANOVA or two-way ANOVA with Sidak *post-hoc* test was used to establish statistical significance where appropriate. $P<0.05$ was considered significant. A Mann Whitney-U test was used to assess statistical differences between independent healthy patient samples and baseline samples of patients taking netazepide. A Wilcoxon signed ranked test with Bonferroni correction was subsequently used to determine significant differences between repeated samples from the same patients as not all data were normally distributed and $P<0.0125$ was considered significant after correction.

3 Results

3.1 Gastrin-induced miR-222 promotes gastric tumour development by suppressing p27

3.1.1 Abstract

Elevated circulating concentrations of the hormone gastrin contribute to the development of gastric adenocarcinoma and types-1 and -2 gastric neuroendocrine tumours (NETs). MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate proteins which in turn influence various biological processes. We hypothesised that gastrin induces the expression of specific gastric miRNAs within CCK2 receptor (CCK2R) expressing cells and that these mediate functionally important actions of gastrin. miRNA PCR arrays were used to identify changes in miRNA expression following G_{17} treatment of human gastric adenocarcinoma cells stably transfected with CCK2R (AGS_{GR}). miR-222 was further investigated using primer assays and samples from hypergastrinaemic mice and humans. Chemically synthesised mimics and inhibitors were used to assess cellular phenotypical changes associated with miR-222 dysregulation. Gastrin increased miR-222 expression in AGS_{GR} cells, with maximum changes observed at 10nM G_{17} for 24hrs. Signalling occurred via CCK2R and the PKC and PI3K pathways. miR-222 expression was increased in the serum and gastric corpus mucosa of hypergastrinaemic INS-GAS mice and miR-222 expression was further increased in gastric corpus biopsies of INS-GAS mice with *H. felis* infection. miR-222 expression decreased in the serum and gastric corpus biopsies of hypergastrinaemic patients with autoimmune atrophic gastritis and type-1 gastric NETs following treatment with the CCK2R antagonist netazepide. Gastrin-induced miR-222 overexpression resulted in reduced expression and cytoplasmic mislocalisation of p27, which in turn caused actin remodelling and increased migration in AGS_{GR} cells. These data indicate a novel mechanism contributing to gastrin-associated gastric tumour development. miR-222 may also be a promising biomarker for monitoring gastrin induced premalignant changes in the stomach.

3.1.2 Introduction

Elevated circulating concentrations of the gastric antral hormone gastrin are found in patients who are hypochlorhydric as a result of atrophic gastritis. Autoimmune atrophic gastritis predisposes to the development of type-1 gastric neuroendocrine (carcinoid) tumours (NETs) [664, 665]. Hypergastrinaemia is crucial for the development of these tumours, as surgical antrectomy to remove the source of hypergastrinaemia [666] or treatment with gastrin/CCK2 receptor antagonist drugs can reverse tumour development [459, 460]. *H. pylori* induced atrophic gastritis also results in hypergastrinaemia and this is thought to act as a co-factor during gastric adenocarcinoma development. This is supported by animal studies which have demonstrated accelerated *H. pylori* induced gastric carcinogenesis in transgenic hypergastrinaemic INS-GAS mice [667, 668].

Gastrin contributes to gastric tumour development via several cellular mechanisms. These are in addition to its well established role in regulating gastric acid secretion and include alterations in cell proliferation, apoptosis, migration, differentiation and angiogenesis (reviewed in [669, 670]). Moreover several proteins including Reg [671], MMP7 [191], MMP1 [188] and members of the urokinase plasminogen activator family of proteins [185] show increased expression in the stomach or serum of patients with hypergastrinaemia. Many of these proteins are thought to contribute to gastric tumourigenesis by altering key functions including cell migration and differentiation. Some may also have utility as biomarkers of tumour development. Gastrin exerts its effects in the stomach predominantly as a result of binding to the CCK2 receptor (CCK2R) on ECL cells. Downstream signalling occurs via a number of pathways, including protein kinase C (PKC), MAP kinase (MAPK), and phosphatidylinositol (PI) 3-kinase (PI3K) [669].

MicroRNAs (miRNAs) are a class of endogenous non-protein coding short RNAs that post-transcriptionally regulate approximately 30% of the human genome [470, 476]. They inhibit the

translation, increase cleavage or induce the degradation of target mRNAs depending upon complementary RNA-RNA binding [540]. As miRNAs control a large proportion of the genome, their expression patterns are tissue-specific and dysregulation has been observed in many malignancies [545], suggesting the potential for miRNAs to be biomarkers of cancer diagnosis, prognosis and response to therapies. One gene can be regulated by many miRNAs and likewise one miRNA can regulate several genes, including tumour suppressor genes and oncogenes. This adds an additional layer of functional complexity, as miRNAs can act as both 'oncomiRs' to promote tumour development or 'anti-oncomiRs' to suppress tumour development, depending upon their tissue expression [546]. Moreover, >50% miRNA genes are located within fragile sites and genomic regions associated with deletion, translocation and amplification in cancers, further indicating their significance during carcinogenesis [547].

I hypothesised that gastrin may exert some of its pro-tumourigenic effects in the stomach by altering the expression of specific microRNAs, which in turn alter the expression of downstream proteins regulating key cellular processes involved in gastric tumour progression. I therefore investigated which miRNAs showed altered expression following G_{17} treatment of a CCK2 receptor expressing gastric epithelial cell line. One of the upregulated miRNAs, miR-222, was further investigated using samples obtained from hypergastrinaemic mice and humans and upstream and downstream signalling pathways were defined in AGS_{GR} cells using various inhibitor compounds and siRNA approaches.

3.1.3 Gastrin induces miR-222 expression in AGS_{GR} cells

miScript miRNA PCR arrays were used to identify differentially expressed miRNAs between AGS_{GR} cells treated with and without 10nM G_{17} for 24hrs (N=6, n=2). Three miRNAs showed increased expression and three miRNAs showed decreased expression beyond the 2-fold threshold (Figure 10A). However, only miR-376c and miR-222 proved significant with fold changes of 5.2 ($P<0.01$) and 2.3 ($P<0.0001$) respectively (for all miRNA expression profiles see Table 2. in **6. Supplementary data**).

Further validation by qPCR primer assays was performed to confirm these differences and normalised against endogenous RNU62 expression. As miR-376c was of relatively low abundance in both the control and gastrin-treated cells, miR-222 was chosen for further investigation. In parental AGS cells (not stably transfected with the CCK2R), gastrin treatment had no significant effect on miR-222 expression at concentrations of 0-100nM for 2-48hrs. However in AGS_{GR} cells which express the CCK2R, miR-222 expression increased dose and time dependently following gastrin treatment and this was maximal after administering 10nM G_{17} for 24hrs (Figure 10B and 10C) ($P<0.0001$, N=3, n=4).

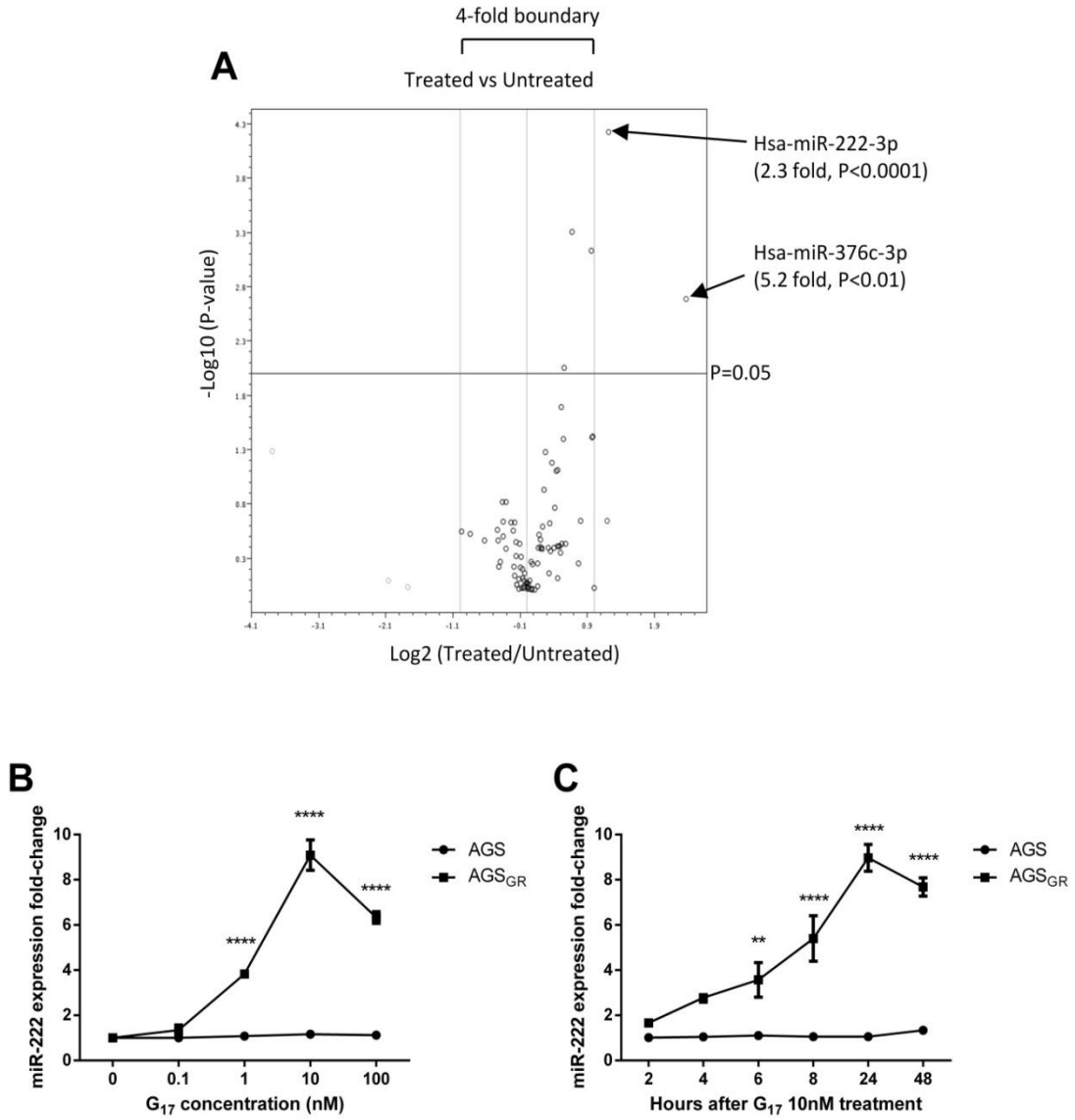

Figure 10. In AGS_{GR} cells treated with 10nM G_{17} compared with untreated controls, miScript miRNA PCR arrays (N=6, n=2) showed 3 miRNAs that increased and 3 miRNAs that decreased in expression beyond the 2-fold threshold (of the 84 biologically relevant arrayed miRNAs), with only miR-222 (P<0.0001) and miR-376c (P<0.01) proving significant (A). qPCR primer assays showed that miR-222 expression increased dose and time dependently in AGS_{GR} cells and was maximal following treatment with 10nM G_{17} for 24hrs in serum free media (P<0.0001, N=3, n=4). However, miR-222 expression did not significantly change following G_{17} treatment of untransfected AGS cells (B, C). Statistical significance was determined using student t-test (A) or two-way ANOVA (B, C) with Sidak *post-hoc* test and P<0.05 was considered significant (**P<0.01, ***P<0.001 and ****P<0.0001 vs untreated controls at the same time point).

3.1.4 Activation of the CCK2 receptor by gastrin leads to increased miR-222 expression via the PKC and PI3K pathways in AGS_{GR} cells

In order to investigate the signalling mechanisms downstream of CCK2R that were responsible for the observed increase in miR-222 expression, we used known inhibitors of these pathways. AGS_{GR} cells were pre-treated with the following signalling pathway inhibitors: Ro-32-0432 (PKC inhibitor), LY294002 (PI3K inhibitor), PD98059 (inhibitor of MAPK activation) and netazepide or YM022 (both CCK2R antagonists) for 20mins prior to treating the cells for 24hrs with 10nM G_{17}. miR-222 expression was evaluated using qPCR primer assays. As miRNAs are regulated by many mRNAs and *vice versa,* miRNAs may be involved in multiple signalling pathways. Small nuclear RNAs, such as the housekeeping gene RNU62 used for normalisation, may therefore also be regulated by the particular pathways investigated. Therefore, an exogenous spike in control (*C.elegans* miR-39) was added to each sample at a known concentration () and used for normalisation. Untreated, 10nM G17 treated and 1% DMSO treated cells were used as negative, positive and vehicle controls.

Gastrin-induced miR-222 overexpression was almost fully reversed when AGS_{GR} cells were pre-treated with Ro-32-0432 (1μM) ($P<0.0001$), LY294002 (20μM) ($P<0.0001$), YM022 (100nM) ($P<0.0001$) and netazepide (100nM) ($P<0.0001$) suggesting that miR-222 expression is increased via activation of the CCK2 receptor and subsequent PKC and PI3K pathways. However, there was only a partial reversal caused by the inhibitor of MAPK activation ($P<0.001$), indicating that this is not the major pathway for miR-222 expression (Figure 11A) (N=3, n=4). Further investigation of downstream signalling was performed by pre-treating AGS_{GR} cells with and without PKC and PI3K inhibitors followed by activation of PKC via PMA 100nM for 24hrs. The activation of PKC stimulated a significant increase in miR-222 expression ($P<0.001$) which was also significantly, but not completely reversed by

pre-treatment with the PI3K inhibitor ($P<0.05$). These data indicate that gastrin-CCK2 receptor activation increases miR-222 expression via both the PKC and PI3K pathways in AGS_{GR} cells (Figure 11B) (N=3, n=4).

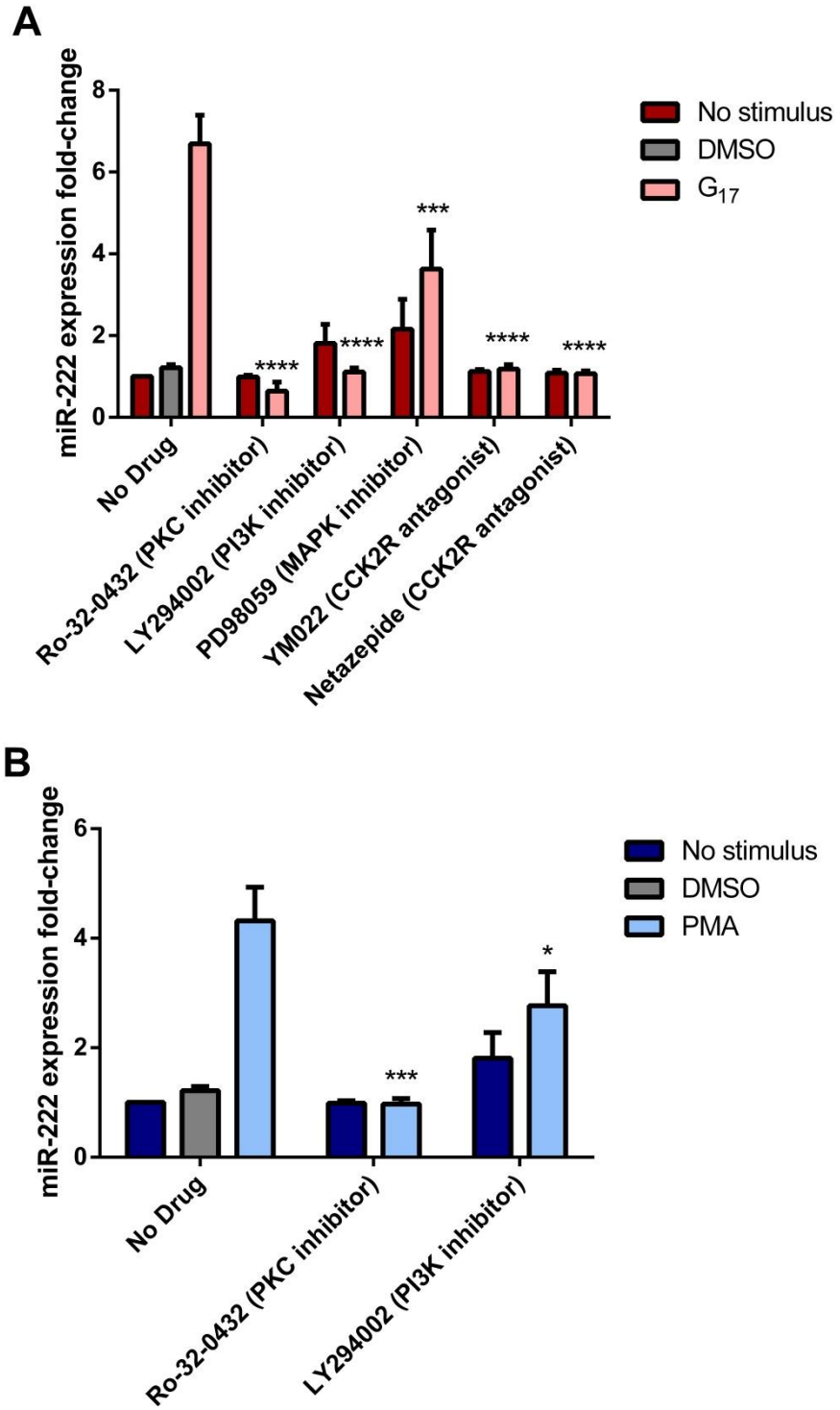

Figure 11. In PCR primer assays, LY294002 (20µM), YM022 (100nM), netazepide (100nM) and Ro-32-0432 (1µM) all completely reversed while PD98089 (20µM) partially reversed the miR-222 overexpression caused by 10nM G_{17} treatment of AGS_{GR} cells for 24hrs (A) (N=3, n=4). Ro-32-0432 (1µM) also completely reversed while LY294002 (20µM) partially reversed the miR-222 overexpression induced by 100nM PMA treatment of the same cell line for 24hrs (B) (N=3, n=4). Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (*P<0.05, ***P<0.001 and ****P<0.0001 vs no drug controls).

3.1.5 miR-222 expression increases with age and further increases with *Helicobacter felis* (*H. felis*) infection, in hypergastrinaemic INS-GAS mice

As previously reported, miRNAs are highly stable in both blood and tissues [587, 588]. miR-222 expression was therefore assessed by qPCR in male FVB/N and transgenic INS-GAS mice on the same genetic background. Mice were culled at 12 and 30 weeks of age (n=10 per group) and gastric corpus mucosal scrapings and serum were used for primer assays. miR-222 expression was significantly increased in both the gastric mucosal scrapings ($P<0.01$) and serum ($P<0.0001$) of 30 week old INS-GAS mice compared with age-matched FVB/N wild-type, with no significant differences being observed between 12 week and 30 week old FVB/N wild-type mice. There was also a statistically significant difference between 12 week INS-GAS and FVB/N wild type mice ($P<0.05$) in the serum (Figure 12A and 12B) (N=3, n=4).

miR-222 expression was additionally assessed in INS-GAS mice that had been infected with *H. felis* for 6 weeks. At 6 weeks of age, mice were given 3 doses of *H. felis* via oral gavage 48hrs apart and culled after a further 6 weeks. Gastric corpus mucosal scrapings were used for miR-222 primer assays. *H. felis* infection further increased miR-222 expression compared with age-matched uninfected FVB/N (0.0001) and INS-GAS mice ($P<0.01$) (n=9 per group) (Figure 12C) (N=3, n=4).

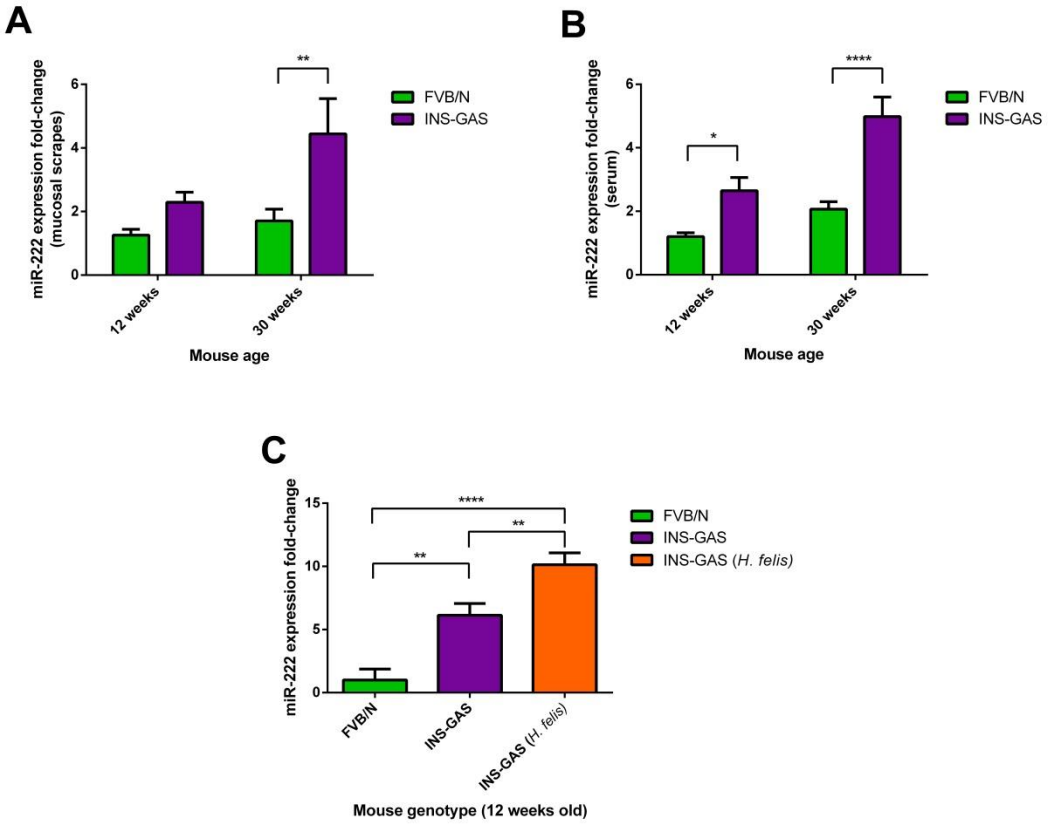

Figure 12. qPCR primer assays showed that miR-222 expression was significantly increased in mucosal scrapings taken from the gastric corpus (A) and in the serum (B) of 30 week old male hypergastrinaemic INS-GAS mice relative to age and sex-matched FVB/N mice, with significant differences also being observed between 12 week INS-GAS and FVB/N mice in the serum (n=10 per group) (N=3, n=4). miR-222 expression was further significantly increased in 6 week *H. felis* infected INS-GAS mice compared with age-matched FVB/N and INS-GAS uninfected mice (n=9 per group) (C) (N=3, n=4). Statistical significance was determined using either one-way (C) or two-way ANOVA (A, B) with Sidak *post-hoc* test and $P<0.05$ was considered significant (*$P<0.05$, **$P<0.01$ and ****$P<0.0001$ between groups).

3.1.6 miR-222 expression is increased in the serum and gastric corpus of patients with hypergastrinaemia and type-1 gastric neuroendocrine tumours, and is significantly reduced by netazepide treatment

We also assessed miR-222 expression by qPCR in both gastric corpus biopsies and serum samples from patients with autoimmune atrophic gastritis, hypergastrinaemia and type-1 gastric NETs who had been enrolled on a phase-2 clinical trial to assess the short and long term effects of the CCK2R antagonist netazepide [459]. In addition to the initial 12 week trial, the results of which have been reported, patients were treated for an additional 12 months and showed ongoing histological and biochemical evidence of tumour regression (manuscript in preparation).

In both the short-term (12-week treatment with 12-week follow-up) and longer-term (12-month treatment) regimens, there was a significant but small increase in miR-222 expression in the gastric corpus biopsies of hypergastrinaemic patients before taking netazepide relative to normogastrinaemic controls ($P<0.001$ short term, $P<0.0125$ long term), which significantly decreased whilst patients were taking 50mg netazepide daily ($P<0.0125$) and returned to baseline after cessation of treatment, (Figure 13A and 13B)(N=8, n=3).

miR-222 expression was also significantly increased in the serum of the same hypergastrinaemic patients with a 5.7 fold increase in the short-term study ($P<0.0001$) and a 5-fold increase in the longer study ($P<0.0001$), when compared to healthy controls. Similarly, whilst patients were taking netazepide, serum miR-222 expression significantly decreased and returned towards baseline after cessation of treatment (Figure 13C and 13D) (N=8, n=3).

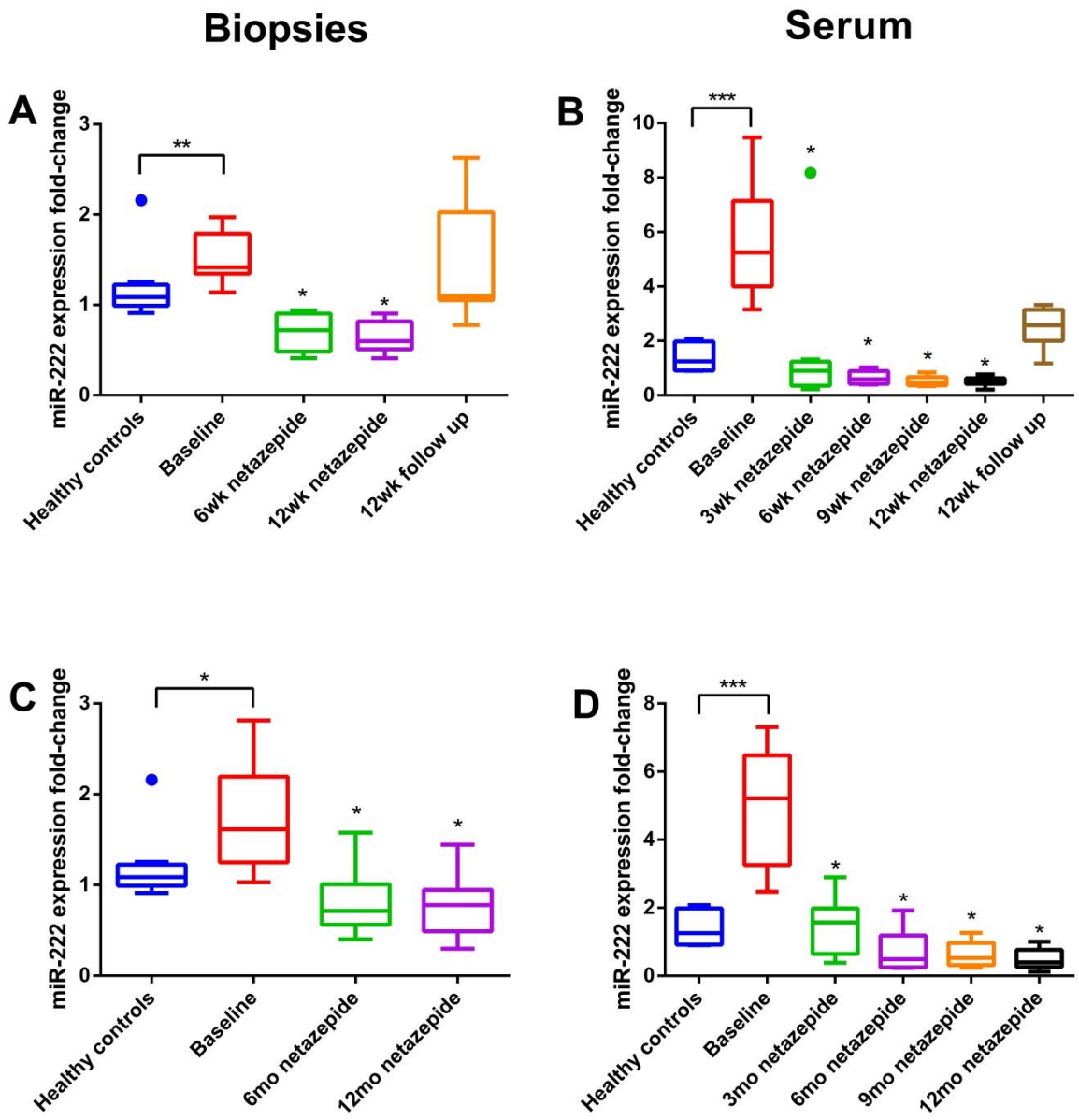

Figure 13. In both gastric corpus biopsies (A, C) and serum samples (B, D) from patients with hypergastrinaemia and type-1 gastric neuroendocrine tumours (n=8), qPCR miR-222 expression was significantly higher at baseline compared with normogastrinaemic healthy controls who had a normal stomach at endoscopy (n=10). miR-222 expression decreased whilst patients were taking netazepide and returned to baseline after cessation of treatment, in short (12 weeks, A and B) and long (1 year, C and D) term studies. Statistical significance was determined using a Mann Whitney U test between healthy controls and baseline, and by Wilcoxon signed rank test with Bonferroni correction between treatment visits. P<0.0125 was considered significant after correction (*P<0.0125, **P<0.001 and ***P<0.0001).

3.1.7 miR-222 overexpression decreases proliferation and increases migration and the extension of long processes in AGS_{GR} cells

Gastrin has been shown to both stimulate and inhibit AGS cell proliferation and previous studies by Varro *et al.* suggested that the expression of the cell surface receptor CCK2R has been associated with reduced cell growth in AGS_{GR} cells [663]. Chemically synthesised miR-222 mimics at concentrations >10nM also significantly reduced AGS_{GR} cell proliferation after 48hrs transfection in a dose dependent manner (Figure 14A) (N=3, n=3). Alternatively, chemically synthesised miR-222 inhibitors (>50nM) significantly reversed the anti-proliferative effects of 24hrs G_{17} treatment after 72hrs transfection in AGS_{GR} cells, again in a dose-dependent manner (Figure 14B) (N=3, n=3).

Gastrin has previously been shown to increase the migration of AGS_{GR} cells dose dependently after 8hrs treatment with concentrations of 30pM to 3nM G_{17} [672]. Incubation of AGS_{GR} cells with 10nM G_{17} for 8hrs significantly stimulated cell migration in scratch wound assays (Figure 15A) when compared to untreated AGS_{GR} cells. Chemically synthesised miR-222 mimics at concentrations 10-100nM also significantly increased AGS_{GR} cell migration dose dependently (Figure 15B) (N=3, n=3). By contrast, chemically-synthesised miR-222 inhibitors significantly reversed 10nM G_{17}-induced AGS_{GR} cell migration at concentrations 50-100nM and completely reversed gastrin-stimulated (10nM) migration at a concentration of 500nM (Figure 15C) ($P<0.0001$, N=3, n=3).

Incubation of AGS_{GR} cells with 1nM G_{17} has also previously been shown to induce cell scattering and the extension of long processes which were maximal after 6hrs treatment [673]. Similar responses were observed in AGS_{GR} cells treated with 10nM G_{17} for 6hrs (Figure 16A), with visible morphological changes observed with the extension of long processes (arrows). Chemically synthesised miR-222 mimics dose dependently increased the extension of long processes in AGS_{GR} cells and this was significant at concentrations >10nM

(Figure 16B) ($P<0.0001$, N=3, n=3). Chemically synthesised miR-222 inhibitors dose dependently reversed the extension of long processes induced by 10nM G_{17} in AGS_{GR} cells, which was significant after treatment with concentrations >50nM (Figure 16C) ($P<0.001$, N=3, n=3).

AGS_{GR} cells transfected with miR-1 (10-100nM) also showed a significant increase in the extension of long processes ($P<0.01$ and $P<0.001$), compared with untreated controls. However, miR-1 is physiologically expressed at low levels in AGS_{GR} cells and has previously been associated with cell differentiation in cardiomyocytes [674] and skeletal muscle [675], which may account for the changes in cell morphology observed in the scattering assays.

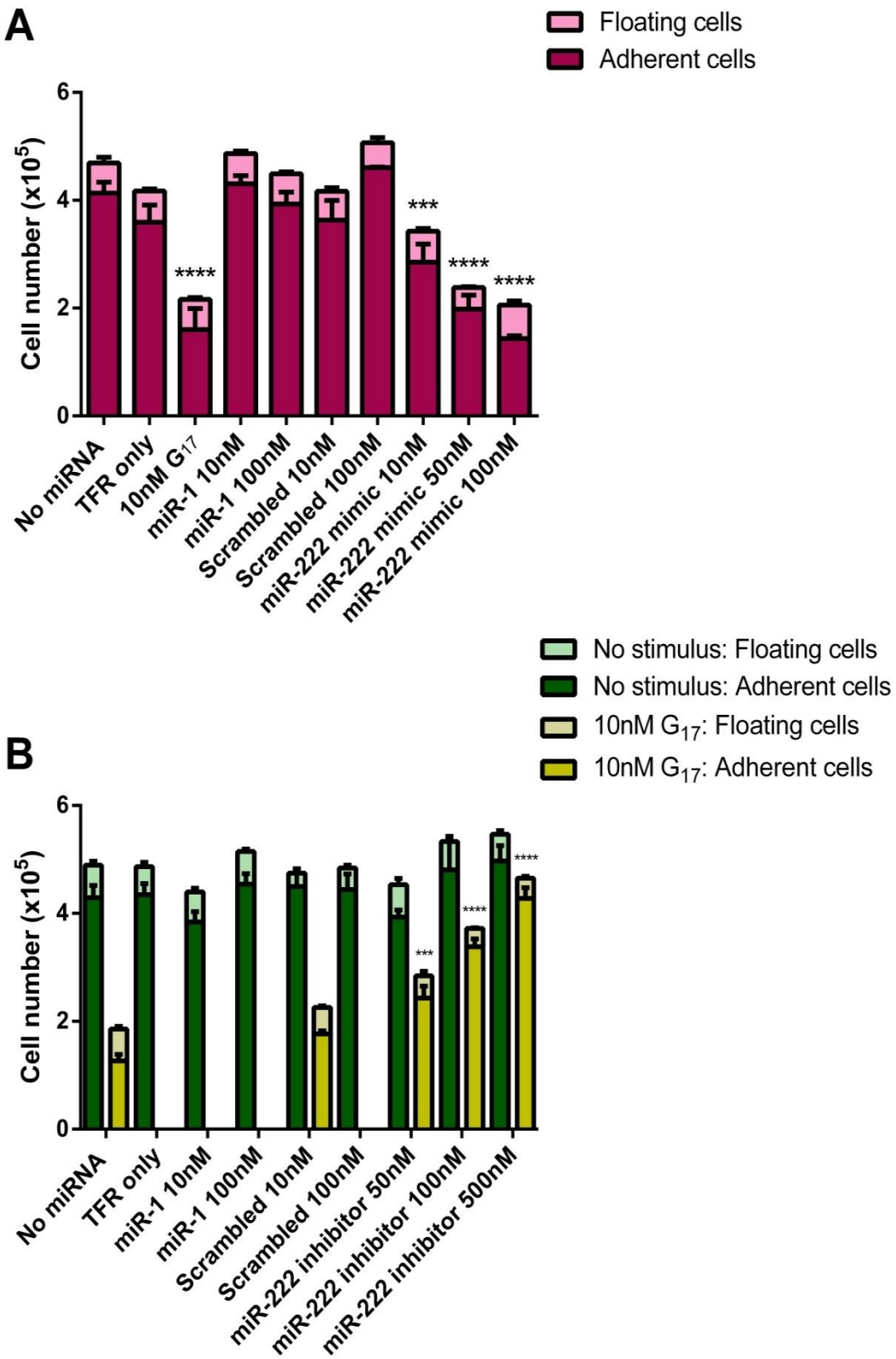

Figure 14. Haemocytometer counts showed that transfection (48hrs) with a chemically synthesised miR-222 mimic significantly reduced AGS_{GR} cell growth in a dose dependent manner after 10nM concentration (A) (N=3, n=3). Chemically synthesised miR-222 inhibitors (72hrs) dose dependently reversed the anti-proliferative effects of 10nM G_{17} for 24hrs which was significant after 50nM concentration (B) (N=3, n=3). The miR-222 mimic and miR-222 inhibitor were not cytotoxic as the number of floating cells did not differ from the untreated controls. Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (***P<0.001 and ****P<0.0001 vs no miRNA controls).

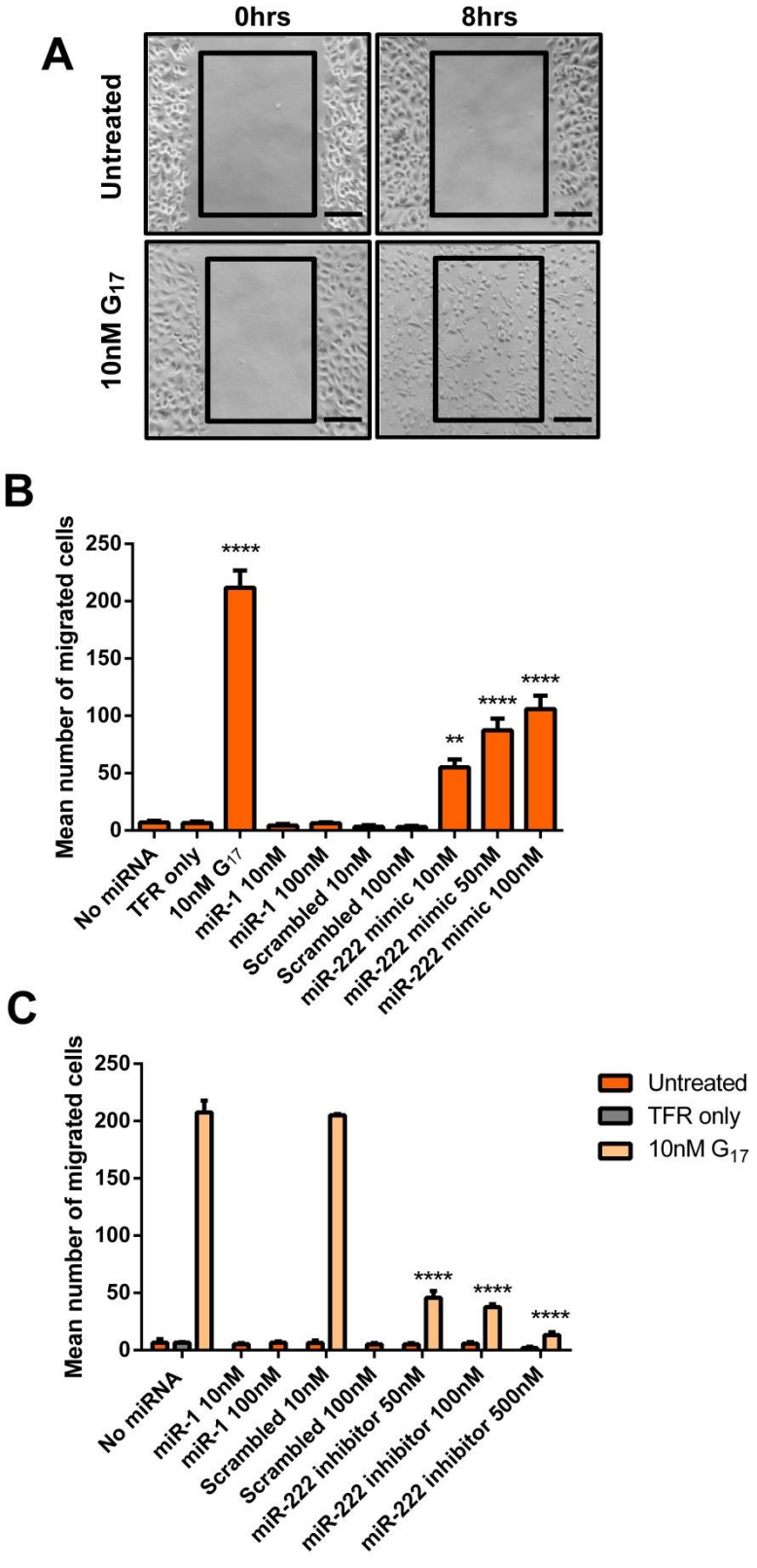

Figure 15. Scratch wound assays were performed to assess cell migration (A) following G_{17} treatment of AGS_{GR} cells (scale bar 50μm). Chemically synthesised miR-222 mimics significantly increased migration (B) in a dose dependent manner with statistical significance determined using one way ANOVA with Sidak *post-hoc* test (N=3, n=3). Whereas miR-222 inhibitors significantly reduced the migration (C) that were stimulated by 10nM G_{17} treatment of AGS_{GR} cells, in a dose dependent manner and statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test (N=3, n=3). P<0.05 was considered significant (**P<0.01 and ****P<0.0001 vs no miRNA controls).

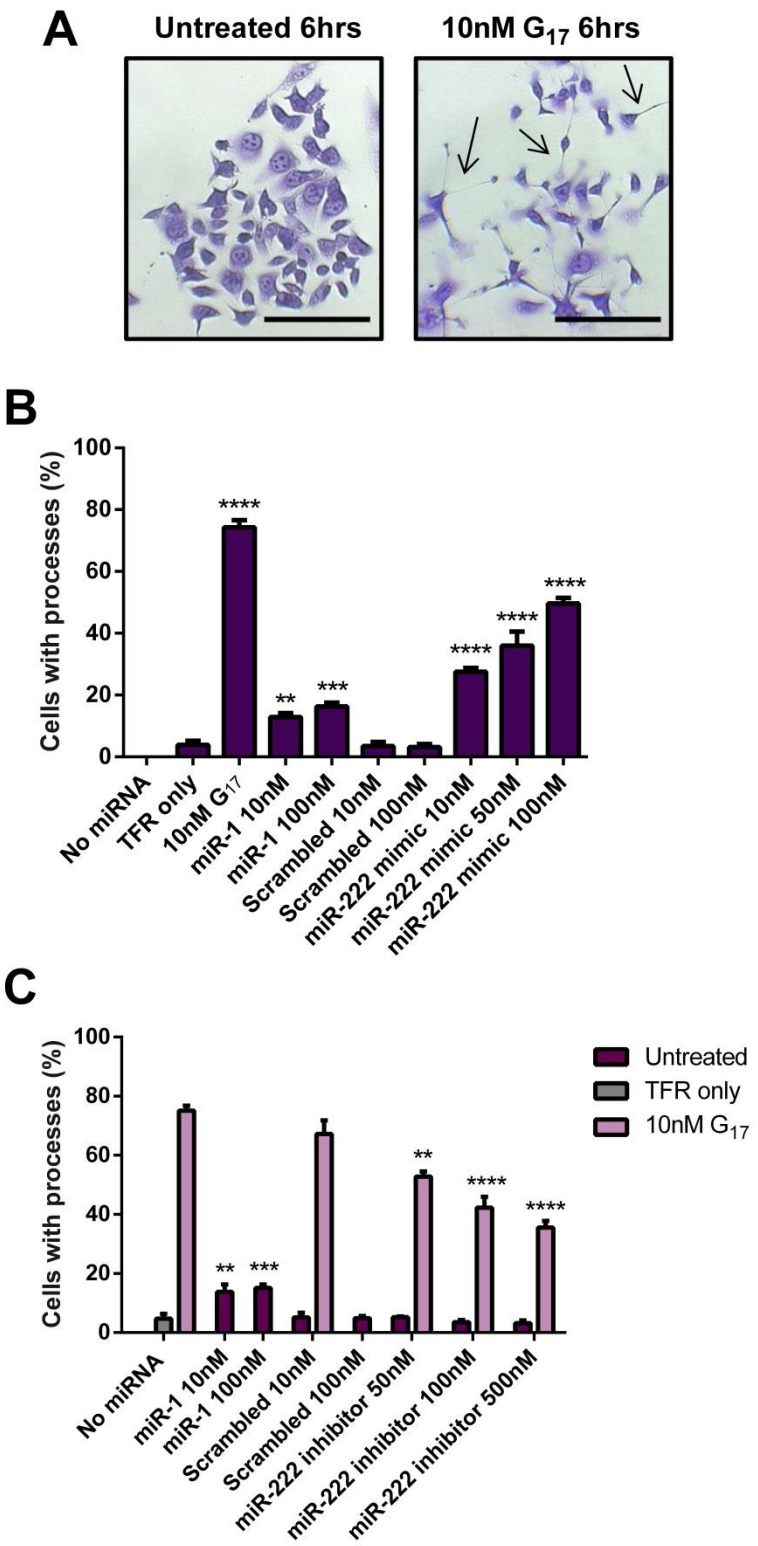

Figure 16. Scattering assays were performed to assess the expression of long processes (A) following G_{17} treatment of AGS_{GR} cells (scale bar 100µm). Chemically synthesised miR-222 mimics significantly increased the extension of long processes (A) in a dose dependent manner with statistical significance determined using one way ANOVA with Sidak *post-hoc* test (N=3, n=3). Whereas miR-222 inhibitors significantly reduced the extension of long processes (C) that were stimulated by 10nM G_{17} treatment of AGS_{GR} cells, in a dose dependent manner and statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test (N=3, n=3). P<0.05 was considered significant (**P<0.01, ***P<0.001 and ****P<0.0001 vs no miRNA controls).

3.1.8 Gastrin-induced miR-222 overexpression decreases the expression of p27 *in vitro* and *in vivo*, via the PKC and PI3K pathways

In AGS_{GR} cells, qPCR primer assays showed that p27 mRNA expression decreased in dose (Figure 17A) and time (Figure 17B) dependent manners following G_{17} treatment, which was maximal after 10nM G_{17} for 24hrs ($P<0.0001$, N=3, n=4). Western blots indicated that AGS_{GR} cells incubated with G_{17} also showed dose (Figure 17C) and time (Figure 17D) dependent decreases in p27 protein expression. This was again significant after treating cells with 10nM G_{17} for 24hrs ($P<0.05$, N=3).

Immunofluorescence visualisation showed a dose dependent increase in cytoplasmic abundance of p27 that was significant at a concentration 0.1nM G_{17} for 24hrs. A reciprocal dose dependent decrease in nuclear p27 expression was also observed after 0.1nM G_{17} for 24hrs (Figure 18A) ($P<0.001$, N=3, n=6). p27 shuttling between the nucleus and cytoplasm was observed <6hrs after 10nM G_{17} ($P<0.001$). After 8hrs 10nM G_{17}, there was a significant decrease in the nuclear abundance and increase in the cytoplasmic abundance of p27 in a time-dependent manner (Figure 18B) ($P<0.0001$, N=3, n=6). Representative images were taken at 8hrs and 24hrs after 10nM G_{17} treatment for visual comparisons (Figure 19).

Western blotting showed thatthe decreased p27 protein expression caused by 10nM G_{17} for 24hrs was completely reversed by pre-treatment of cells with Ro-32-0432 (1μM), LY294002 (20μM), YM022 (100nM) and netazepide (100nM) and partially reversed by PD98059 (20μM) (Figure 20) ($P<0.05$, N=5).

AGS_{GR} cells transfected with a chemically synthesised miR-222 mimic showed a dose dependent decrease in p27 protein expression (Figure 21A); this was significant at miR-222 mimic concentrations >50nM ($P<0.01$, N=3). The chemically synthesised miR-222 inhibitor at 500nM concentration also significantly reversed the decrease in p27 protein expression caused by 10nM G_{17} (Figure 21B) ($P<0.01$, N=3).

In 30 week old male hypergastrinaemic INS-GAS mice, p27 mRNA expression was also significantly decreased relative to age and sex-matched FVB/N wild-type mice ($P<0.05$), with no significant differences shown between 12 week INS-GAS and FVB/N mice (n=10 per group) (Figure 22). These data suggest that gastrin stimulates CCK2R activation leading to increased miR-222 expression which in turn decreases p27 mRNA and protein expression via the PKC and PI3K signalling pathways.

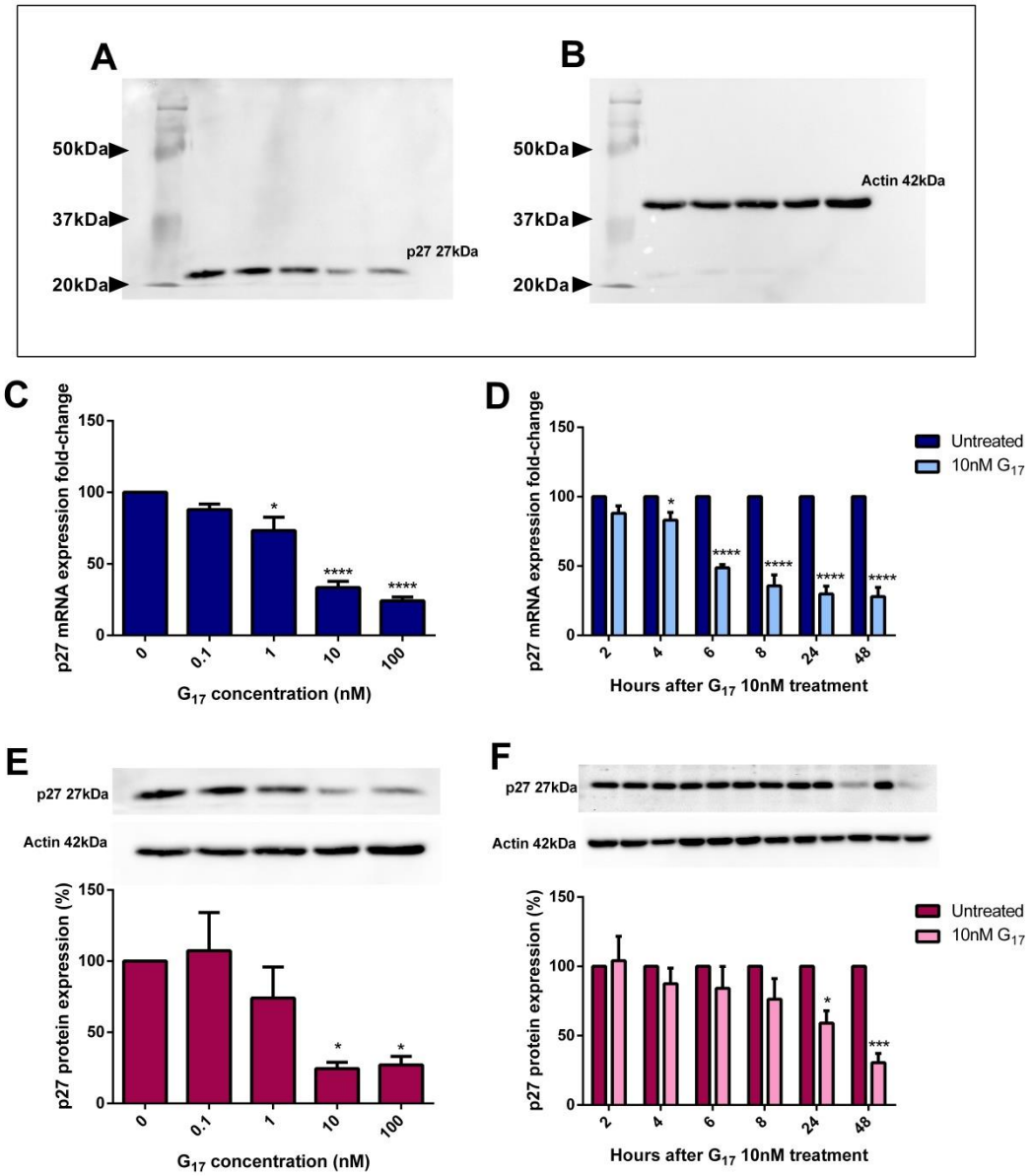

Figure 17. Representative western blots for p27 (A) and after stripping and re-probing the same blot for actin (B) which confirm correct band sizes. p27 mRNA expression significantly decreased dose (C) and time (D) dependently in AGS_{GR} cells treated with gastrin (N=3, n=4). p27 protein expression dose (E) and time (F) dependently decreased following gastrin treatment of AGS_{GR} cells (N=3). p27 decreased expression was maximal after 10nM G_{17} for 24hrs. Statistical significance determined using one way ANOVA (C, E) with Sidak *post-hoc* test and P<0.05 was considered significant (*P<0.05 and ****P<0.0001 vs untreated control) or two-way ANOVA (D, F) with Sidak *post-hoc* test and P<0.05 was considered significant (*P<0.05, ***P<0.001 and ****P<0.0001 vs untreated control at the same time point).

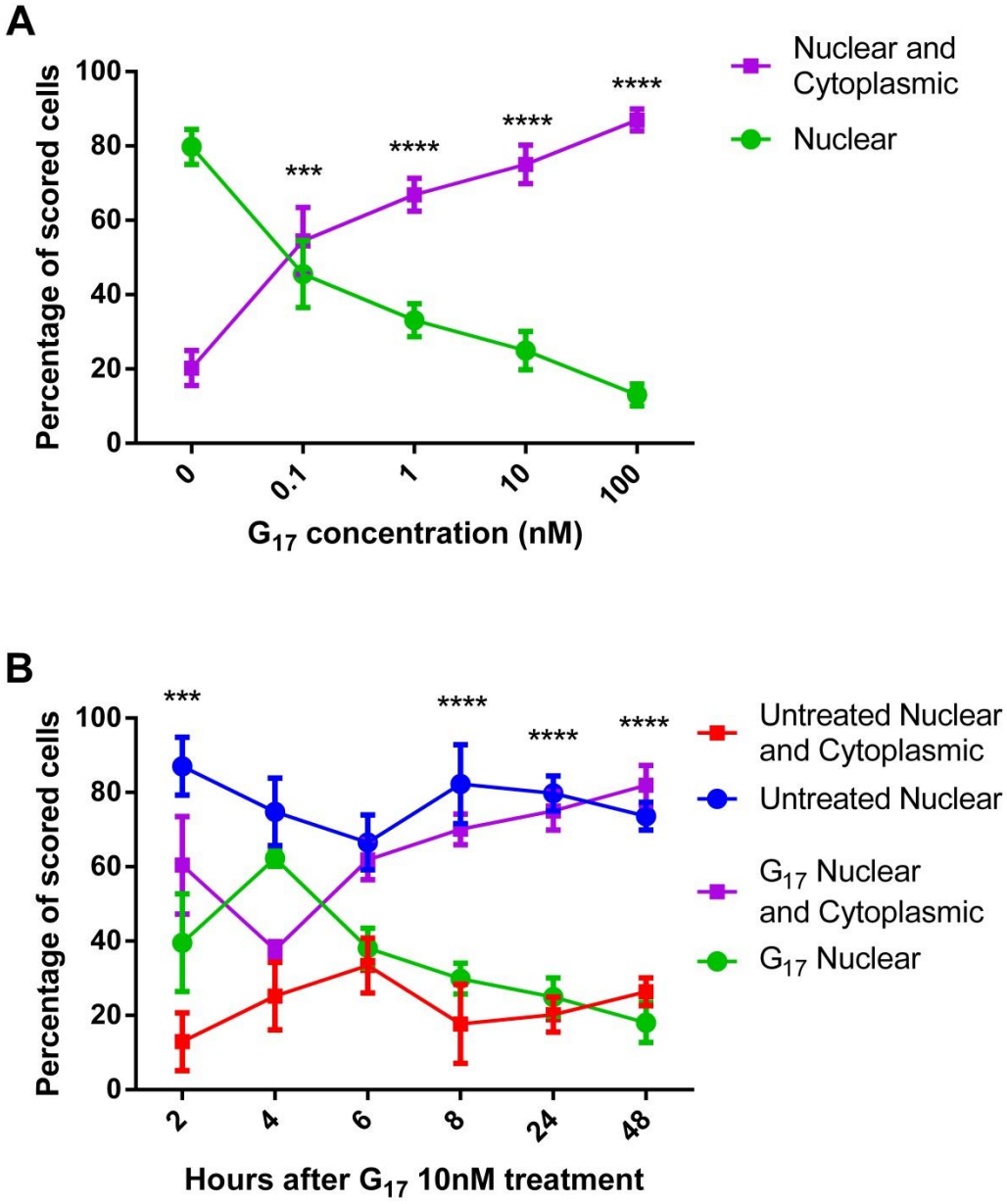

Figure 18. Immunofluorescence staining of AGS_{GR} cells= showed that the nuclear expression of p27 decreased and cytoplasmic expression of p27 increased dose dependently with G_{17} treatment, which was significant after 0.1nM concentration 24hrs (A) (N=3, n=6). 10nM G_{17} treatment of AGS_{GR} cells (2-6hrs) showed the shuttling of p27 with a significant increase in cytoplasmic mislocalisation of p27 after 8hrs, in a time-dependent manner (B) (N=3, n=6). Statistical significance determined using two-way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (***P<0.001 and ****P<0.0001 vs untreated control at the same time point).

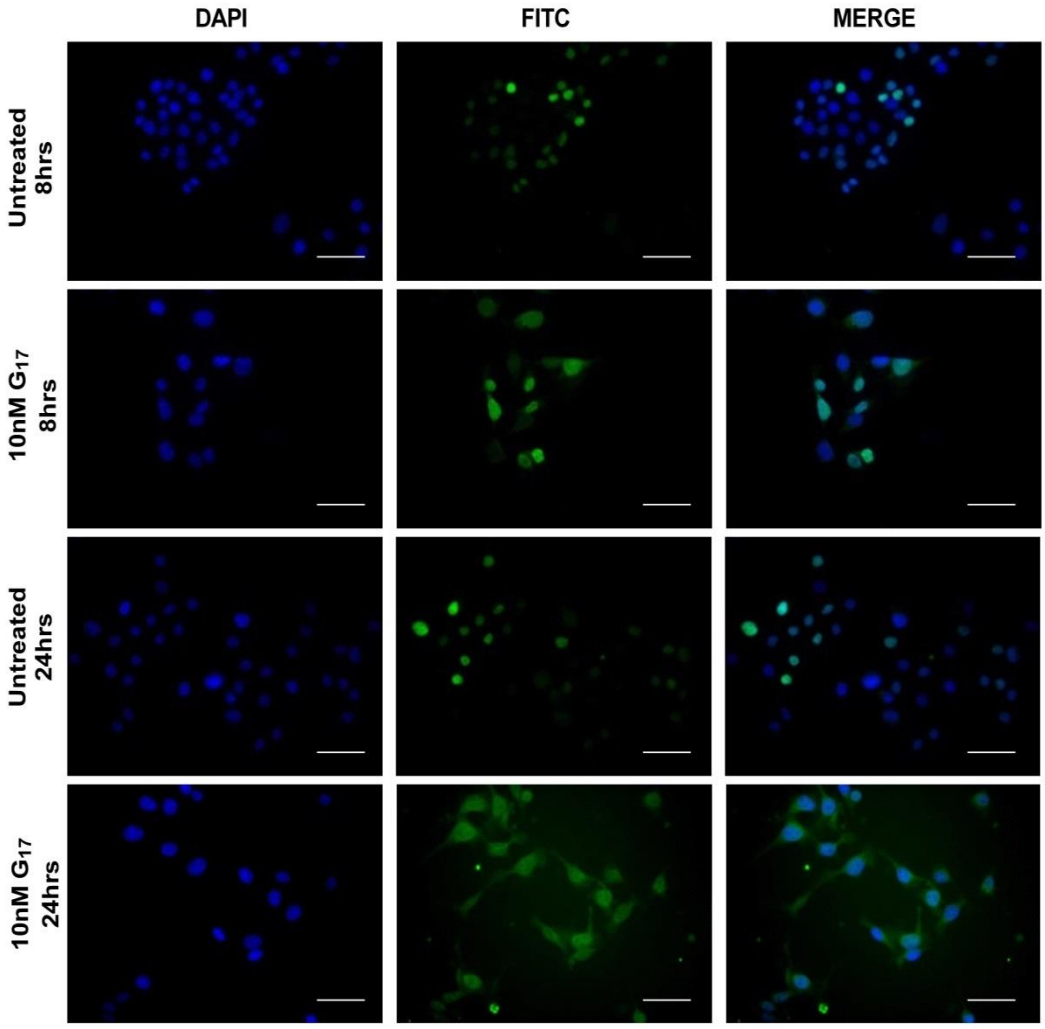

Figure 19. Representative images were taken of cellular p27 localisation by immunofluorescence staining with mouse monoclonal anti-p27 antibody (1:500) followed by FITC conjugated rabbit anti-mouse horseradish peroxidase secondary (1:500) antibody (green) in AGS_{GR} cells with and without 10nM G_{17} treatment for 8hrs and 24hrs. Nuclear staining with DAPI was used as an intensity control (blue) (scale bar 100µm) (N=3, n=6). Incubation with rabbit anti-mouse FITC conjugated HRP secondary antibody alone was used as a non-specific binding (negative) control.

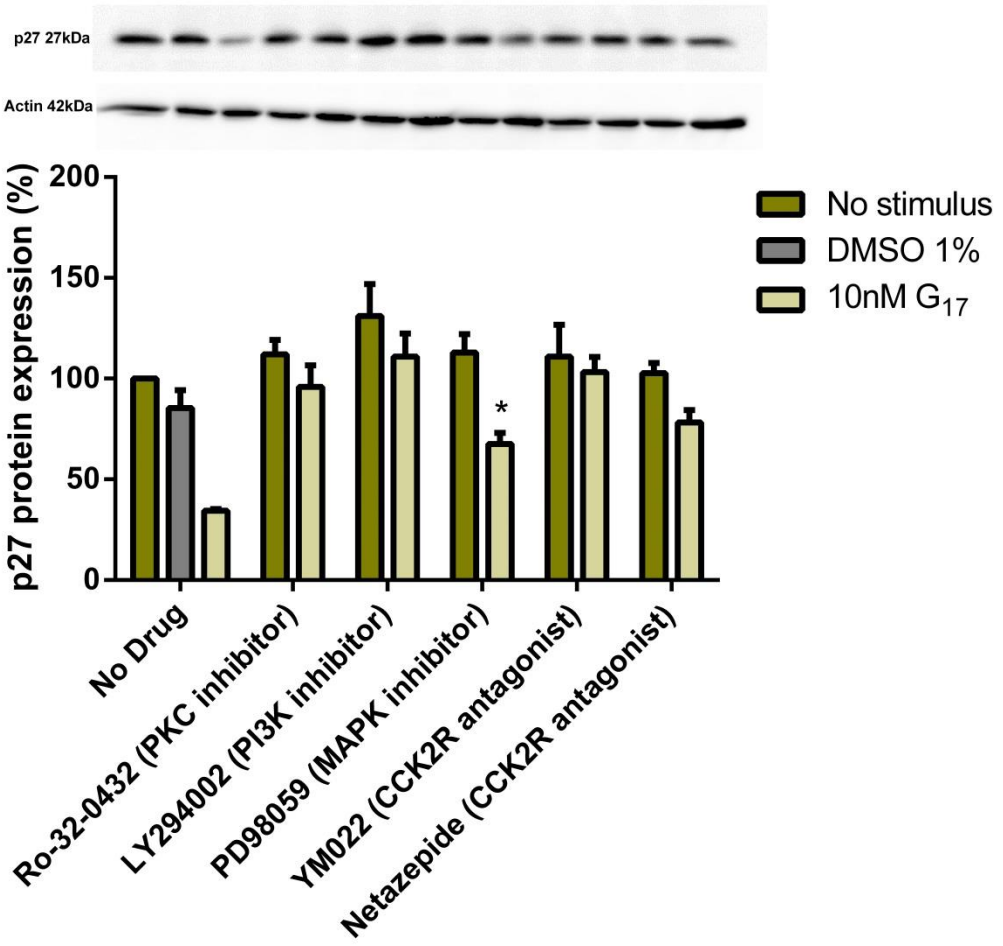

Figure 20. Western blot analysis indicated that LY294002 (20μM), YM022 (100nM), netazepide (100nM) and Ro-32-0432 (1μM) all completely reversed while PD98089 (20μM) partially reversed the reduction in p27 expression caused by 10nM G17 treatment for 24hrs. Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and $P<0.05$ was considered significant. (*$P<0.05$ vs no drug controls) (N=5).

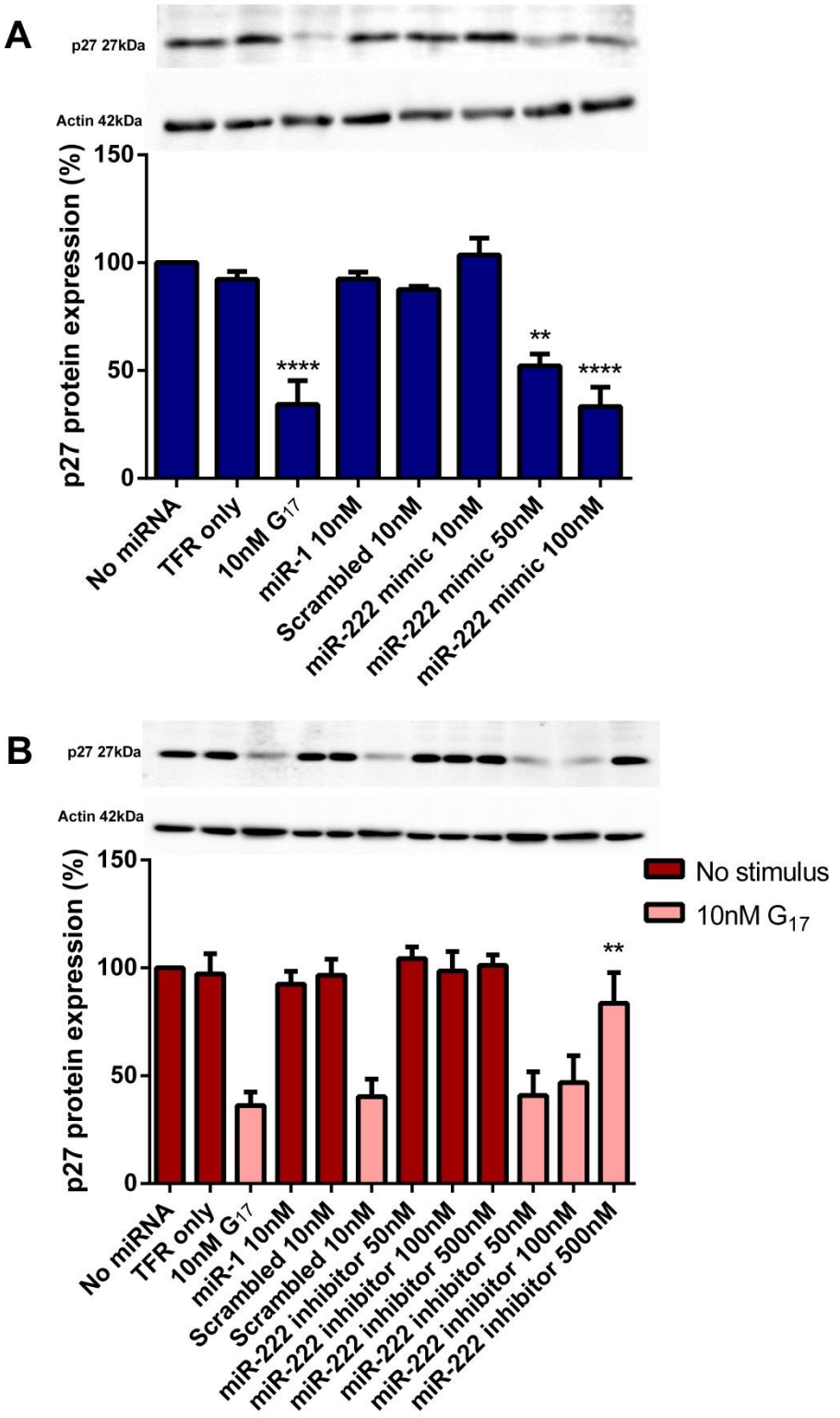

Figure 21. Using primer assays, a chemically synthesised miR-222 mimic significantly reduced p27 expression (A) after 50nM concentration, whereas a chemically synthesised miR-222 inhibitor (500nM) significantly reversed the reduced p27 expression that was caused by 10nM G_{17} treatment for 24hrs (B) in AGS_{GR} cells. Statistical significance was determined using one way ANOVA (A) with Sidak *post-hoc* test and P<0.05 was considered significant (**P<0.001 and ****P<0.0001 vs scrambled 10nM) or two-way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (**P<0.01 vs scrambled 10nM).

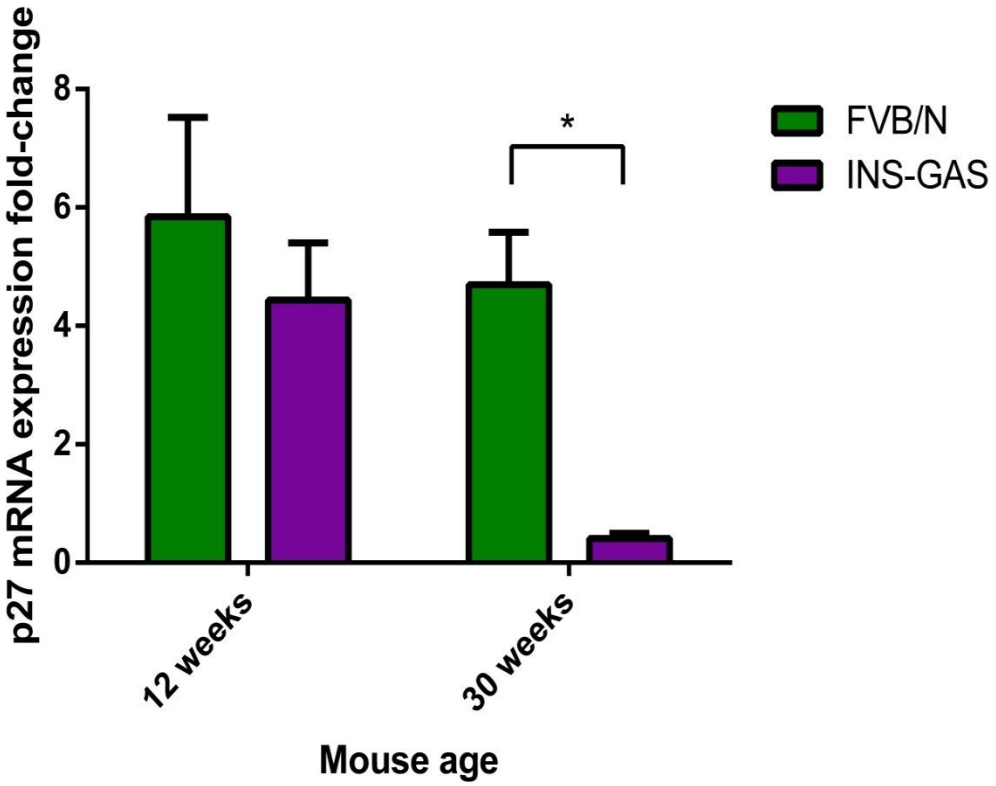

Figure 22. qPCR analysis of p27 mRNA expression also significantly decreased with age in the gastric corpus mucosa of 30 week old hypergastrinaemic INS-GAS mice compared with age-matched FVB/N wild-type mice, with no significant differences between 12 week old INS-GAS and FVB/N mice (n=10 per group). Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and $P<0.05$ was considered significant (*$P<0.05$).

3.1.9 The reduced p27 expression induced by gastrin decreases proliferation and increases migration and the extension of long processes in AGS_{GR} cells

AGS_{GR} cells were transfected with CDKN1B (p27) siRNA at concentrations between 10-50nM using 0.25µl-2.5µl transfection reagent for 24-72hrs for optimisation and confirmed by western blotting to determine the function of gastrin-inhibited p27 protein expression.

Optimised western blots confirmed that p27 expression was significantly decreased after 48hrs transfection with 25nM siRNA using 0.5µl transfection reagent by ~75%, in a dose dependent manner (Figure 23) ($P<0.0001$, N=3). Therefore, a p27 siRNA concentration of 25nM (using 0.5µl transfection reagent) for 48hrs was used in future cell based assays.

The reduced p27 protein expression induced by 25nM p27 siRNA significantly inhibited AGS_{GR} cell growth (Figure 24A) ($P<0.0001$, N=3, n=3) and increased cell migration (Figure 24B and 24D) ($P<0.0001$, N=3, n=3) and the extension of long processes (Figure 24C and 24E) ($P<0.0001$, N=3, n=3) that were assessed using haemocytometer counts, scratch wound assays and scattering assays respectively.

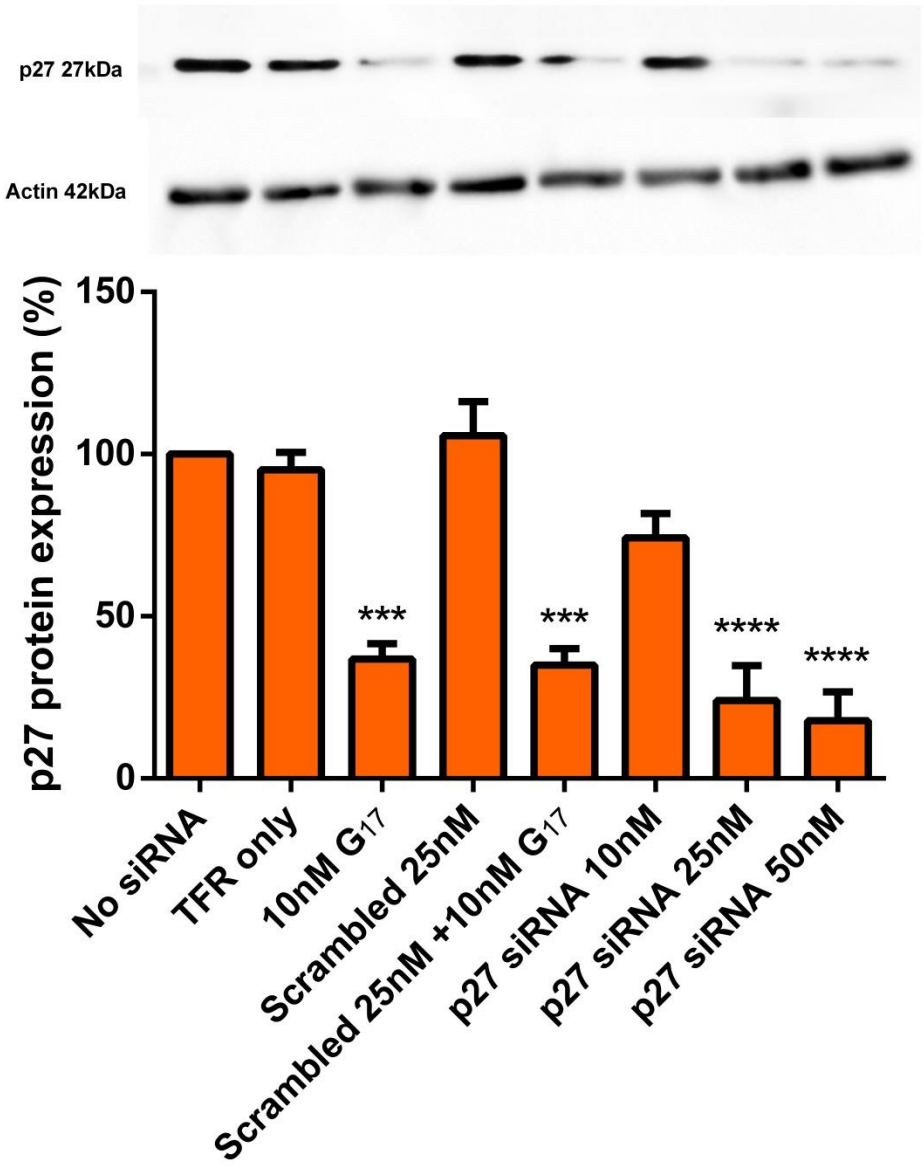

Figure 23. Western blot analysis confirmed that p27 25nM siRNA knockdown for 48hrs resulted in decreased protein expression. Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (***P<0.001 and ****P<0.0001 vs scrambled 25nM) (N=3).

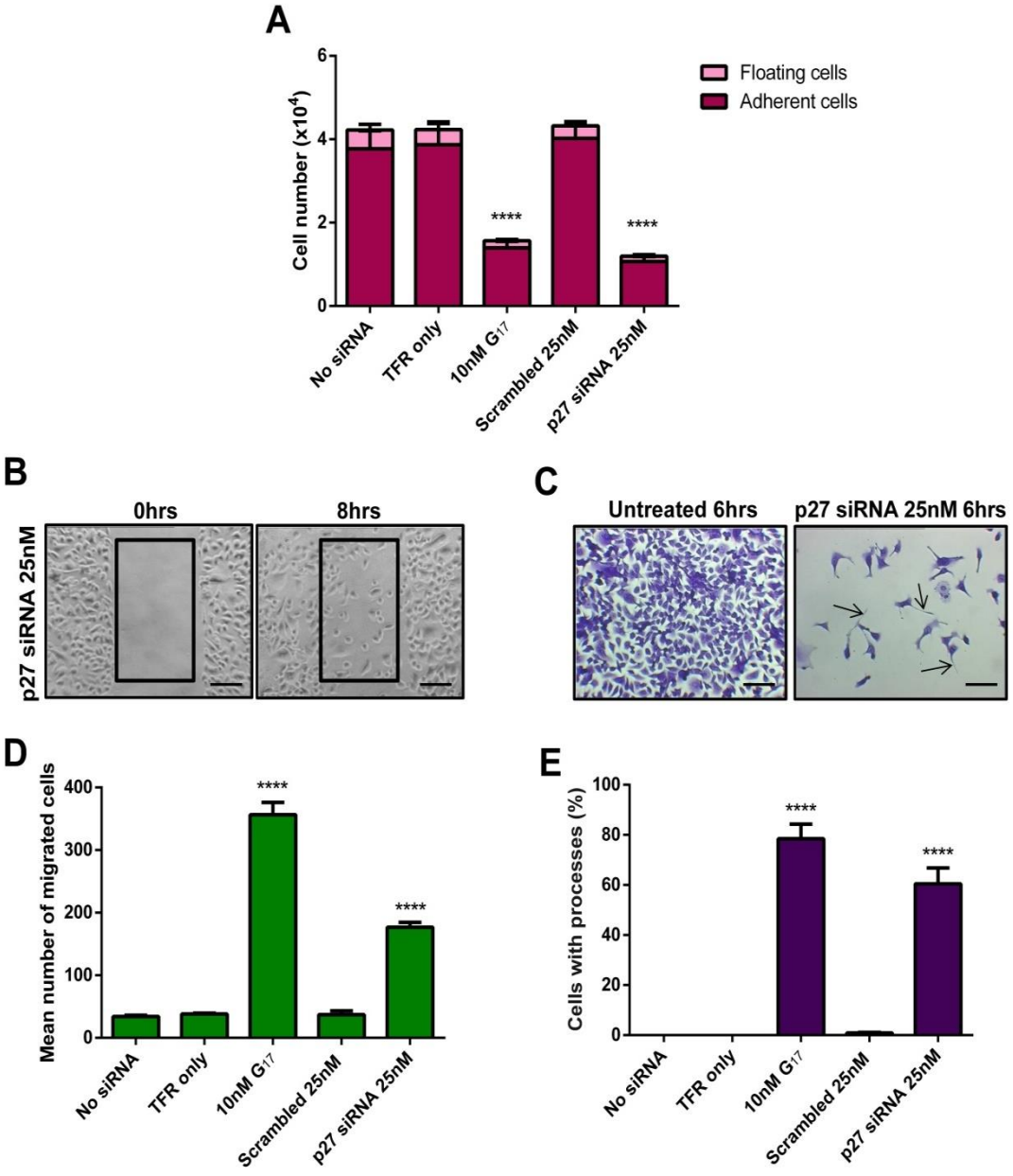

Figure 24. p27 25nM siRNA knockdown for 48hrs significantly decreased cell growth (A) and increased cell migration (B,D) and the extension of long processes (C,E) in AGS_{GR} cells (scale bar 50µm). Statistical significance was determined using one way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (****P<0.0001 vs scrambled 25nM) (scale bar 50µm).

3.1.10 Discussion

In summary I have demonstrated that miR-222 expression is increased in AGS_{GR} cells following G_{17} treatment and that the abundance of this miRNA is also increased in the gastric mucosa and serum of hypergastrinaemic mice and humans, with a further increase observed in *H. felis* infected INS-GAS mice. Increased gastrin-induced miR-222 expression leads to decreased proliferation and increased migration and the extension of long processes in AGS_{GR} cells, of which increased migration and morphological changes are associated with gastric tumour development. miR-222 exerts these effects at least in part by decreasing p27 expression and causing this protein to be mislocalised in the cytoplasm (Figure 25).

There are few previous reports of the regulation of microRNAs by gastrin. However miR-449 expression was shown to be significantly downregulated in the antrum of gastrin knockout mice relative to wild-type counterparts [676]. The transcription factor E2F1 promotes miR-449 transcription which inhibits the oncogenic genes *CDC25A* and *CDK6*. Reduced expression of *CDC25A* and *CDK6* causes dephosphorylation of the retinoblastoma protein (pRB), which arrests the cell cycle and reduces further E2F1 release [677, 678]. Dysregulation of the miR-449/pRB-E2F1 regulatory loop therefore increases E2F1 activity and promotes cell cycle progression and inhibits apoptosis in gastric cancer. miR-146a expression is also up-regulated in the stomach of gastrin-knockout mice and also in 73% of tested gastric cancers. It targets CARD10 and COPS8, resulting in reduced expression of NF-κB [679].

Several microRNAs have, however, been shown to be dysregulated in gastric adenocarcinoma and in the *H. pylori* infected stomach (reviewed in [572, 680, 681]). These miRNAs influence a number of cellular pathways that are involved in gastric carcinogenesis

including apoptosis, proliferation and metastasis. Moreover certain miRNAs have also shown promise as biomarkers of both diagnosis and prognosis in gastric cancer.

Among the miRNAs dysregulated in gastric cancer is miR-222. miR-221 and miR-222 are encoded in tandem from a gene cluster located on chromosome Xp11.3. Overexpression of miR-222 occurs in several other malignancies including breast, lung, papillary thyroid, prostate and glioblastoma [682-686]. In the GI tract, miR-222 is upregulated in cancers of the oesophagus, stomach, colon, liver and pancreas and shows decreased expression in cholangiocarcinoma and gastrointestinal stromal tumours (reviewed in [687]). miR-222 expression is increased in the plasma of patients with gastric cancer relative to patients with chronic active gastritis and healthy controls. Higher levels are associated with more advanced disease [688] and reduced 5 year survival [689]. miR-222 expression is also increased in gastric cancer tissue-derived mesenchymal stem cells [690] and in the stomachs of *H. pylori* infected individuals [691, 692].

miR-222 has several downstream mRNA targets including $p27^{kip1}$, p57, PUMA, PTEN, Bim and MMP1 (reviewed in [693]). Specifically in the stomach, increased miR-222 expression in *H. pylori* infected AGS cells post-transcriptionally regulates RECK and promotes cancer-cell growth and invasion [692, 694]. miR-222 also targets the tumour suppressor PTEN in SGC7901 gastric cancer cells [695]. This negatively regulates the Akt pathway and promotes progression through the cell cycle via the inhibition of p27 and p57 [689]. miR-222 overexpression is also associated with reduced expression of VGLL4 in human gastric cancer cell lines and tissues suggesting that miR-222 inhibits the translation of VGLL4 and promotes YAP-TEAD activation, which is sufficient to increase tumour proliferation, epithelial-mesenchymal transition and invasion [694].

One of the best characterised downstream targets of miR-222 is $p27^{kip1}$ (p27). Several studies have suggested that miR-222-induced inhibition of p27 influences tumour

development. There are few data linking p27 expression in the stomach specifically to gastrin, but several investigators have previously studied the importance of p27 during *H. pylori* induced gastric carcinogenesis. *H. pylori* infection decreases the expression of the cell cycle inhibitor protein p27 at both transcriptional and post-translational levels. *H. pylori* modulates the G-protein coupled delta opioid receptor (DOR) which regulates the histone acetylation of the p27 gene (CDKN1B) [696]. In addition $CagA^+$ *H. pylori* strains decrease the transcription of p27 through activation of the PI3K/Akt pathway [697]. *H. pylori* infection also promotes the threonine/serine phosphorylation of p27 allowing its accumulation in the cytoplasm [698]. This impairs the ubiquitination of p27 which accelerates its degradation via a proteasome-dependent pathway during cell cycle progression [699]. *p27*-null mice show increased susceptibility to *H. pylori* induced gastric preneoplastic pathology [700]. Patients with *H. pylori*-induced chronic gastritis also show decreased gastric expression of p27, which is reversed following eradication of the bacterium [701, 702]. Long-term exposure to *H. pylori* in animal [703] and cell culture [704] models has also been shown to promote an apoptosis-resistant phenotype associated with decreased p27 expression [705].

p27 has a number of well described functions that may influence tumour development (reviewed in [706]). When located in the nucleus it is predominantly a cell-cycle inhibitor. The AGS_{GR} cells used in this study show an unusual response to gastrin stimulation with direct inhibition instead of the expected stimulation of proliferation [663]. Similarly, in other cell lines such as Chinese Hamster Ovary (CHO) and human pancreatic cells (Panc-1 and MiaPaca-2) there is decreased proliferation in response to gastrin treatment [707, 708]. The CCK2R is coupled to heterotrimeric G-proteins[709] and other G-protein coupled receptors (GPCRs) such as m3 muscarinic acetylcholine receptors, which have also been associated with both positive and negative effects on cellular proliferation in response to mitogenic stimuli. Carbachol has been shown to stimulate proliferation in mouse embryonic fibroblasts that have been transfected with the m3 muscarinic cholinergic receptor

(NIH3T3/m3) during cell quiescence and inhibit cell growth if the cells were in the G_1 phase of the cell cycle [710]. These data suggest that CCK2R coupling, downstream signalling and subsequent biological response are specific to cell-type and environment.

Varro *et al*. has previously suggested that the proliferative response of AGS cells was attributable to the activation of the epidermal growth factor receptor (EGFR) due to the release of growth factors, such as heparin-binding epidermal growth factor (HB-EGF), during epithelial structural remodelling via the activation of the PKC pathway. However activation of this pathway can also be induced by phorbol esters, such as phorbol-12-myristate-13-acetate (PMA) which mimics the action of diacyl-glycerol (DAG), and causes cell cycle arrest in the G_1 phase [663]. In AGS_{GR} cells PKC activation appears to predominantly induce cell cycle inhibition, which is consistent with the reduced cell growth observed in this study and therefore the decreased expression of p27 caused by gastrin may have a negligible effect on the cell-cycle, in this context.

When p27 is located in the cytoplasm it has been shown to regulate cell migration and invasion in a cell cycle independent manner [706]. Thr^{198} phosphorylation of p27 increases its cytoplasmic localisation, inhibits RhoA (Ras homolog gene family, member A) activation and increases cell motility [711]. Our observation of cytoplasmic mislocalisation of p27 following gastrin treatment is therefore consistent with the increase in migration and change in morphology that was observed in AGS_{GR} cells following treatment with gastrin and a miR-222 mimic.

In conclusion, gastrin induced the expression of miR-222 in CCK2R bearing cells. Increased amounts of miR-222 were also detected in the gastric mucosa and sera of hypergastrinaemic patients with type-1 gastric NETs and in *H. felis* infected INS-GAS mice. This miRNA therefore has potential utility as a biomarker of hypergastrinaemia and of type-1 gastric NETs. Measurement of serum miR-222 abundance may also be useful for

monitoring the response to treatment with CCK2R antagonists such as netazepide. Further work is however needed to investigate whether increased serum miR-222 abundance is specific to patients with type-1 gastric NETs or whether it is also increased in patients who have other causes for hypergastrinaemia such as long-term proton pump inhibitor use. Gastrin-induced miR-222 upregulation appears to be functionally important. Increased miR-222 expression results in decreased abundance of p27 mRNA and protein and causes p27 mislocalisation into the cytoplasm. This mechanism appears to contribute to the increased migration and actin remodelling observed in AGS_{GR} cells following G_{17} treatment (Figure 25). It is therefore possible that similar mechanisms may also be important during the development of gastrin-related gastric tumours *in vivo*.

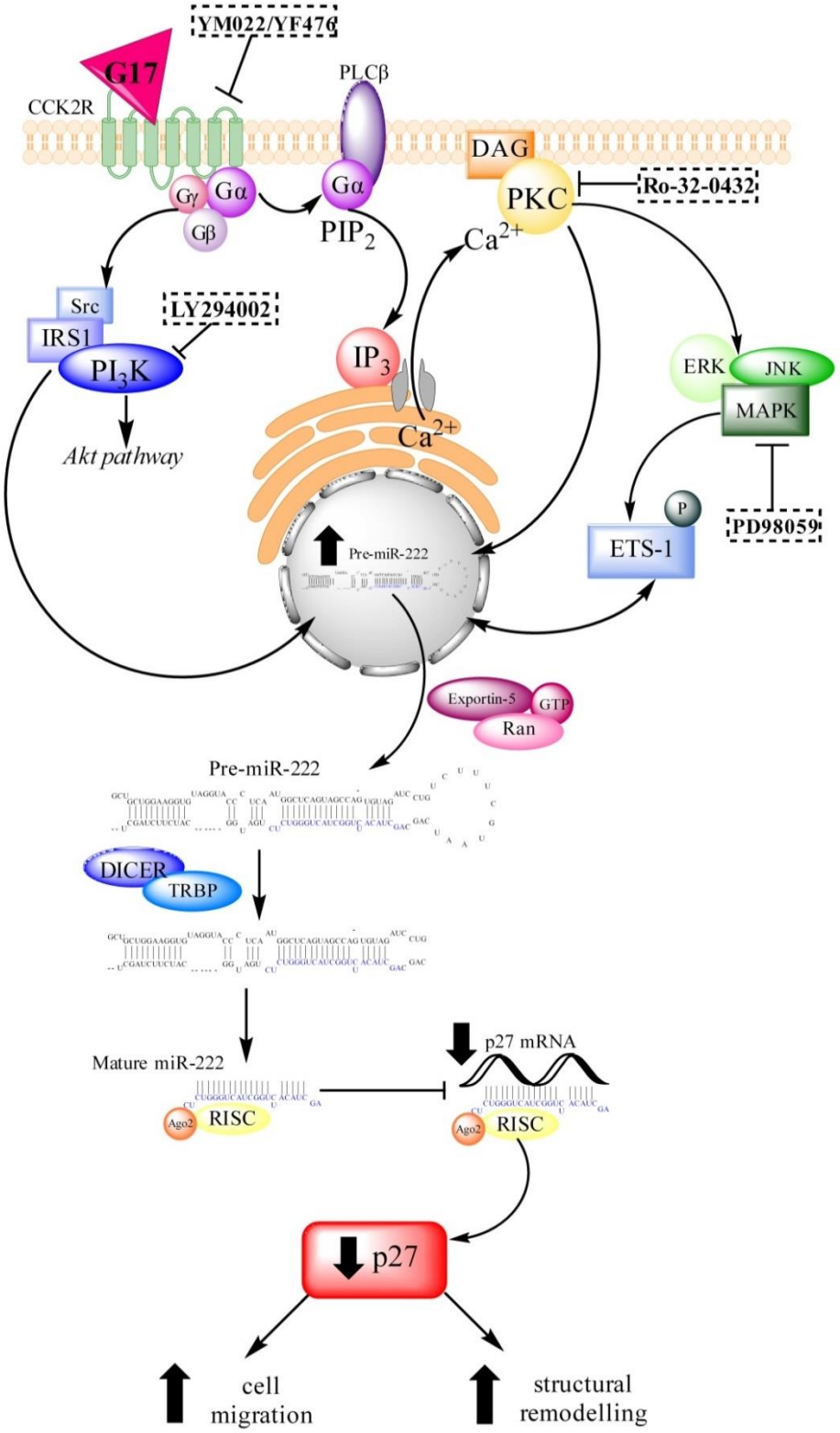

Figure 25. A schematic diagram of the signalling pathways that are activated by gastrin-CCK2R binding. miR-222 transcription is increased via the PKC and PI3K pathways and partially via the MAPK pathway. The pri(mary)-miR-222 transcript is cleaved by the RNAse-II enzyme Drosha into a hairpin structure (pre-miR-222) which is transported from the nucleus to the cytoplasm via exportin-5. Pre-miR-222 is cleaved by a second RNase II enzyme, Dicer, into mature miR-222 which associates with RISC to target imperfect complementary mRNA sequences. Mature miR-222 inhibits the translation of p27 which increases cell migration and epithelial mesenchymal transition in AGS_{GR} cells.

3.2 Gastrin-induced PAPPA2 overexpression decreases cell growth and promotes migration and structural remodelling in AGS_{GR} cells

3.2.1 Abstract

Microarray studies that investigated differentially expressed genes in patients with hypergastrinaemia and type-1 gastric neuroendocrine tumours (NETs) previously identified pregnancy associated plasma protein (PAPP)-A2 as a potential gastrin regulated gene [4]. PAPPA2 has been shown to increase the bioavailability of insulin-like growth factors (IGF) types-I and –II in the circulation [712]. IGFs are important for cell growth and development [713], however altered circulating concentrations have also been observed in numerous pathologies including gastric cancer [714, 715].

Gastrin-induced activation of the CCK2R has been shown to contribute to epithelial-mesenchymal transition (EMT) which includes the transformation of gastric epithelial cells, increased proliferation, migration and metastasis [189, 191] and hypergastrinaemia has also been strongly associated with an increased risk of developing type-1 gastric NETs [180, 463, 716]. Therefore changes in PAPPA2 expression may correlate with the extent of tissue remodelling, increased cell growth and/or migration that has previously been associated with chronic hypergastrinaemia. Using qPCR and immunofluorescence staining we examined whether gastrin stimulated the expression of PAPPA2 in AGS_{GR} cells. Functional analyses were performed using PAPPA2 siRNAs to examine the potential role of PAPPA2 as a biomarker of gastric carcinogenesis.

Gastrin treatment dose and time dependently increased PAPPA2 mRNA and protein expression in AGS_{GR} cells, which was maximal after 10nM G_{17} for 24hrs. The knockdown of PAPPA2 expression (both mRNA and protein) via siRNA significantly increased the anti-proliferative effects and decreased the migratory response and the extension of long processes caused by 10nM G_{17} in AGS_{GR} cells. These data indicate that the upregulation of the protease PAPPA2 is partially dependent on gastrin and may have a role in the development of gastric tumours, via the activation of the CCK2R and possibly indirectly by

increasing the bioavailability of IGFs. Therefore, PAPPA2 provides promise as a novel biomarker of gastrin-associated conditions and the monitoring of structural remodelling of the gastric mucosa. However, further studies within the circulation would strength the potential clinical application of PAPPA2 as a biomarker.

3.2.2 Introduction

The peptide hormone gastrin exerts many of its biological activity through the activation of gastrin receptors (CCK2R) located on enterochromaffin-like (ECL) cells within the stomach. The gastrin/CCK2 receptor signalling pathway has been extensively investigated with respect to acid secretion [717-719] however less in known about the mechanisms by which gastrin exerts other trophic effects in the stomach such as cell migration and tissue remodelling.

In a previous cDNA microarray and qPCR study from our group, differentially expressed genes were identified in gastric corpus biopsies taken from hypergastrinaemic patients with type-1 gastric NETs who were treated daily with the gastrin/CCK2 receptor antagonist netazepide (50mg) for both short (12 weeks with a 12 week follow up) and long term regimens (1 year) (Figure 26). Gastrin receptor antagonism with netazepide caused a 30% reduction in tumour size and number, suggesting that gastrin/CCK2R activation is strongly associated with the development of gastric NETs [459]. In these patients, PAPPA2 expression was initially high at baseline (before treatment), but this decreased upon treatment with netazepide, with levels returning to baseline upon cessation of the drug, indicating a possible correlation between gastrin/CCK2 receptor activation and PAPPA2 expression [4].

Pregnancy-associated plasma protein A2 (PAPPA2/pappalysin) is an insulin-like growth factor-binding protein (IGFBP) protease that is predominantly expressed in the placenta, but is also found at low levels in the gall bladder, kidney and stomach[720]. Evidence suggests that PAPPA2 is upregulated as a consequence of hypertensive disorders including preeclampsia [721, 722], HELLP (Haemolytic anaemia, Elevated Liver enzymes, and Low Platelet count) syndrome [721-723] and cancers of the uterus [724] and lung [725]. In human placental trophoblast cell lines (BeWo), PAPPA2 was shown to be regulated by hypoxia and TNF-α,

with 47-fold and a 6-fold increases in expression respectively [726]. These are factors that are known to be highly expressed as a consequence of placental pathology.

The PAPPA2 enzyme primarily cleaves insulin-like growth factor binding protein-5 (IGFBP5), and to a lesser extent IGFBP3. This extends the half-life and reduces the turnover rate of insulin-like growth factors (IGFs). IGFs are responsible for several important biological processes including bone formation [727] and the growth and permeability of the placenta [728]. The latter is crucial during pregnancy and therefore the observed increased expression of IGF regulatory proteins such as PAPPA2 is to be expected. However, the increased bioavailability of IGFs and subsequent signalling cascades have also been previously associated with various pathological conditions including breast [729], colorectal [730, 731], prostrate [732, 733] and stomach cancers [714, 715].

Previous studies by Varro *et al.* have suggested that some matrix metalloproteinases (MMPs), particularly MMP7 also cleave IGFBP5 and are significantly increased in both hypergastrinaemic mice and humans in order to facilitate the expansion of the extracellular matrix surrounding ECL cell populations in type-1 gastric neuroendocrine tumours (NETs) [191]. Other studies have also suggested that MMP7 abundance increases as a consequence of *H. pylori* infection [190, 734, 735]; a known cause of hypergastrinaemia. Interestingly, during epithelial-mesenchymal transition (EMT) and stromal remodelling, which have been shown to be stimulated by high concentrations of gastrin, there is an increased abundance of myofibroblasts which release IGFBP5 [734, 736]. Different substrate bioavailability for proteases such as PAPPA2 during early gastric pathological changes may cause different levels of PAPPA2 expression and this is a potential biomarker of carcinogenesis in the stomach.

Therefore, I have further investigated the relationship between PAPPA2 expression and cellular phenotypic alterations that have previously shown to contribute to gastric

carcinogenesis such as cell migration, the extension of long processes and proliferation. Using gastric adenocarcinoma cells that express the CCK2R (AGS_{GR}) with and without gastrin treatment, I assessed both PAPPA2 mRNA and protein expression as they may provide a potential biomarker for the detection of gastric neoplastic conditions and/or monitoring the effects of therapy.

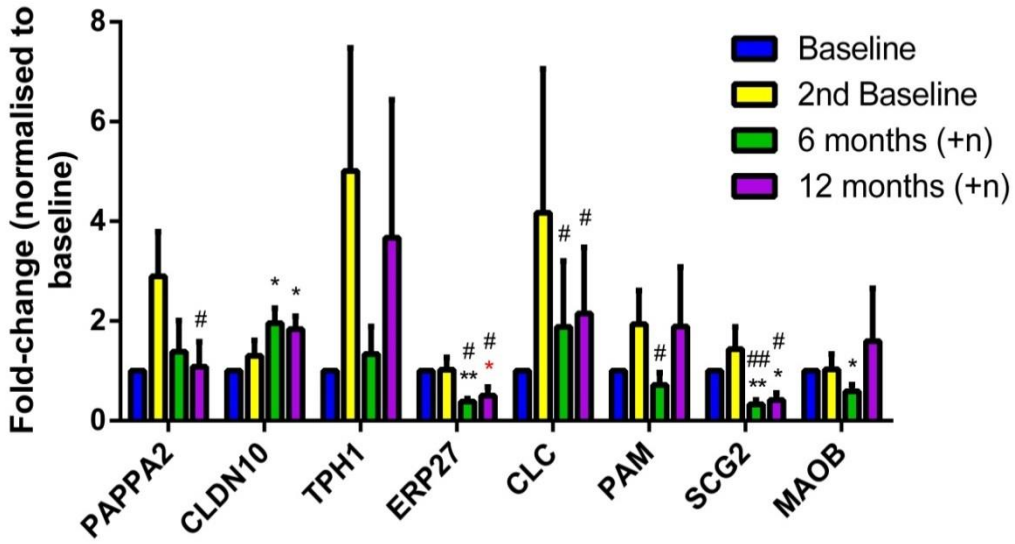

Figure 26. The abundance of several potential novel biomarkers in gastric corpus biopsies, relative to GAPDH expression. Statistical significance was determined using a Wilcoxon signed rank test. *P<0.05 compared to baseline, *P=0.055 compared to baseline (**P<0.01 compared to baseline, #P<0.05 compared to 2nd baseline, and ##P<0.01 compared to 2nd baseline and +n= patients taking netazepide[4]).

3.2.3 Gastrin increases PAPPA2 mRNA and protein expression in AGS_{GR} cells

Previous studies have identified PAPPA2 as a potential biomarker of type-1 gastric NETs as PAPPA2 mRNA expression was high before treatment in patients with hypergastrinaemia and type-1 gastric NETs, reduced whilst patients were taking 50mg oral daily dose of the CCK2R antagonist netazepide and returned to baseline after cessation of treatment [4]. To investigate whether gastrin affects PAPPA2 expression, dose response and time-course experiments were initially assessed using qPCR in AGS_{GR} cells treated with and without gastrin.

PAPPA2 mRNA dose (Figure 27A) and time (Figure 27B) dependently increased with gastrin treatment (N=3, n=4). Further validation using immunocytochemical staining confirmed that PAPPA2 protein expression was primarily nuclear in AGS_{GR} cells and was significantly increased in a dose and time dependent manner (Figure 30) in response to gastrin treatment. Representative immunofluorescence images were taken for visual comparisons between untreated and 10nM G_{17} treatment 24hrs (Figure 28) and untreated and 10nM G_{17} treatment for 6hrs and 24hrs in AGS_{GR} cells (Figure 29) (N=3, n=6). Incubation with swine anti-rabbit FITC conjugated HRP secondary antibody alone was used as a non-specific binding (negative) control.

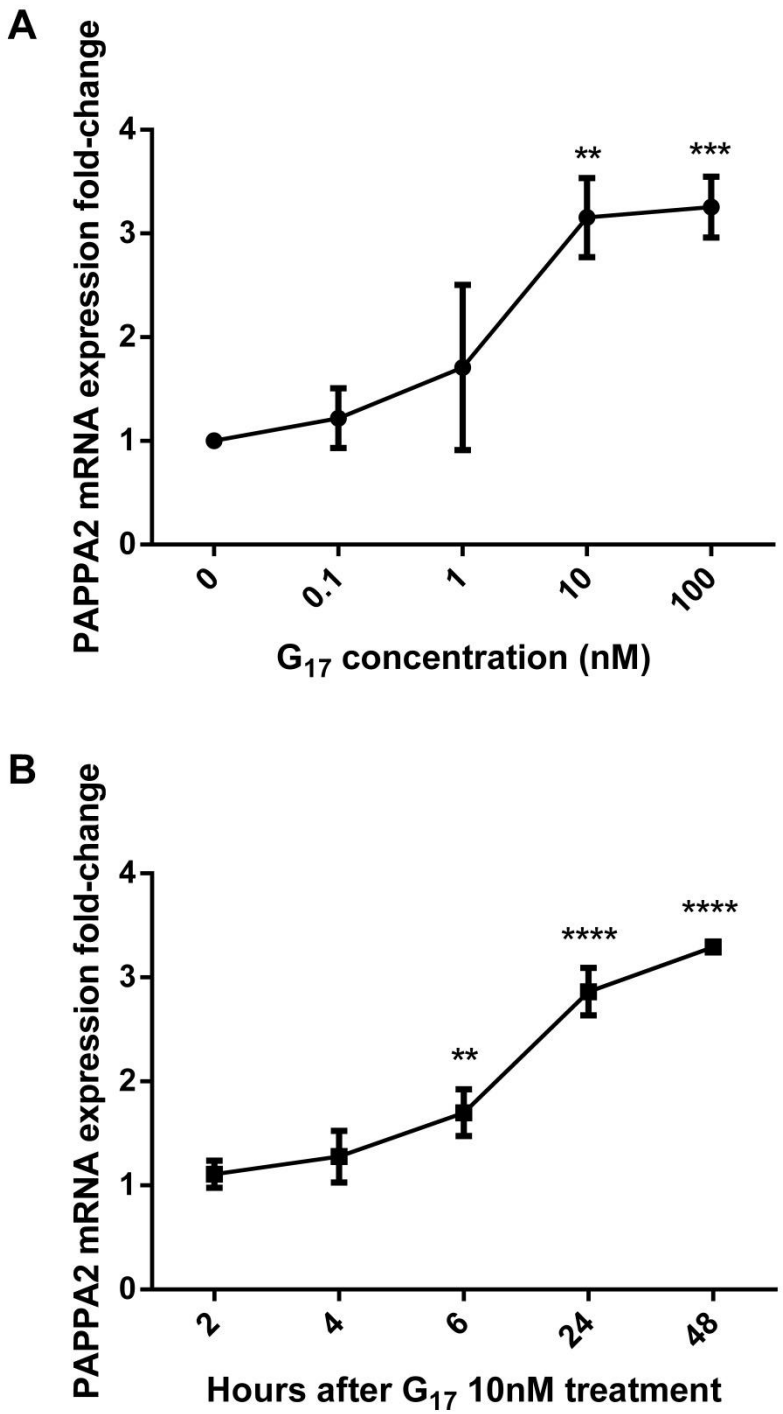

Figure 27. Gastrin increases PAPPA2 mRNA expression in as dose (A) and time (B) dependent manner which is maximal after 10nM G_{17} treatment for 24hrs in AGS_{GR} cells. Statistical significance was determined using one-way ANOVA (A) with Sidak *post-hoc* test and P<0.05 was considered significant (**P<0.01 and ***P<0.001 vs untreated control) or two-way ANOVA (B) with Sidak *post-hoc* test and P<0.05 was considered significant (**P<0.01, and ****P<0.0001 vs untreated control at the same time point) (N=3, n=4).

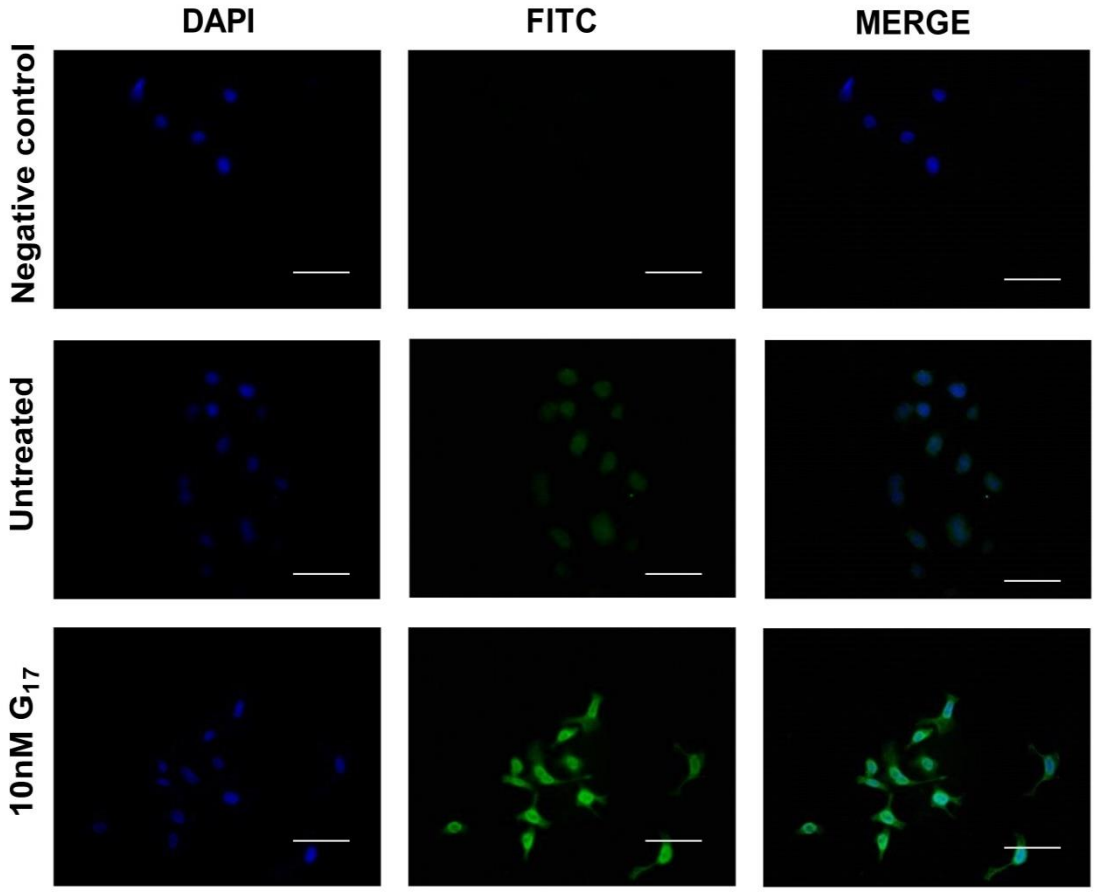

Figure 28. Representative immunofluorescence staining with rabbit anti-plac3 (PAPPA2) antibody (1:500) followed by swine anti-rabbit FITC conjugated HRP secondary antibody (1:500) showed an increase in primarily nuclear PAPPA2 protein expression (FITC: green) with 10nM G_{17} treatment compared to untreated controls in AGS_{GR} cells after 24hrs. Nuclear staining (DAPI: blue) was used as an intensity control between treated and untreated AGS_{GR} cells (scale bar 100µm). Incubation with swine anti-rabbit FITC conjugated HRP secondary antibody alone was used as a non-specific binding (negative) control (N=3, n=6).

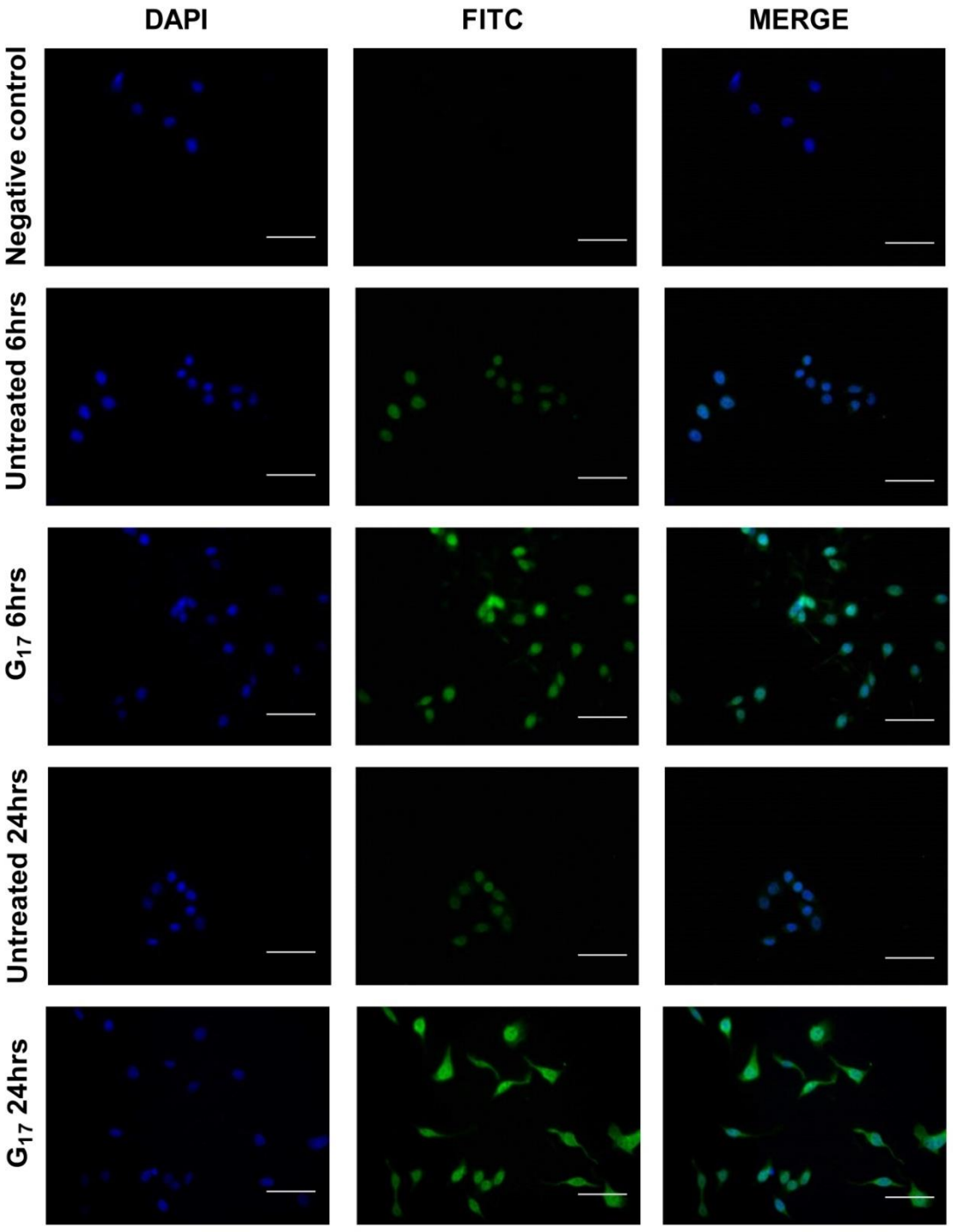

Figure 29. Representative immunofluorescence staining with rabbit anti-plac3 (PAPPA2) antibody (1:500) followed by swine anti-rabbit FITC conjugated HRP secondary antibody (1:500) showed an increase in primarily nuclear PAPPA2 protein expression (FITC: green) with 10nM G_{17} treatment after 6hrs that was maximal after 24hrs compared to time-matched untreated controls in AGS_{GR} cells. Nuclear staining (DAPI: blue) was used as an intensity control between treated and untreated AGS_{GR} cells (scale bar 100µm). Incubation with swine anti-rabbit FITC conjugated HRP secondary antibody alone was used as a non-specific binding (negative) control (N=3, n=6).

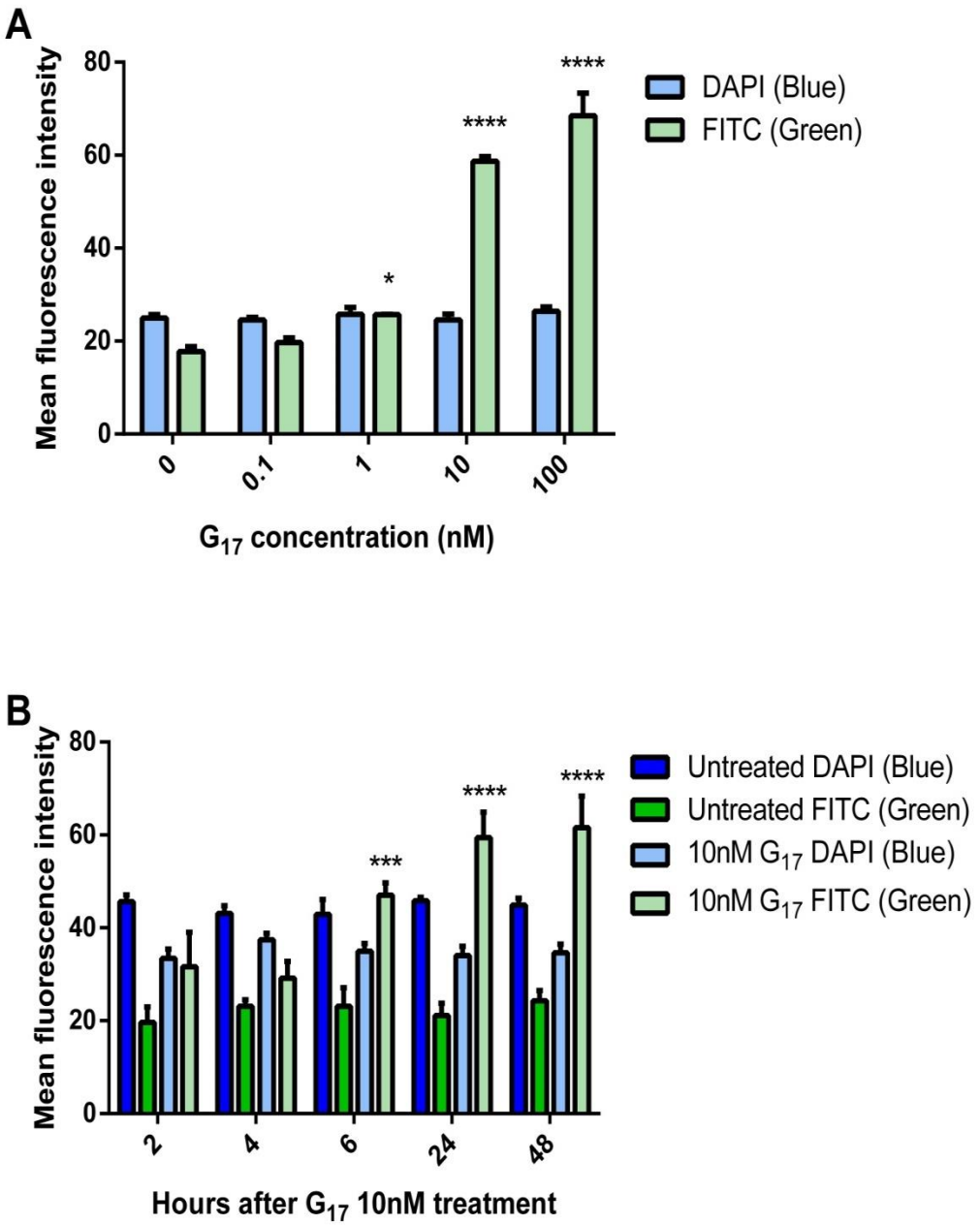

Figure 30. Relative fluorescence intensities measured using AxioVision Rel. 4.8 software showed that PAPPA2 protein expression (primarily nuclear) increased dose (A) and time (B) dependently in AGSGR cells treated with gastrin (N=3, n=6). Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (*P<0.05, ***P<0.001 and ****P<0.0001 vs untreated controls at the same time point).

3.2.4 PAPPA2 siRNA significantly reduces gastrin-induced PAPPA2 mRNA and protein expression in AGS_{GR} cells, by 90% and 80% respectively.

Immunofluorescence and qPCR were used to confirm the optimal conditions for the successful knockdown of PAPPA2 mRNA and protein in AGS_{GR} cells, in order to assess the functional consequence(s) of gastrin-induced PAPPA2 expression in the stomach.

AGS_{GR} cells that had been transfected with a non-targeting scrambled (25nM 48h) siRNA followed by 10nM G_{17} for 24hrs showed a significant increase in PAPPA2 mRNA (Figure 31A $P<0.001$, Figure 32A $P<0.0001$ and Figure 33A $P<0.0001$) and protein (Figure 31C $P<0.0001$, Figure 32C $P<0.0001$ and Figure 33C ($P<0.0001$, 33E $P<0.0001$ and 33G $P<0.0001$) expression and were used as a positive control.

PAPPA2 siRNA (25nM 48hrs) successfully reduced gastrin-induced PAPPA2 mRNA expression by >90% at all volumes of transfection reagent (TFR) (0.25-2.5µl) (Figure 31B) ($P<0.0001$, N=3, n=4). However, PAPPA2 protein expression was only significantly reduced with 0.5µl TFR (Figure 31D) ($P<0.0001$, N=3, n=6) as higher volumes resulted in limited numbers of adherent AGS_{GR} cells which indicated cytotoxicity (data not shown).

PAPPA2 siRNA at concentrations of 10nM (for 48hrs with 0.5µl TFR) significantly reduced gastrin-induced PAPPA2 mRNA expression ($P<0.05$) which was maximal at 25nM with a reduction of 87% (Figure 32B) ($P<0.0001$, N=3, n=4). PAPPA2 siRNA dose-dependently reduced PAPPA2 protein expression which was significant at 25nM (for 48hrs with 0.5µl TFR) with an 80% reduction (Figure 32D) ($P<0.0001$, N=3, n=6) in AGS_{GR} cells.

PAPPA2 siRNA (25nM with 0.5µl TFR) significantly reduced gastrin-induced PAPPA2 mRNA expression by 88% after 48hrs (Figure 33B) ($P<0.0001$, N=3, n=4) and also reduced protein expression significantly (by 50%) after 24hrs (Figure 33D) ($P<0.0001$, N=3, n=6) in a time-dependent manner.

Representative immunofluorescence images were taken for visual comparisons between AGS_{GR} cells transfected with and without PAPPA2 siRNA using 0.5µl TFR for 48hrs (Figure 34) (N=3, n=6). Incubation with swine anti-rabbit FITC conjugated HRP secondary antibody alone was used as a non-specific binding (negative) control

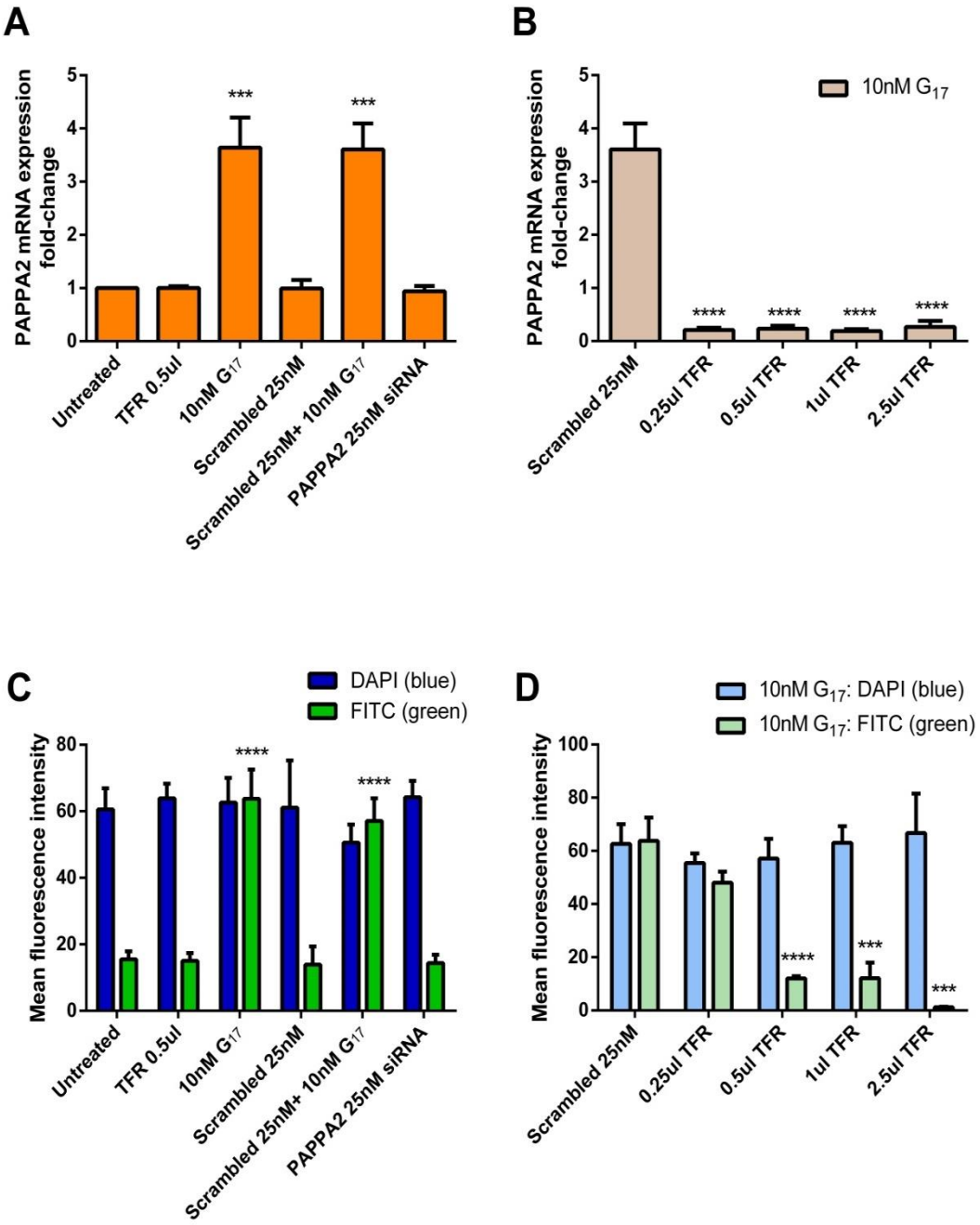

Figure 31. PAPPA2 mRNA (A) and protein (C) expression were significantly increased in AGS_{GR} cells transfected with and without a non-targeting scrambled control siRNA (25nM) for 48hrs followed by 10nM G_{17} treatment for 24hrs. Transfection with PAPPA2 25nM siRNA alone did not significantly affect basal PAPPA2 mRNA or protein expression. PAPPA2 25nM siRNA that had been transfected with all volumes of transfection reagent (TFR) (0.25µl-2.5µl) decreased gastrin-induced PAPPA2 mRNA expression (B) (N=3, n=4) and 0.5µl TFR significantly reduced gastrin-induced PAPPA2 protein expression (D) (N=3, n=6). Statistical significance was determined using one-way ANOVA (A and B) or two-way ANOVA (C and D) with Sidak *post-hoc* test and $P<0.05$ was considered significant (***$P<0.001$ and ****$P<0.0001$ vs scrambled 25nM+ 10nM G_{17}).

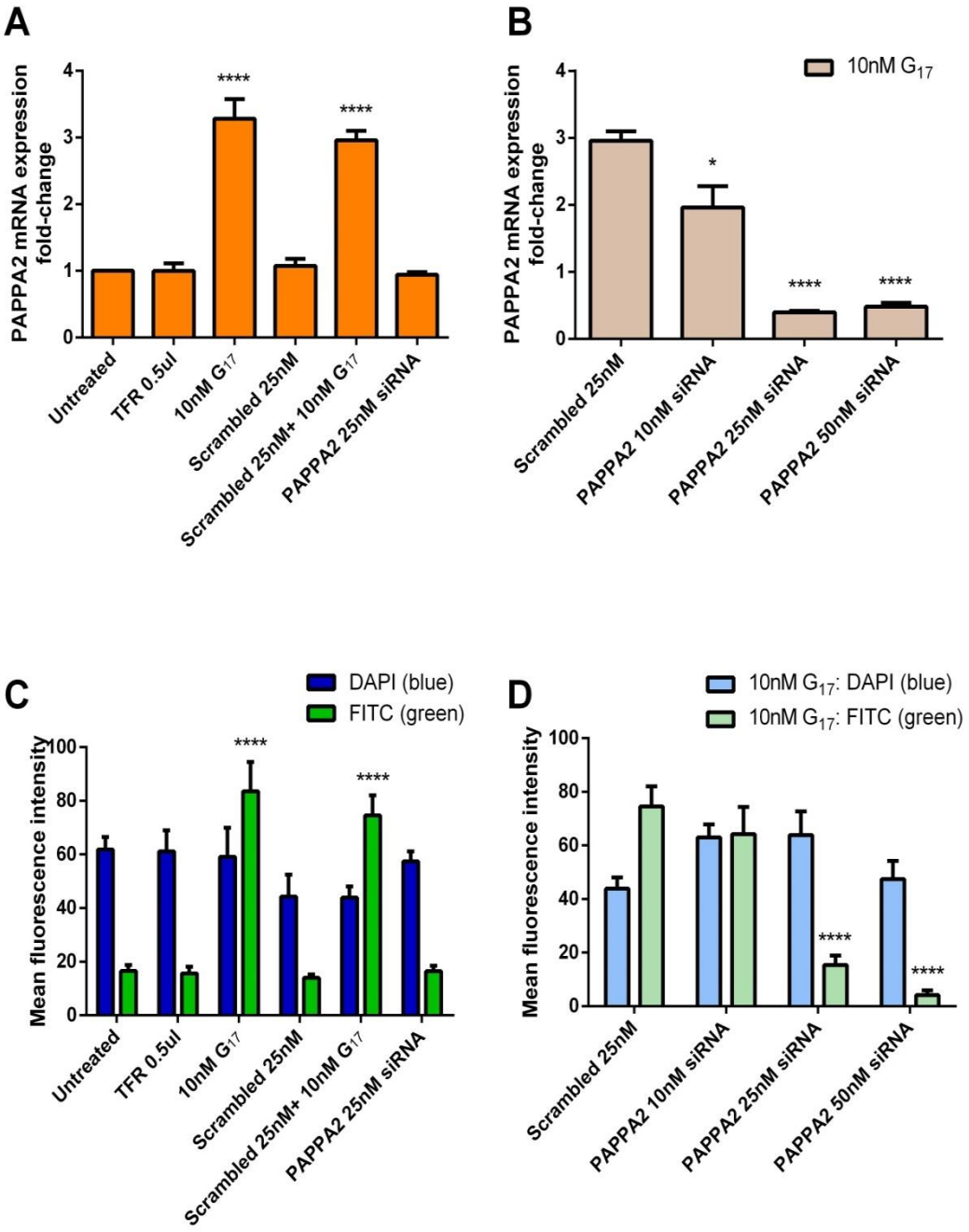

Figure 32. PAPPA2 mRNA (A) and protein (C) expression were significantly increased in AGS_{GR} cells transfected with and without a non-targeting scrambled control siRNA (25nM) for 48hrs followed by 10nM G_{17} treatment for 24hrs. Transfection with PAPPA2 25nM siRNA alone did not significantly affect basal PAPPA2 mRNA or protein expression. AGS_{GR} cells that had been transfected with 10nM PAPPA2 siRNA significantly decreased gastrin-induced PAPPA2 mRNA expression (B) (N=3, n=4) and PAPPA2 25nM siRNA significantly reduced gastrin-induced PAPPA2 protein expression (D) (N=3, n=6). Statistical significance was determined using one-way ANOVA (A and B) or two-way ANOVA (C and D) with Sidak *post-hoc* test and P<0.05 was considered significant. (*P<0.05 and ****P<0.0001 vs scrambled 25nM+ 10nM G_{17}).

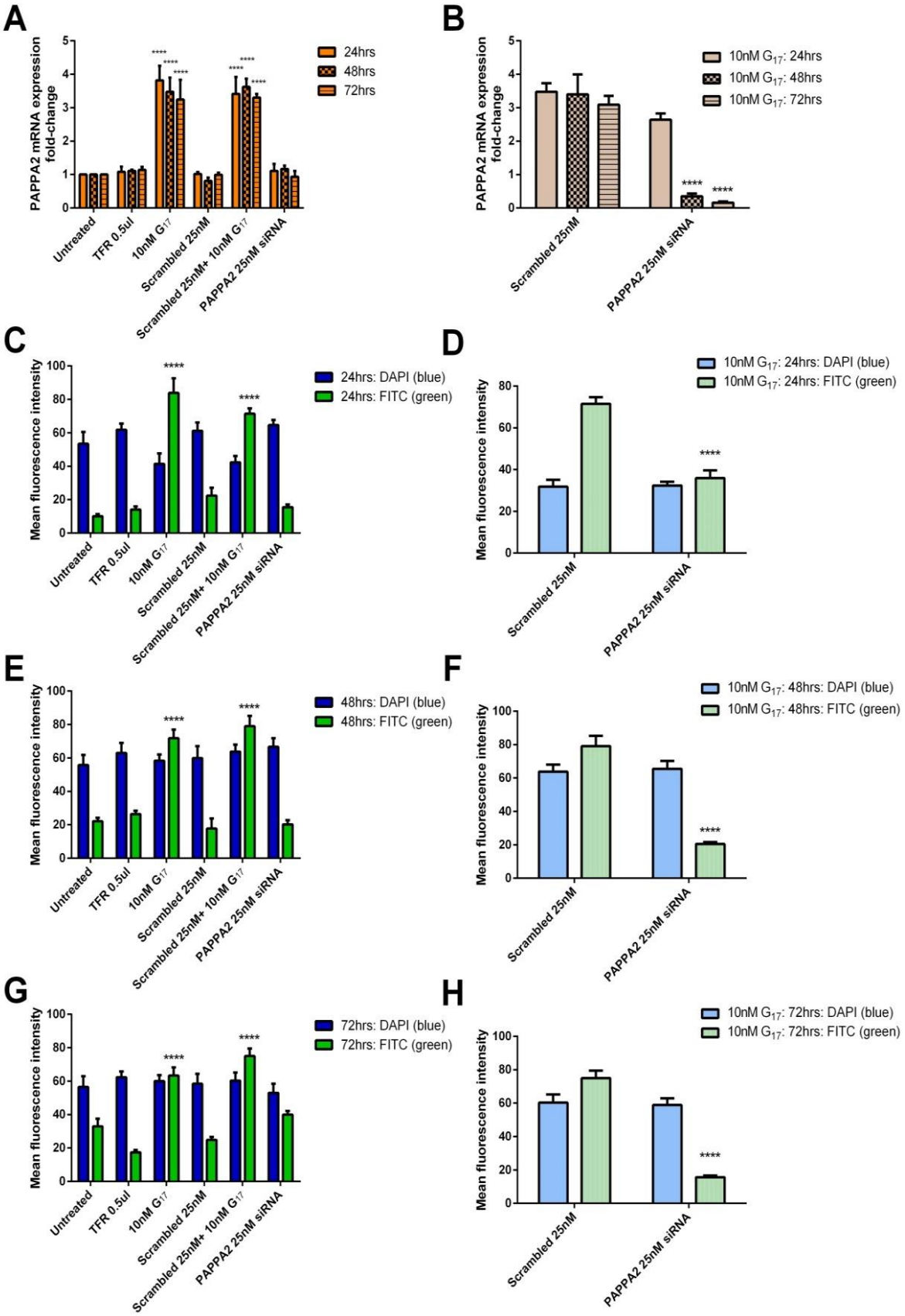

Figure 33. PAPPA2 mRNA (A) and protein (C,E and G) expression were significantly increased in AGS_{GR} cells transfected with and without a non-targeting scrambled control siRNA (25nM) for 24-72hrs followed by 10nM G_{17} treatment for 24hrs. Transfection with PAPPA2 25nM siRNA alone did not significantly affect basal PAPPA2 mRNA or protein expression. AGS_{GR} cells that had been transfected with PAPPA2 25nM siRNA significantly decreased gastrin-induced PAPPA2 mRNA expression after 48hrs (B) (N=3, n=4) and protein expression in a time-dependent manner which was significant after 24hrs (D, F and H) (N=3, n=6). Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and $P<0.05$ was considered significant (****$P<0.0001$ vs scrambled 25nM+ 10nM G_{17}).

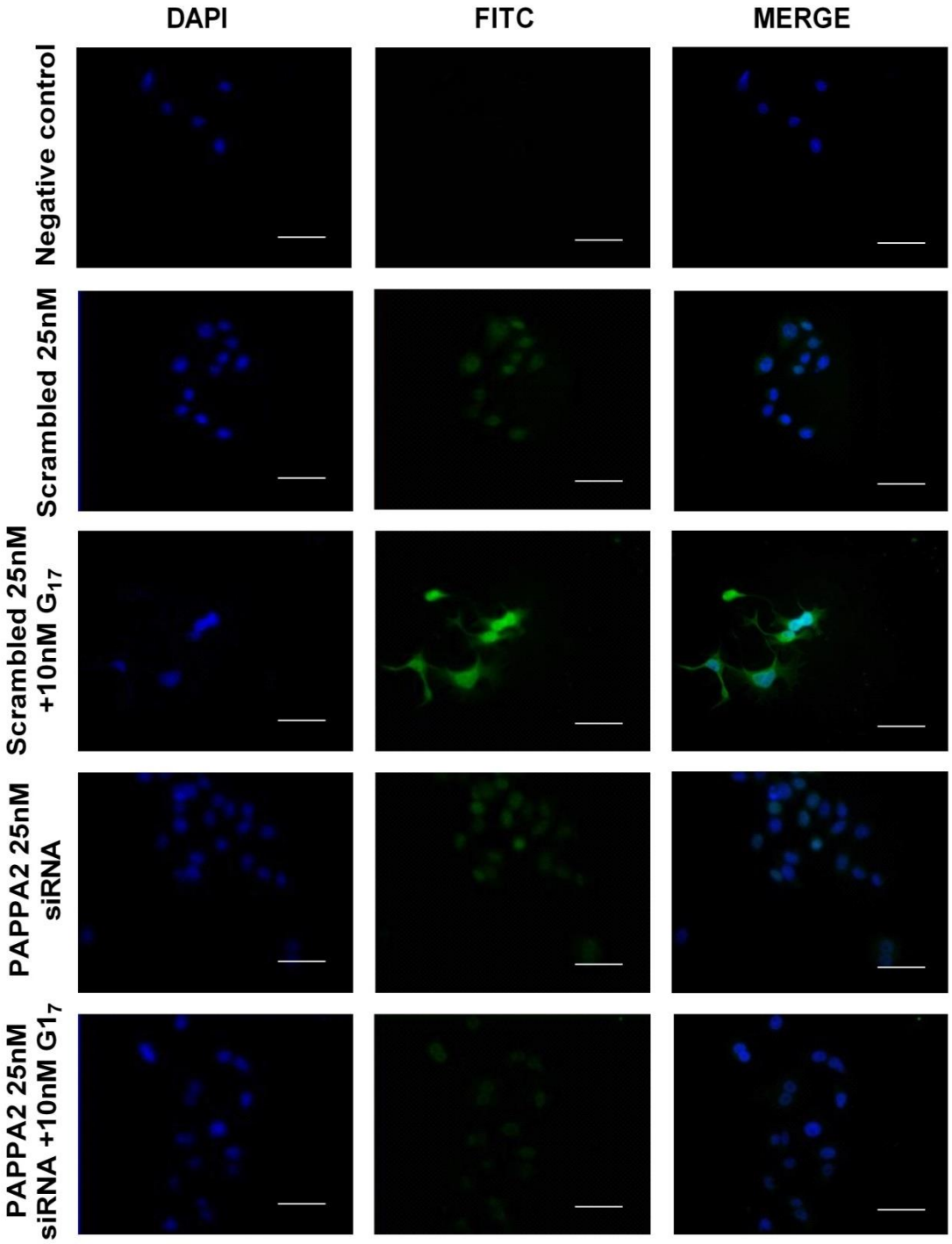

Figure 34. Representative immunofluorescence staining with rabbit anti-plac3 (PAPPA2) antibody (1:500) followed by swine anti-rabbit FITC conjugated HRP secondary antibody (1:500) showed an increase in primarily nuclear PAPPA2 protein expression (FITC: green) after 48hrs transfection with a non-targeting scrambled siRNA (25nM) followed by 10nM G_{17} for 24hrs in AGS_{GR} cells. Cells transfected with PAPPA2 25nM siRNA (0.5µl TFR for 48hrs) showed significantly decreased gastrin-induced PAPPA2 protein expression. PAPPA2 25nM siRNA transfection without gastrin treatment did not significantly affect basal levels of PAPPA2 protein expression. Nuclear staining (DAPI: blue) was used as an intensity control between treated and untreated AGS_{GR} cells (scale bar 100µm). Incubation with swine anti-rabbit FITC conjugated HRP secondary antibody alone was used as a non-specific binding (negative) control (N=3, n=6).

3.2.5 PAPPA2 siRNA significantly inhibits the anti-proliferative effects of gastrin in AGS_{GR} cells

PAPPA2 is a member of a large family of matrix metalloproteinases that are essential for postnatal tissue and bone development in mice [737]. Therefore we assessed phenotypical changes associated with gastrin induced PAPPA2 expression using PAPPA2 siRNA knockdown in AGS_{GR} cells.

Cell proliferation was significantly inhibited by 10nM G_{17} treatment for 24hrs in AGS_{GR} cells ($P<0.0001$), with minimal cytotoxic effects being observed as the number of floating cells (indicative of apoptosis) was not affected. The knockdown of PAPPA2 using PAPPA2 25nM siRNA (with 0.5µl TFR) for 48hrs prior to 10nM G_{17} treatment, significantly reversed the inhibition of AGS_{GR} cell growth induced by gastrin after 24hrs ($P<0.05$) with no observed cytotoxic effects as the number of floating cells was again unaffected (Figure 35) (N=3, n=3).

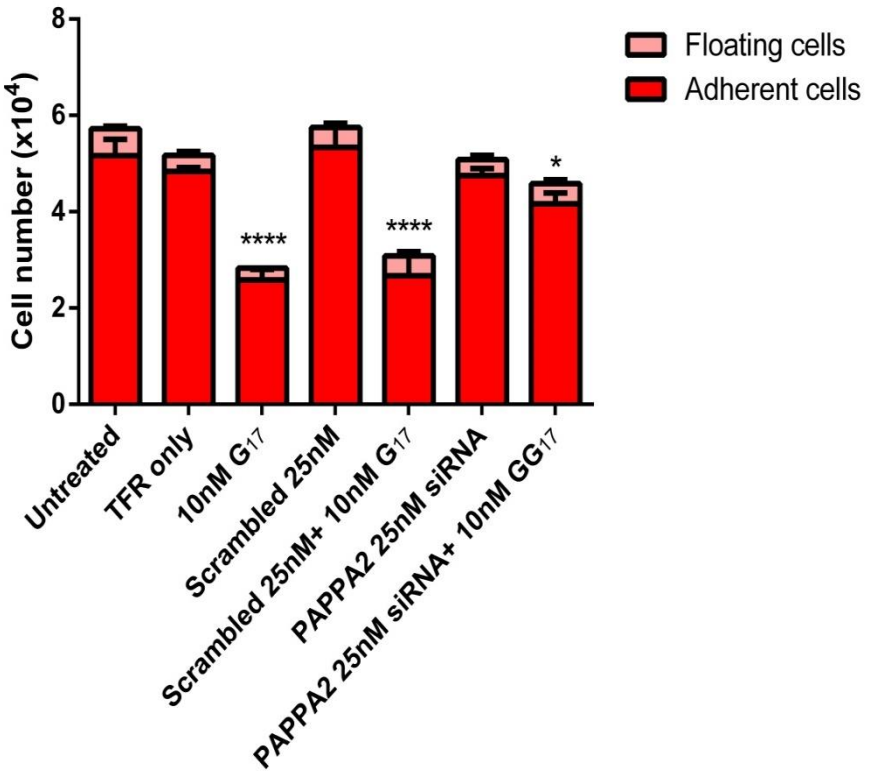

Figure 35. Haemocytometer counts showed that 10nM G_{17} for 24hrs inhibited AGS_{GR} cell growth compared with untreated controls. AGS_{GR} cells transfected with a non-targeting scrambled siRNA (25nM) or PAPPA2 siRNA (25nM) alone showed no significant effect on cell proliferation. AGS_{GR} cells transfected with a non-targeting scrambled siRNA (25nM) for 48hrs followed by 10nM G_{17} for 24hrs showed significantly inhibited cell growth. PAPPA2 25nM siRNA (48h) significantly reduced the anti-proliferative effects of 10nM G_{17} for 24hrs in AGS_{GR} cells. No significant differences were observed between floating cell number for any treatment, indicating negligible cytotoxic effects (N=3, n=3). Statistical differences were determined using two-way ANOVA with Sidak *post-hoc* test and $P<0.05$ was considered significant (*$P<0.05$ and ****$P<0.0001$ vs untreated controls).

3.2.6 PAPPA2 siRNA significantly inhibits the extension of long processes induced by gastrin in AGS_{GR} cells

Previous studies have suggested that IGFBPs, particularly IGFBP-5, are substrates for PAPPA2 and are cleaved to release IGFs [712], which have been shown to regulate many cellular processes including tissue remodelling and cell differentiation. Gastrin has previously been shown to induce the remodelling of the actin cytoskeleton via the extension of long processes in gastric cancer cells [673] and the loss of gastric gland structure *in vivo* has been associated with the development of premalignant gastric atrophy [292]. Therefore we investigated the effects of gastrin-induced PAPPA2 expression on gastric epithelial cell morphology in AGS_{GR} cells.

Gastrin (10nM for 6hrs) induced the extension of long processes in AGS_{GR} cells and was therefore used as a positive control. AGS_{GR} cells transfected with 25nM PAPPA2 siRNA for 48hrs showed a significant decrease in the proportion demonstrating extension of long processes following treatment with 10nM G_{17} for 6hrs ($P<0.0001$). PAPPA2 siRNA 25nM alone did not cause any significant effects on cell morphology (Figure 36A) (N=3, n=3).

Representative images were taken of AGS_{GR} cells transfected with and without scrambled 25nM siRNA or PAPPA2 25nM siRNA with and without 10nM gastrin treatment 6hrs for visual comparisons of cell morphology (Figure 36B) (N=3, n=3).

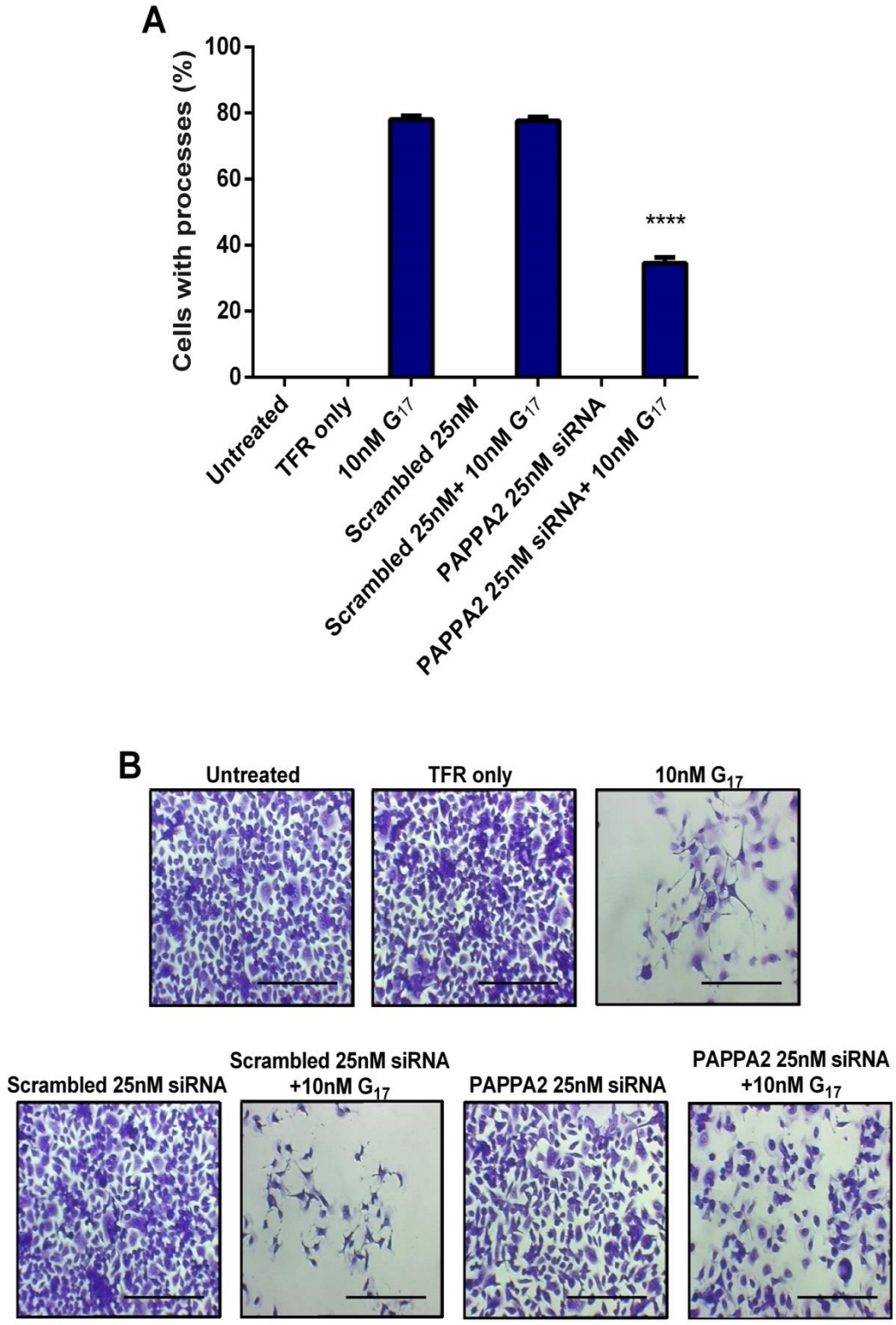

Figure 36. AGS_{GR} cells treated with 10nM G17 for 24hrs and cells transfected with a non-targeting scrambled siRNA (25nM) for 48hrs before 10nM G_{17} for 6hrs showed an increase in the extension of long processes. The gastrin-induced extension of long processes was significantly reduced by PAPPA2 25nM siRNA transfection for 48hrs prior to gastrin treatment. Cells transfected with a non-targeting scrambled siRNA (25nM) or PAPPA2 siRNA (25nM) alone showed no significant change in cell morphology (A). Cells were stained with 0.3% crystal violet after 6hrs G_{17} treatment and representative images were taken (scale bar 150µm) (N=3, n=3). Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (****P<0.0001 vs scrambled 25nM+ 10nM G_{17}).

3.2.7 PAPPA2 siRNA significantly inhibits the migratory response stimulated by gastrin in AGS_{GR} cells

The expression of IGFBP-4 and -5 have been shown to be increased during the first trimester of pregnancy and are involved in the regulation of cell migration [738], suggesting an important role for this protein in cell growth and development. An increase in IGFBPs (PAPPA2 substrate) may cause an increase in PAPPA2 expression during periods of tissue reorganisation, which may occur during the development of gastric neoplasia. Therefore, we assessed whether gastrin-stimulated PAPPA2 expression was positively associated with AGS_{GR} cell migration.

Gastrin treatment alone (10nM) stimulated AGS_{GR} cell migration after 8hrs and was therefore used as a positive control. Transfection with PAPPA2 siRNA 25nM for 48hrs had no effect on AGS_{GR} cell migration, however PAPPA2 siRNA at a concentration of 25nM for 48hrs significantly reduced the migratory response of AGS_{GR} cells caused by 10nM G_{17} treatment for 8hrs (Figure 37A) ($P<0.0001$, N=3, n=3).

Representative images were taken of AGS_{GR} cells transfected with and without scrambled 25nM siRNA or PAPPA2 25nM siRNA with and without 10nM gastrin treatment 8hrs for visual comparisons of cell morphology, at 0hrs and 8hrs post-gastrin treatment (Figure 37B and 37C) (N=3, n=3).

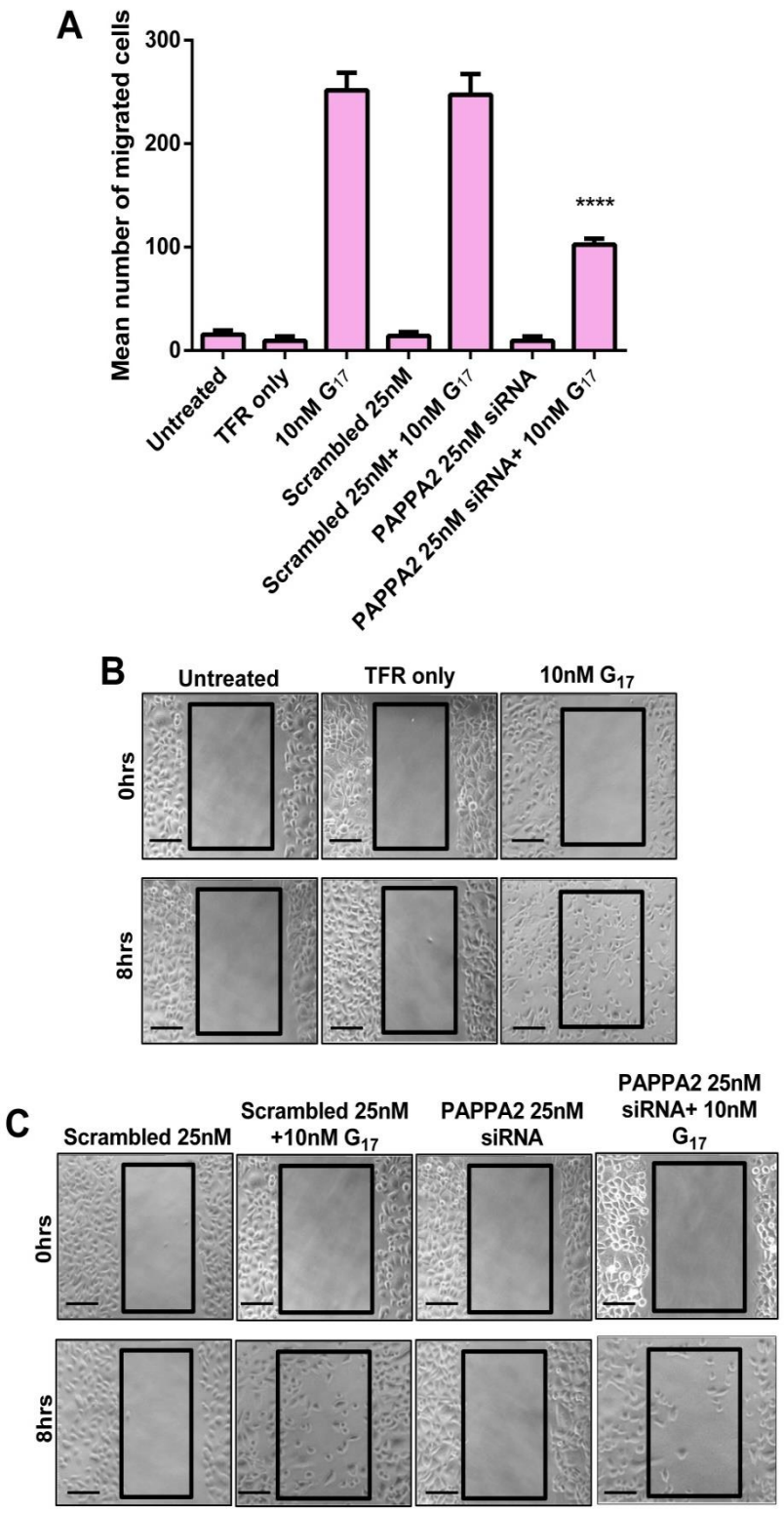

Figure 37. Gastrin (10nM for 8hrs) increased cell migration in AGS_{GR} cells that were transfected with or without a non-targeting scrambled siRNA (25nM) for 48hrs prior to treatment. Transfection with PAPPA2 25nM siRNA for 48hrs significantly reduced the migratory response caused by 10nM G_{17} for 8hrs. Cells transfected with a non-targeting scrambled siRNA (25nM) or PAPPA2 siRNA (25nM) alone showed no significant alteration in cell migration (A). Representative images of scratch wound assays were taken at 0hrs and 8hrs after gastrin treatment for visual comparison (scale bar 50μm) (B and C) (N=3, n=3). Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and $P<0.05$ was considered significant (****$P<0.0001$ vs scrambled 25nM+ 10nM G_{17}).

3.2.8 Discussion

In the current study we have evaluated whether PAPPA2 has a potential role as a novel biomarker for gastric neoplasia. This study is based on a previous microarray study by Moore *et al.* [4] which identified 8 genes that were increased in hypergastrinaemic patients with type-1 gastric NETs one of which was PAPPA2. This increased PAPPA2 mRNA expression subsequently reduced when the patients were taking the CCK2R antagonist netazepide and returned to baseline upon cessation of treatment indicating that PAPPA2 expression may be associated with CCK2R activation. The findings of this study support this hypothesis as PAPPA2 mRNA and protein expression significantly increased with gastrin treatment in AGS_{GR} cells; this was maximal at 10nM G_{17} for 24hrs. After confirming the microarray results with qPCR and immunofluorescence, functional analysis was undertaken using short interfering RNAs (siRNAs) and suggested that gastrin-induced PAPPA2 overexpression may contribute in part, to the cellular morphological changes, migration and invasion that are associated with gastric carcinogenesis.

PAPPA2 is a homologue of PAPPA which is 1542 amino acids in length and comprises a putative pre-propeptide, an elongated zinc-binding motif (HEXXHXXGXXH) [739], a strongly conserved Met-turn[740] and a lin-notch repeat (LNR) module [741]. The proteolytic domain consists of five complement control protein modules (CCP1-5) [742, 743] and selectin, and two more LNR modules [741] which mediate cell surface adhesion and regulate tissue differentiation, respectively. Although PAPPA and PAPPA2 are encoded on different chromosomes (9 and 1 respectively), studies have identified a high sequence homology (44% identical and 62% similar amino acids) suggesting that the two proteins are a related subfamily of metzincin metalloproteinases that are distinct from other metalloproteinases such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase family of enzymes (ADAMs) [744, 745].

Matrix metalloproteinases (MMPs) are essential for the maintenance of the extra cellular matrix and structural integrity of cells by regulating the turnover of connective tissue such as collagens, proteoglycans and accessory ECM proteins. Recent studies have also suggested a potential role for MMPs in the release of growth factors, chemokines and cytokines [746] which contribute to vascular remodelling and immune and inflammatory responses. MMPs have been shown to cleave IGFBPs and to liberate IGFs, increasing their bioavailability. Liberated IGFs bind to their corresponding cell surface receptors (IGFRs) which activate the PI3K/Akt pathway [747] and are associated with malignant transformation [748, 749]. IGFBP5 upregulation has previously been observed in rhabdomyosarcoma [750], prostate cancer [751], smooth-muscle derived uterine leiomyoma [752], and in ~50% of gastric cancers[714], indicating a potential role in neoplastic development.

Previous studies have suggested that infection with *H. pylori* increases the production and secretion of MMP7 from gastric epithelial cells [190, 735, 753]. Secreted MMP7 liberates IGF-II from IGFBP5, which has been shown to be released from sub-epithelial cells, and stimulates the expansion and migration of the surrounding gastric microenvironment [734, 736]. Subsequent studies suggested that hypergastrinaemia also causes increased MMP7 expression, as well as MMP1 [188] and MMP9 [189], *in vitro* [191, 734, 736] and in mice and humans *in vivo* [112]. Patients with increased gastric MMP7 expression secondary to hypergastrinaemia (>100pM) have been shown to possess an increased risk of developing type-1 gastric NETs [754] and this has also been associated with poorer prognosis [755-757], possibly due to increased tissue remodelling, cell migration and invasion. Additionally in colonic myofibroblasts, MMP7 has been shown to increase the abundance of other MMP precursors and active enzymes including MMP3 and MMP8 [736] which may contribute to a negative clinical outcome.

Metalloproteinase activity is tightly regulated by tissue inhibitors of metalloproteinases (TIMPs) usually via the binding of the TIMP N-terminal domain with the active-site of the enzyme inhibiting substrate binding [758, 759]. However, Rapti *et al*. reported that both ADAM10 and ADAM17 are inhibited by TIMP3, though the binding of the N-terminus to ADAM10 is insufficient for complete inhibition [760], which suggests that other inhibitory mechanisms may also occur. The C-terminal domain of TIMPs has been shown to stabilise interactions with specifc pro-MMPs such as TIMPs -2, -3 and -4 with pro-MMP2, allowing the potential for the binding of a second MMP at the N-terminus. Although the functional significance of multiple MMP binding is unclear, the formation of the TIMP2/pro-MMP2/MT1-MMP ternary complex has been shown to be essential for the activation of proMMP2 [761], which suggest an alternative role for TIMPs in the activation of MMPs.

H. pylori infection has been shown to increase the abundance of TIMPs, specifically TIMP1, 3 and 4 in human gastric corpus biopsies. Immunohistochemical analysis indicated that all three TIMPs were localised to glandular epithelia. Only TIMPs 3 and 4 were localised in the surface epithelia and TIMP3 alone showed strong immunoreactivity within the stroma, which suggests distinct roles and regulatory mechanisms for each anti-protease. *H. felis* infection in mice has also been shown to increase the abundance of TIMP1 and TIMP3 mRNAs [762]. TIMPs have also been shown to exert MMP independent biological functions such as the promotion or inhibition of cell growth, apoptosis, the promotion of angiognenesis and increased synaptic plasticity [763] through the direct binding of cell surface receptors [764]. Therefore, alterations in the ratio of MMPs:TIMPs caused by *H. pylori* infection may be a factor that helps to determine clinical outcome.

Similarly, PAPPA2 has also been shown to cleave IGFBPs, primarily IGFBP5, whose concentrations are increased in the plasma during all stages of pregnancy and peak during the later stages [765]. Proteolysis of IGFBP5 liberates free IGF-I which activates the IGF-1R.

The IGF-1R has been shown to be essential for cell growth and survival and *IGF1R* deletion or *IGFBP5* overexpression in mice is associated with severe growth deficiency and high morbidity at birth [713, 766]. However, aberrant activation of the IGF-1R has been associated with neoplasia and cancer development [767-770], whilst decreased IGF signalling has been shown to reduce tumour proliferation [771-773].

During early placental development, environmental conditions are relatively hypoxic (2-4% oxygen) resulting in increased villus trophoblast proliferation and differentiation. Uterine invasion by villus trophoblasts results in direct contact between the maternal and foetal circulations after 10 weeks of gestation [774] and this is promoted by growth factors such as IGF-I which are liberated from their binding proteins by proteases [775, 776]. Although both PAPPA and PAPPA2 cleave IGFBPs, low PAPPA expression has previously been associated with placental pathology [777-780], whereas high PAPPA2 expression has been associated with complicated pregnancies such as pre-eclampsia [722] and HELLP syndrome [726], as a consequence of placental hypoxia and tissue injury.

Christians *et al.* previously identified low levels of *PAPPA2* gene expression in a variety of other mouse tissues. This was strongest in the stomach, skin and embryo with expression also being observed in the kidney, lung, brain, heart, testis prostate and pancreas [781]. Whole-exome sequencing of patients with lung adenocarcinoma found that an increase in PAPPA2 gene mutations was indicative of a better prognosis with significantly prolonged survival rates being observed in a Japanese population. Mutations in the *PAPPA2* gene may decrease PAPPA2 protein expression which results in elevated levels of IGFBP5 and decreases subsequent IGF-I signalling [725]. However, the proteolytic activity of PAPPA2 is relatively unknown in these tissues.

In the work presented in this chapter, I have identified that PAPPA2 gene and protein expression is increased in a human gastric adenocarcinoma cell line in response to gastrin treatment. Similar to MMPs, gastrin-induced PAPPA2 overexpression was significantly

associated with decreased cell proliferation and increased migration and cell remodelling, known cofactors for gastric tumourigenesis. Metalloproteinases have been shown to share structural homology and also have many overlapping functions that have previously made clinical application difficult [782, 783]. However, their potential use as biomarkers of disease and disease progression may be of clinical significance.

The normal gastric mucosa progresses through a series of premalignant stages during cancer development from atrophy through metaplasia to dysplasia. This involves biological processes that have been associated with hypergastrinaemia and in part, PAPPA2 expression. Therefore, PAPPA2 expression changes may correspond with early stages of neoplasia and if so could potentially provide an indication for an increased risk of gastric cancer development. Previously, PAPPA2 expression has been successfully detected in human serum as an effort to improve prenatal screening [721, 784]. Future serological studies are therefore indicated to assess whether PAPPA2 has a potential application as a novel non-invasive biomarker for gastric disease.

3.3 The effects of three novel gastrin receptor antagonist compounds TR1, TR2 and TR3 on gastrin- induced premalignant changes *in vitro*

3.3.1 Abstract

Type-1 gastric neuroendocrine tumours (NETs) develop as a consequence of chronic autoimmune atrophic gastritis (CAG) in conjunction with hypergastrinaemia. Gastrin is a regulatory peptide that binds to the cholecystokinin subtype-2 receptor (CCK2R) and can promote tumour development via dysregulation of cell proliferation, migration and actin remodelling. For most patients tumour resection is potentially curative, however this is not always possible. Several CCK2 receptor antagonists have previously been developed, but very few have achieved clinical application due to low oral absorption, potency and/or selectivity issues. Using a variety of different cell-based assays, I have examined whether the three novel CCK2 receptor antagonists TR1, TR2 and TR3, inhibit gastrin-induced biological processes in AGS_{GR} and AR42J cells and have compared these effects with the previously developed benzodiazepine analogue CCK-2 receptor antagonists YM022 and netazepide (YF476). All three compounds inhibited gastrin-induced cell growth, migration, branching morphogenesis and clonogenic survival *in vitro* and did not induce cellular toxicity or influence other cellular phenomena when administered alone. Of the three compounds TR2 was the most potent followed by TR1 and TR3 was the least potent. However, all three compounds were less potent than YM022 and netazepide. These data suggest that TR2, but not TR1 or TR3, could potentially be developed as a specific CCK2R antagonist to treat gastrin related conditions if further studies confirm that it has increased oral bioavailability and selectivity compared to its previously established competitors.

3.3.2 Introduction

Gastrin is a hormone that is essential for gastric homeostasis and alterations in its secretion or processing may influence the initiation and progression of various diseases [130]. The physiological functions of gastrin have been largely investigated within the stomach, primarily focussing on acid secretion following CCK2 receptor (CCK2R) binding which aids food digestion [15, 467, 717, 718]. However, gastrin also plays a central role in regulating gastric mucosal growth and differentiation [785]. Previous studies have suggested that pathological changes in the stomach particularly ECL-cell hyperplasia, dysplasia and subsequent neuroendocrine (carcinoid) formation, may develop, in part, due to elevated gastrin concentrations which arise as a consequence of autoimmune atrophic gastritis- induced hypochlorhydria [302].

The organisation of gastric epithelia is maintained by cell proliferation, migration and differentiation. Proliferating cells located in the isthmus of the gastric gland migrate towards the surface or the base and differentiate; adopting multiple cell phenotypes including enterochromaffin-like (ECL), parietal, chief and endocrine cells [154]. Gastrin has both direct and indirect influences on AGS gastric adenocarcinoma cells dependent upon the existence of cell surface receptors, particularly the CCK2R which has been associated with both pro- and anti-proliferative effects of G_{17} [663]. Studies by Håkanson *et al.* examined the trophic effects of hypergastrinaemia on ECL cells and concluded that the gastrin/CCK2 receptor signalling pathway was an essential regulator of cell remodelling [786] which can facilitate type-1 gastric neuroendocrine tumour development.

Tumour cells acquire the ability to reproduce indefinitely, with atypical activation of survival pathways possibly contributing to resistance towards drug treatment. It has previously been shown that gastrin directly inhibits AGS_{GR} cell proliferation by inhibiting progression through the cell cycle in the G_1 phase [663]. However, gastrin treatment has also

been shown to reduce the cytotoxic effects of chemotherapeutic agents such as etoposide [787]. Abdalla reported that gastrin caused a 27% increase in peripheral blood mononuclear cell (PBMC) viability when treated with etoposide [788]; retaining cell reproducibility and increasing survival. By contrast, in other cell lines, specifically rat pancreatic tumour (AR42J) cells, gastrin has been shown to increase cell growth by inducing the progression of the cell cycle into S-phase, which promotes protein and DNA synthesis [339] and down regulation of the CCK2 receptor has shown to inhibit cell growth and induce apoptotic activity [789].

In some patients with hypergastrinaemia there is a progression from a normal mucosa to ECL cell dysplasia and subsequent type-1 gastric neuroendocrine (NETs) formation. The structure of the mucosa is maintained through reciprocal signalling between the gastric epithelium and stromal cells, particularly myofibroblasts, which release cytokines and proteases involved in the regulation of the extracellular matrix (ECM). Gastrin has previously shown to increase the expression of some proteases involved in tissue remodelling, specifically MMP1 [188], MMP7 [191] and MMP9 [189] which increase ECM degradation. Loss of ECM stability activates epithelial-to-mesenchymal transition (EMT) which reduces cell-cell adhesion and may contribute to tumour migration, invasion and angiogenesis.

Multiple gastrin/CCK2 receptor antagonists have previously been investigated; however clinical application has proven disappointing due to low oral bioavailability, potency and/or selectivity. However two clinical trials [460, 460], have recently demonstrated that patients with type-1 gastric neuroendocrine tumours who received a daily oral dose of 50mg netazepide (YF476) for 12 weeks with a 12 week follow-up showed an approximate 30% reduction in tumour size and number. In addition, serum chromogranin A (a biomarker of ECL-cell activity) concentrations were suppressed during therapy, but these returned to

pre-treatment levels after 12 weeks of follow up and serum gastrin levels remained unaffected.

Therefore using a variety of *in vitro* assays, we set out to assess whether three novel potential CCK2 receptor antagonists synthesised by Trio Medicines Ltd (TR1, TR2 and TR3) inhibited gastrin-induced cellular phenomena and have compared their potencies against the previously developed CCK2 receptor antagonists YM022 and netazepide (YF746).

3.3.3 The anti-proliferative effects of gastrin are inhibited by compounds TR1, TR2 and TR3 in AGS_{GR} cells.

To investigate whether pre-treatment with three new gastrin receptor antagonists completely reversed the anti-proliferative effects of gastrin *in vitro*, SRB assays were performed for each drug with and without gastrin treatment. A broad range of concentrations (1, 10, 25, 50, 100, 500, 1000nM) of each compound was initially tested followed by a specific range of concentrations to further characterise the dose response relationship for each drug. SRB stain absorbance was measured at 570nm and cellular content was assessed as a percentage of the negative untreated control absorbance. To validate the minimum concentration at which compounds TR1, TR2 and TR3 inhibited the anti-proliferative effects of gastrin, haemocytometer count assays were also performed. Untreated and G_{17} 10nM alone treatments were used as negative and positive controls respectively and 1% DMSO was used as a vehicle control.

YM022 and netazepide (both at 60nM) completely inhibited the anti-proliferative effects of 10nM G_{17} ($P<0.0001$) and neither YM022 nor netazepide alone (concentrations of 1-100nM) induced cellular toxicity in AGS_{GR} cells (Figure 38)(N=3, n=8). This was validated with haemocytometer counts in AGS_{GR} cells as 60nM of either YM022 or netazepide caused complete reversal of the anti-proliferative effects of 10nM G_{17} with minimal floating cells being observed, indicating no significant cellular toxicity (Figure 39) (N=3, n=3). These drugs were therefore used as positive inhibition controls in future experiments at concentrations of 100nM. Complete reversal of the anti-proliferative effects of 10nM G_{17} was observed when cells were pre-treated with the following minimum concentrations of TR1 (500nM) ($P<0.001$), TR2 (100nM) ($P<0.0001$) and TR3 (500nM) ($P<0.01$) (Figure 40) (N=4, n=8). These findings were validated using haemocytometer counts and treatment with TR1, TR2 and TR3 alone at concentrations 1-1000nM did not induce cellular toxicity, as AGS_{GR} cell growth was not affected (Figure 41).

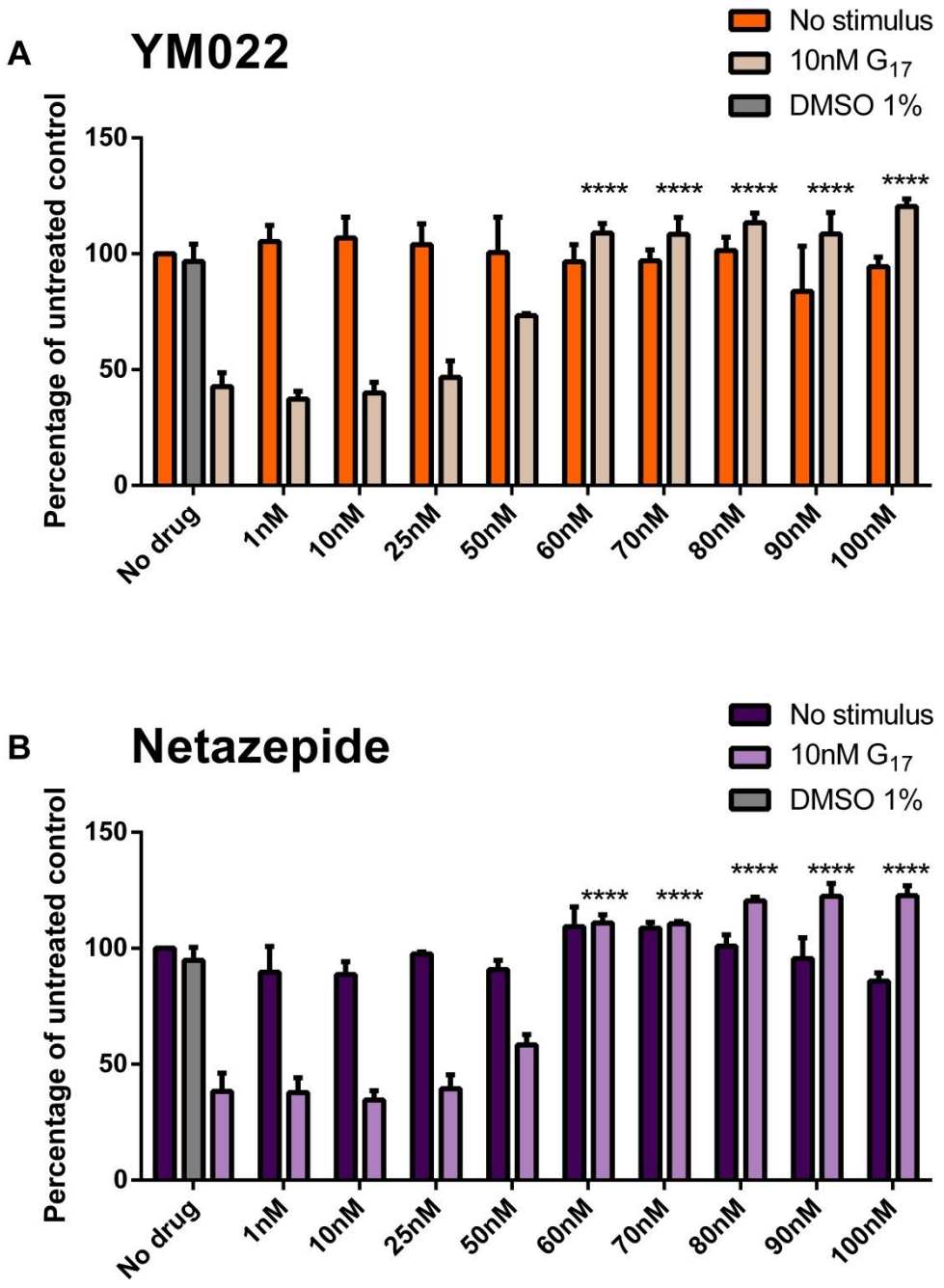

Figure 38. YM022 and netazepide both at 60nM completely reverse the inhibition of AGS_{GR} cell proliferation caused by 10nM G_{17}.YM022 and netazepide alone at concentrations of 1-100nM do not induce toxicity in AGS_{GR} cells (A and B) (N=3, n=8). Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (****P<0.0001 vs no drug controls).

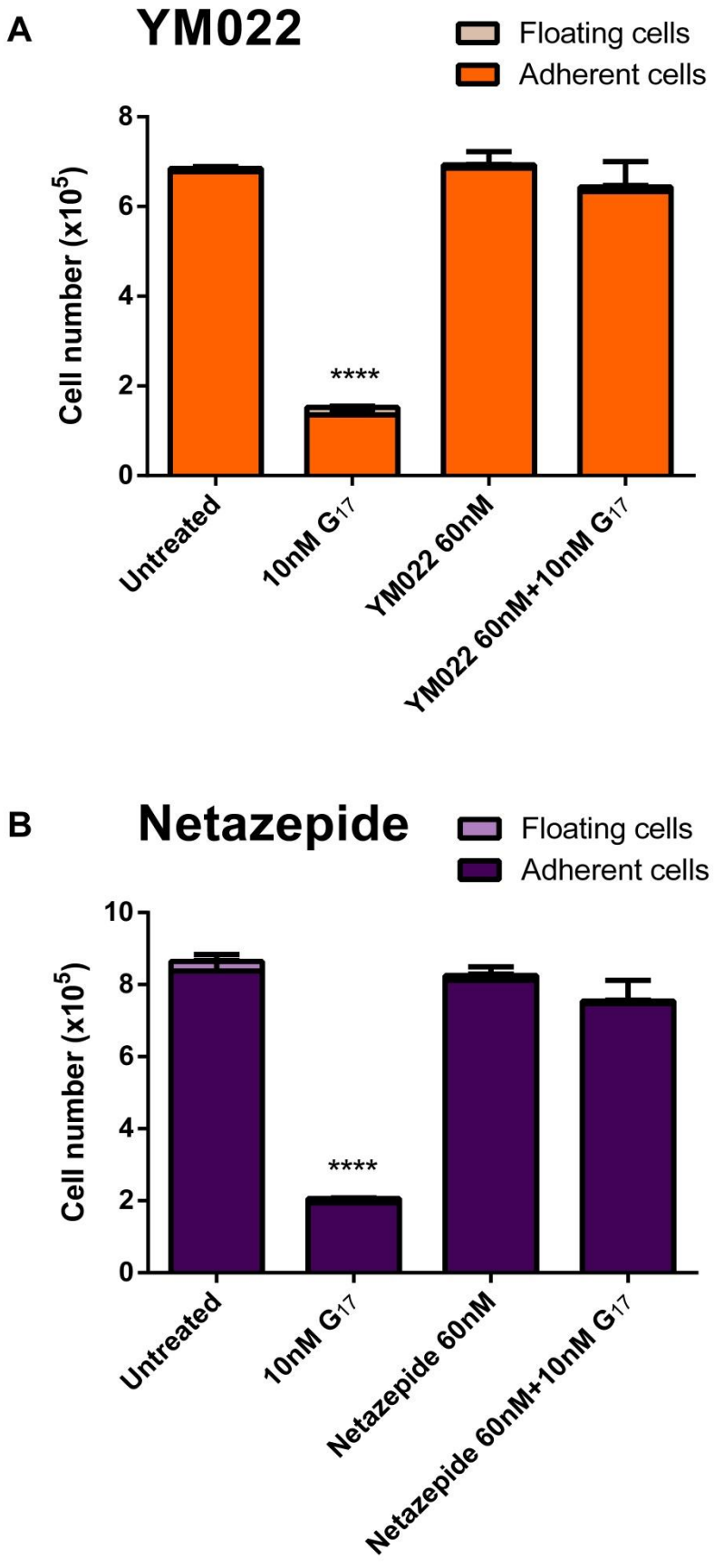

Figure 39. YM022 (A) and netazepide (B) both at 60nM concentration completely reverse the anti-proliferative effects of 10nM G_{17} treatment with no significant cellular toxicity (N=3, n=3). Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (****P<0.0001 vs untreated controls).

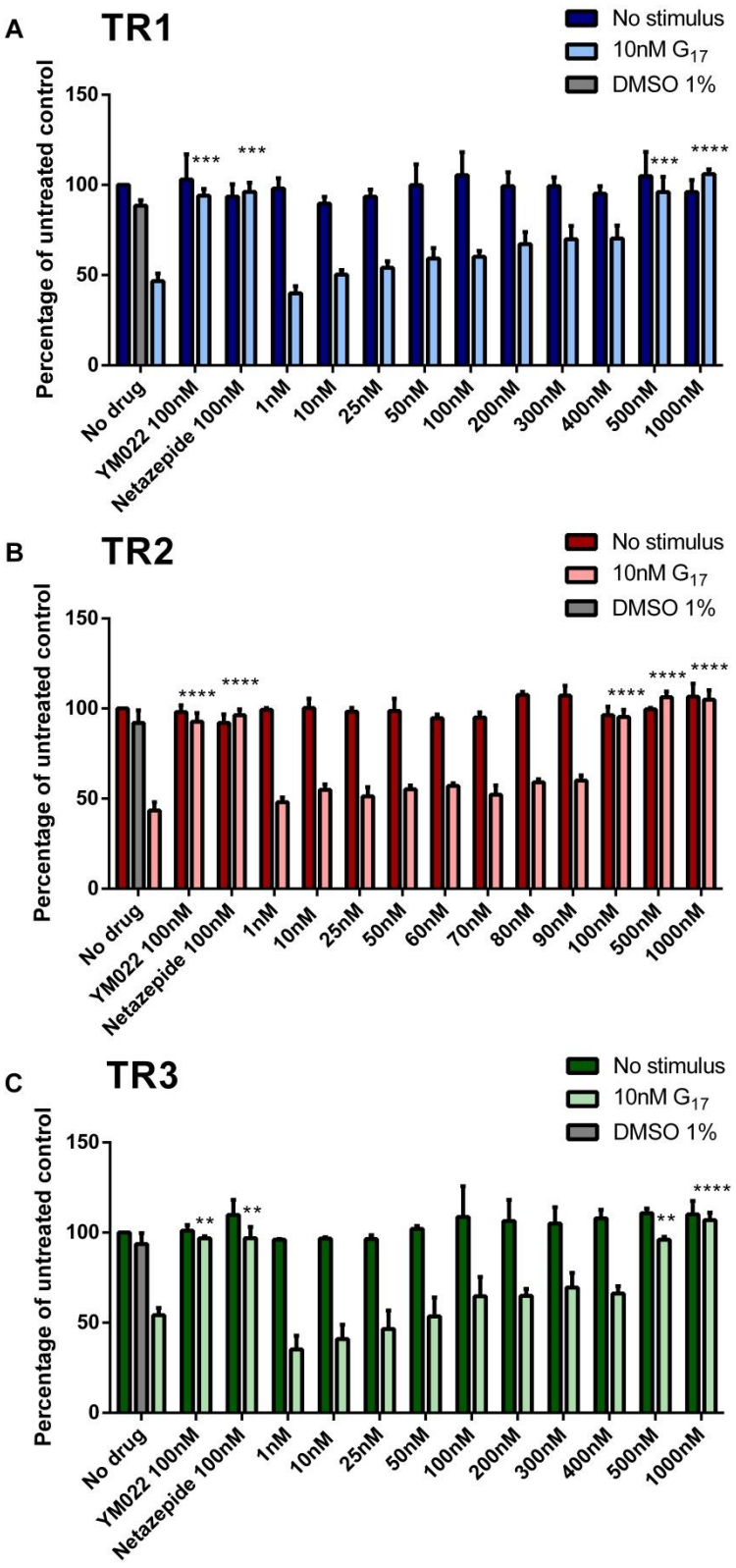

Figure 40. TR1 500nM (A), TR2 100nM (B) and TR3 500nM (C) completely reverse the inhibition of AGS_{GR} cell proliferation caused by 10nM G_{17}. TR1, TR2 or TR3 alone at concentrations of 1-1000nM do not induce toxicity in AGS_{GR} cells (N=4, n=8). Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (**P<0.01, ***P<0.001 and ****P<0.0001 vs no drug controls).

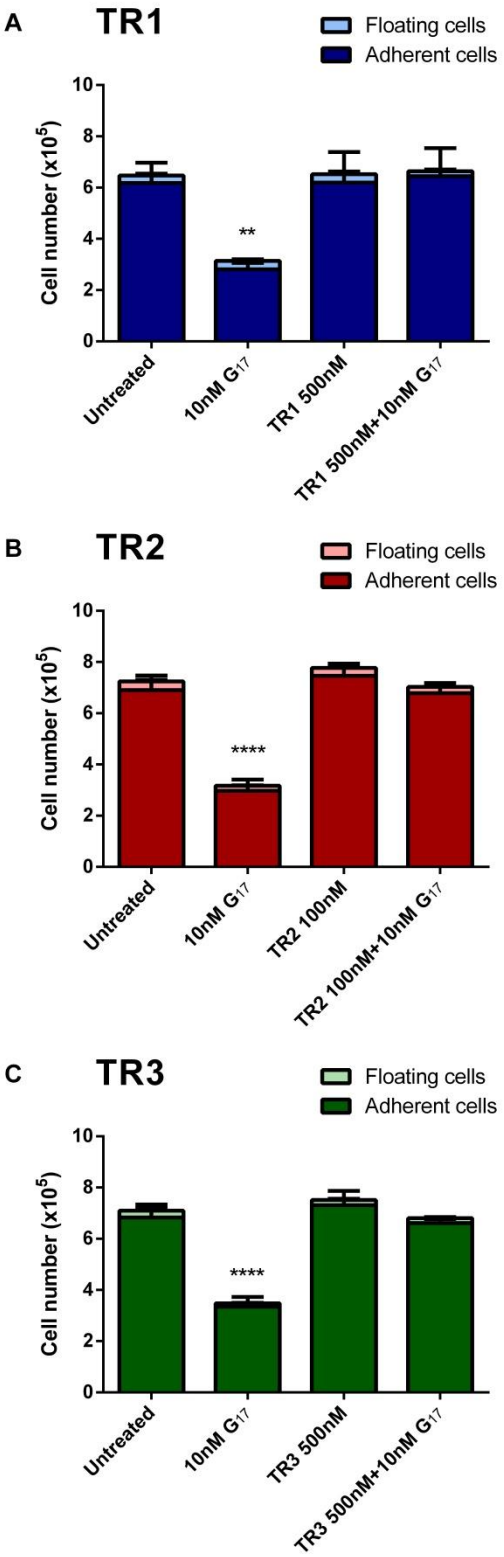

Figure 41. TR1 500nM (A), TR2 100nM (B) and TR3 500nM (C) completely reverse the anti-proliferative effects of 10nM G_{17} treatment with no significant cellular toxicity (N=3, n=3). Statistical significance was determined using two-way ANOVA with Sidak *post-hoc* test and $P<0.05$ was considered significant (**$P<0.01$ and ****$P<0.0001$ vs untreated controls).

3.3.4 The migratory effects of gastrin are inhibited by compounds TR1, TR2 and TR3 in AGS_{GR} cells

Scratch wound migration assays were performed to investigate whether pre-treatment with the three new gastrin receptor antagonists reversed the migratory response stimulated by gastrin in AGS_{GR} cells *in vitro*. For each drug a broad range of concentrations was initially investigated (1, 10, 25, 50, 100nM) followed by a more specific range of concentrations to further characterise the dose response relationship for each drug.

Gastrin stimulated migration was completely inhibited by YM022 and netazepide both at 50nM concentrations (Figure 42 and 44), and were therefore used as positive inhibition controls (N=3, n=3).

Gastrin-stimulated AGS_{GR} cell migration was completely inhibited by TR1 300nM, TR2 80nM and TR3 1000nM (Figure 43 and 44) (N=3, n=3).

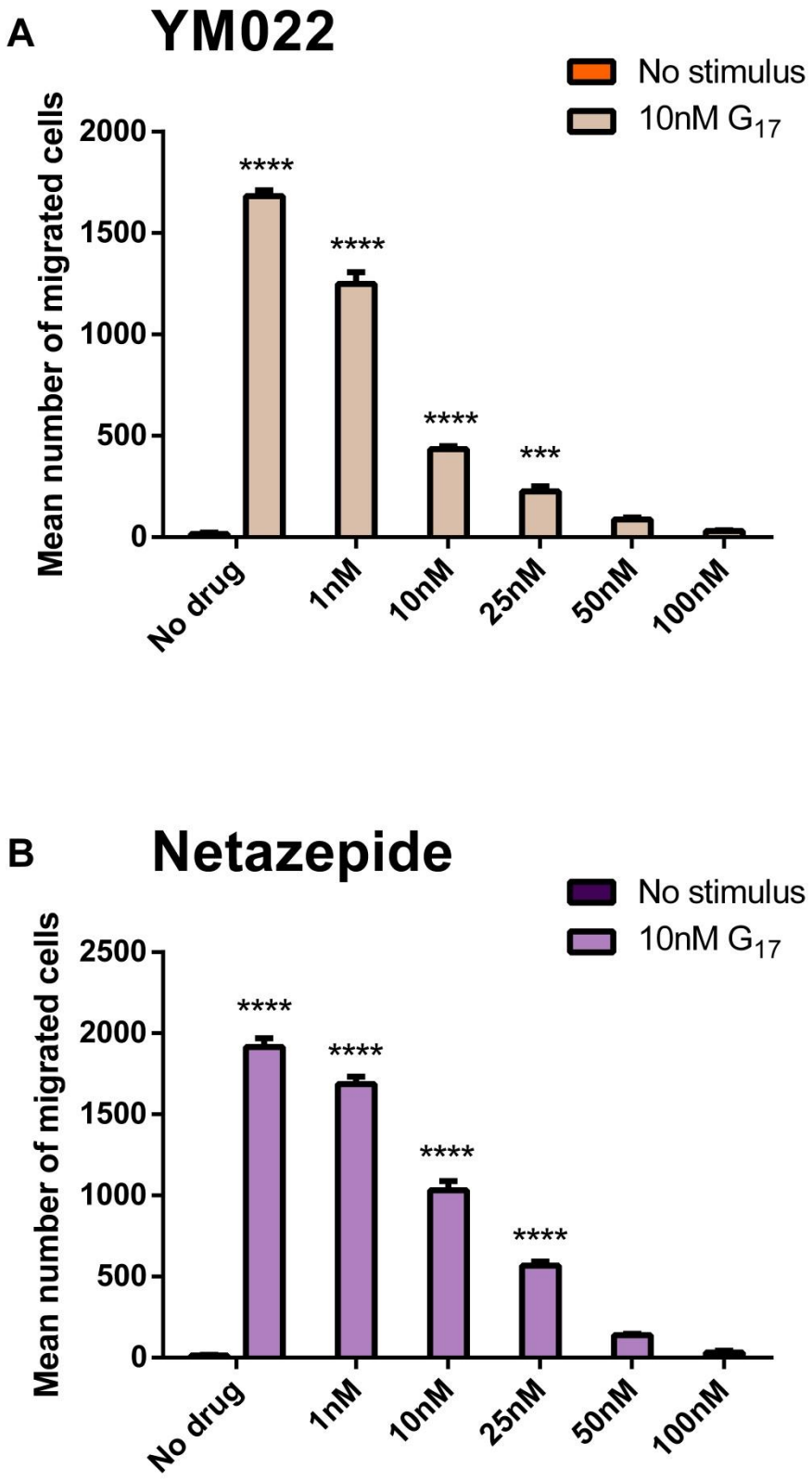

Figure 42. Migratory response in control and gastrin stimulated (10nM, 8hrs) AGS_{GR} cells with and without YM022 (A) or netazepide (B) pre-treatment (N=3, n=3). Statistical significance was determined using two-way ANOVA with Sidak p*ost-hoc* test and P<0.05 was considered significant (***P<0.001 and ****P<0.0001 vs no drug controls).

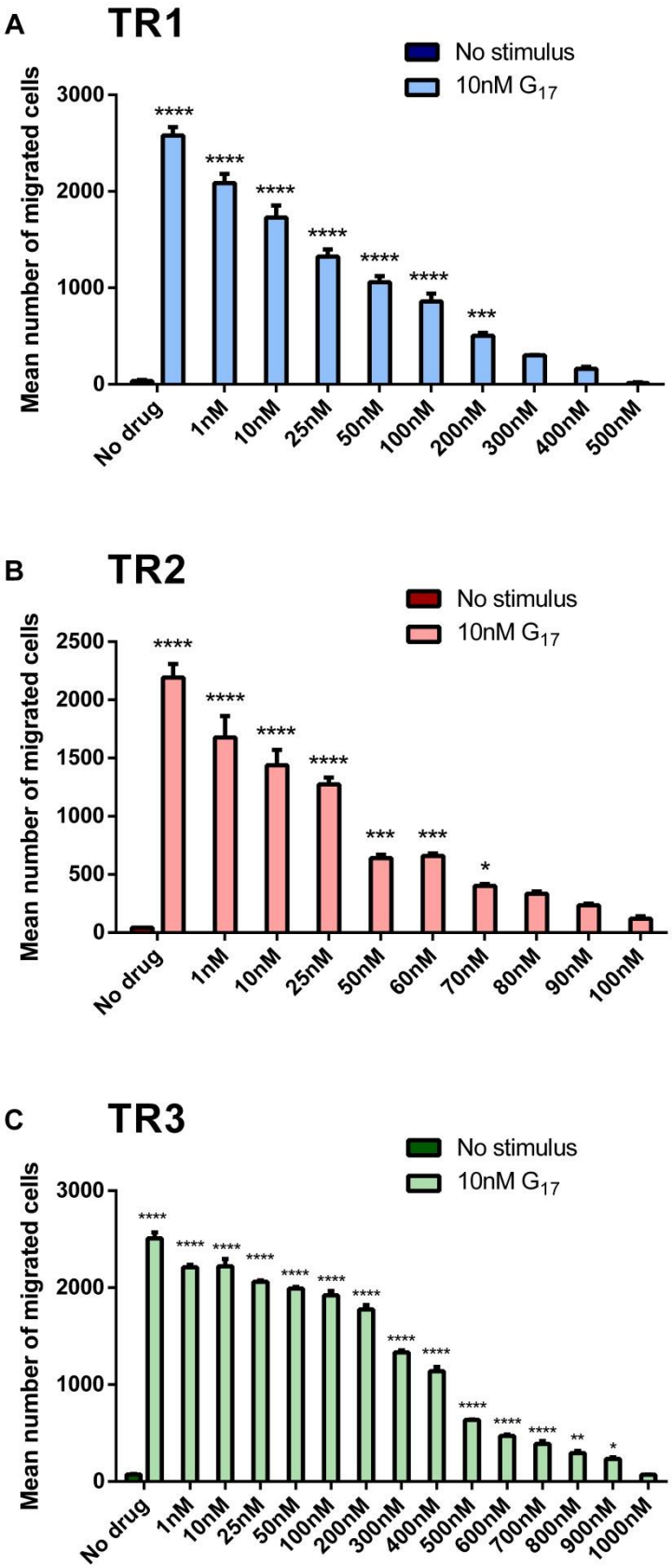

Figure 43. Migratory responses in control and gastrin stimulated (10nM, 8hrs) AGS_{GR} cells with and without TR1 (A) TR2 (B) and TR3 (C) pre-treatment (N=3, n=3). Statistical significance was determined using two-way ANOVA with Sidak p*ost-hoc* test and P<0.05 was considered significant (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs no drug controls).

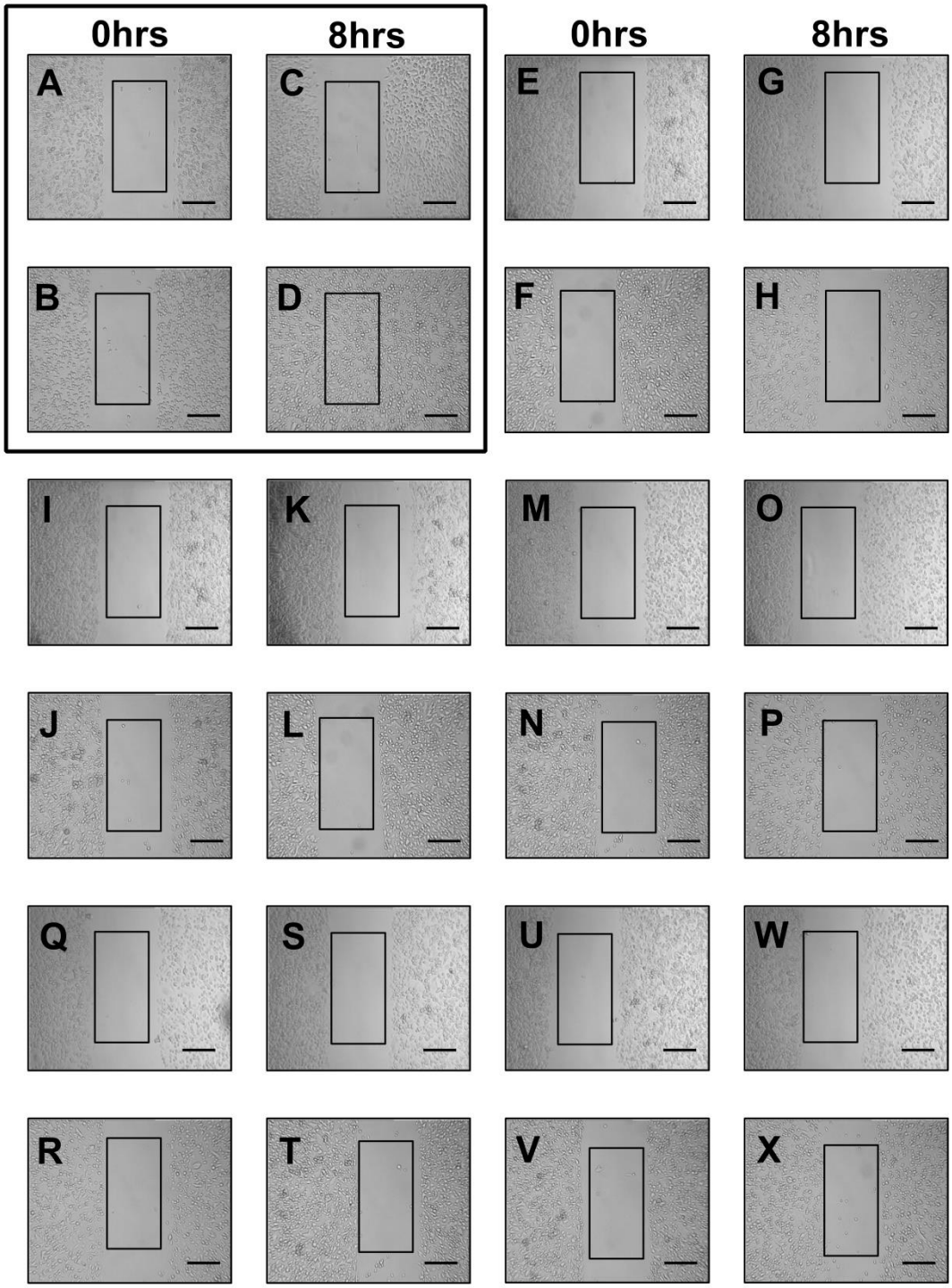

Figure 44. Scratch wounds for 0hrs and 8hrs respectively, in untreated AGS_{GR} cells (A,C) and cells treated with 10nM gastrin (B, D) stimulated cell migration. Treatment with CCK2 receptor antagonists: YM022 50nM (E, G), netazepide 50nM (I, K), TR1 300nM (M, O), TR2 80nM (Q, S) and TR3 1000nM (U, W) alone did not induce AGS_{GR} cell migration. Pre-treatment with CCK2 receptor antagonists: YM022 50nM (F, H), netazepide 50nM (J, L), TR1 300nM (N, P), TR2 80nM (R, T) and TR3 1000nM (V, X) completely inhibited in response to 10nM gastrin treatment at 8hrs (scale bar 50µm) (N=3, n=3).

3.3.5 Gastrin-induced cellular morphological changes are inhibited by compounds TR1, TR2 and TR3 in AGS_{GR} cells

Incubation of AGS_{GR} cells with G_{17} 10nM induced time dependent morphological responses (Figure 45); with the extension of long processes being observed after 6hrs incubation. Therefore, branching morphogenesis assays were performed to investigate whether pre-treatment with compounds TR1, TR2 or TR3 would inhibit this gastrin-induced cellular phenotype in AGS_{GR} cells. An initial dose response was performed for a broad range of concentrations for each drug (1, 10, 25, 50, 100, 500nM) followed by a specific range to further assess the minimum inhibitory concentration. Untreated, 10nM G_{17} treated and 1% DMSO treated were used as negative, positive and vehicle controls.

YM022 (10nM) and netazepide (25nM) completely inhibited, and netazepide 10nM partly inhibited gastrin stimulated branching morphogenesis in AGS_{GR} cells (Figure 46) (N=3, n=3). Gastrin induced morphological changes were significantly inhibited by TR1 10nM, TR2 50nM and TR3 10nM and completely inhibited by TR1 400nM, TR2 400nM and TR3 500nM (Figure 47) (N=3, n=3).

Immunofluorescence visualisation confirmed that gastrin-induced branching morphogenesis was completely inhibited by pre-treatment with CCK2 receptor antagonists: YM022, netazepide, TR1, TR2 and TR3 (Figure 48) at the minimum concentrations for complete inhibition of gastrin that were shown by the branching morphogenesis assays (N=3, n=6).

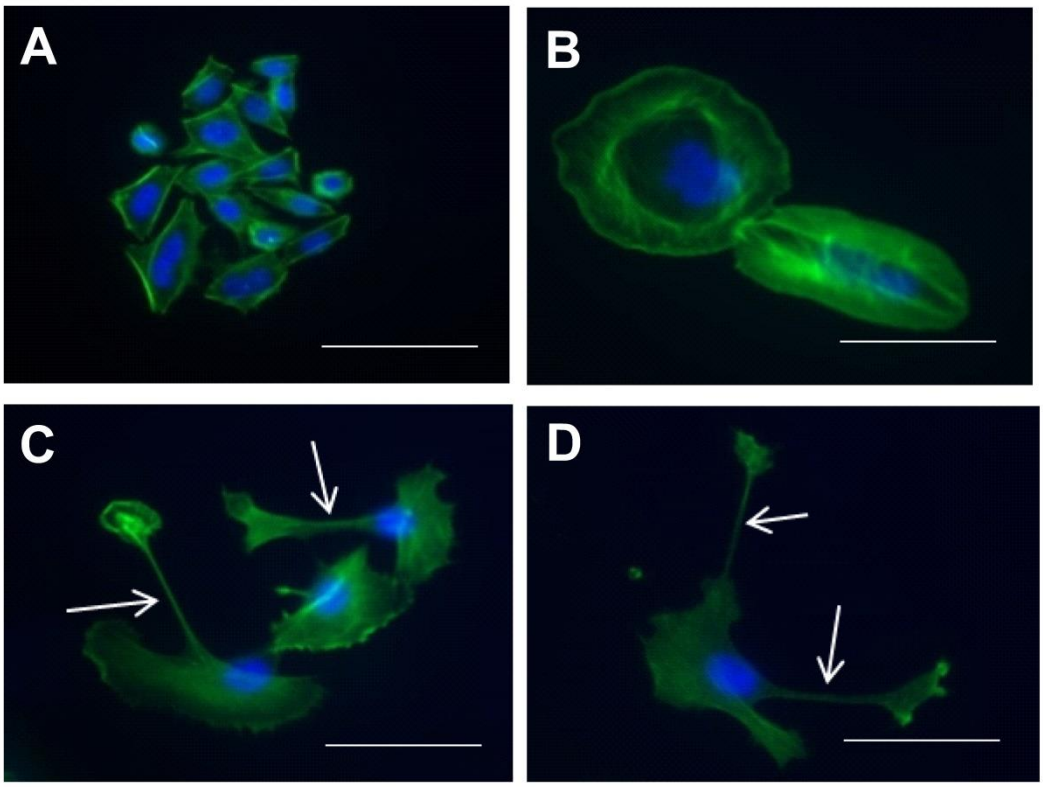

Figure 45. Phalloidin-488 staining of actin shows remodelling of the cytoskeleton and the extension of long processes in AGS_{GR} cells (white arrows). Untreated cells (A) and cells treated with 10nM G_{17} 30mins (B) show gastrin stimulated cell spreading. Cells treated with G_{17} 6hrs (C) and 15hrs (D) show the reorganisation of the actin cytoskeleton and branching morphogenesis in the form of long processes (scale bar 50μm).

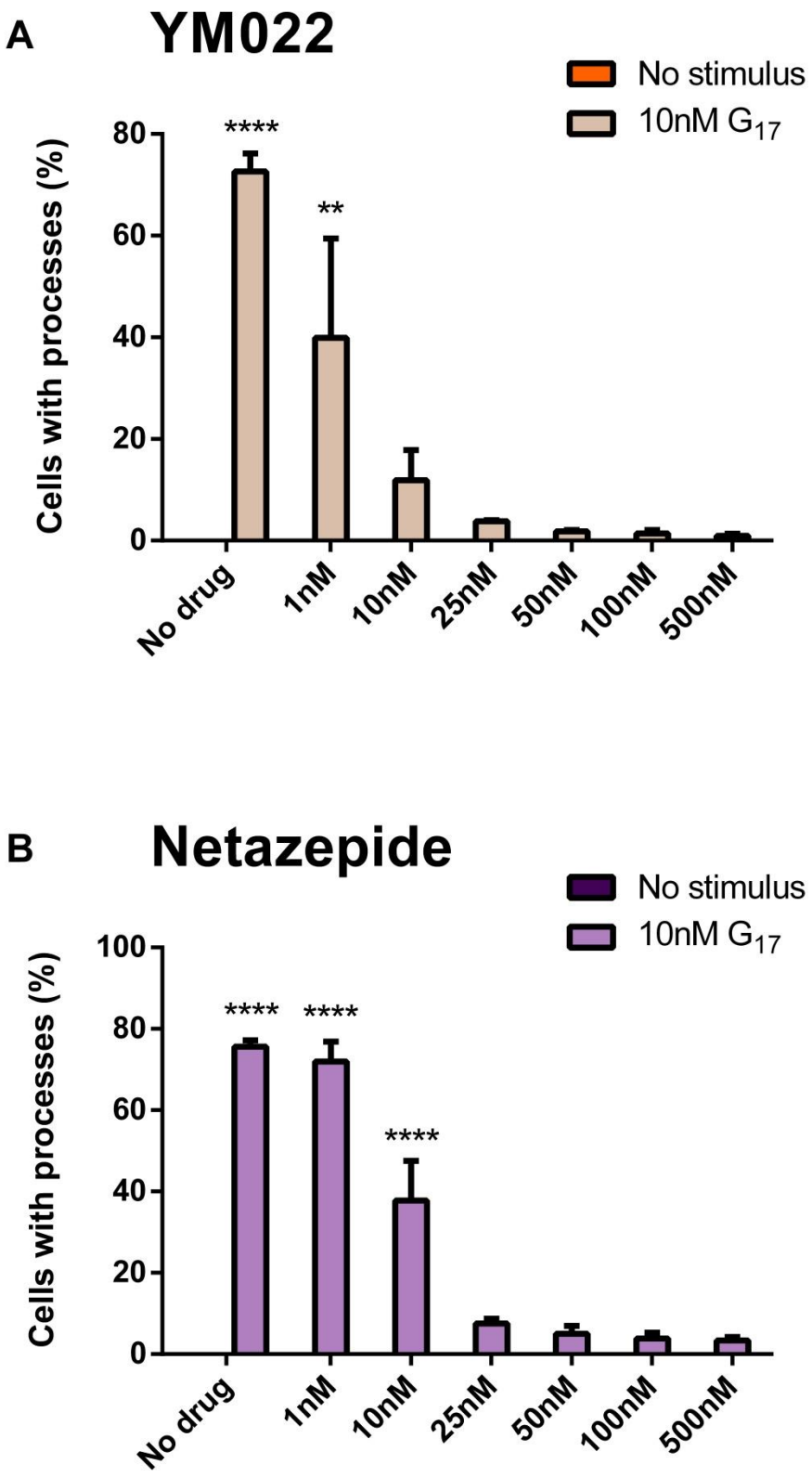

Figure 46. Branching morphogenesis in control and gastrin stimulated (10nM, 6hrs) AGS_{GR} cells with and without YM022 (A) and netazepide (B) pre-treatment (N=3, n=3). Each experiment was run three times and values are expressed as mean percentage of total cells ± SEM. Statistical significance was determined using two way ANOVA with Sidak *post-hoc* test and $P<0.05$ was considered significant (**$P<0.01$ and ****$P<0.0001$ vs no drug controls).

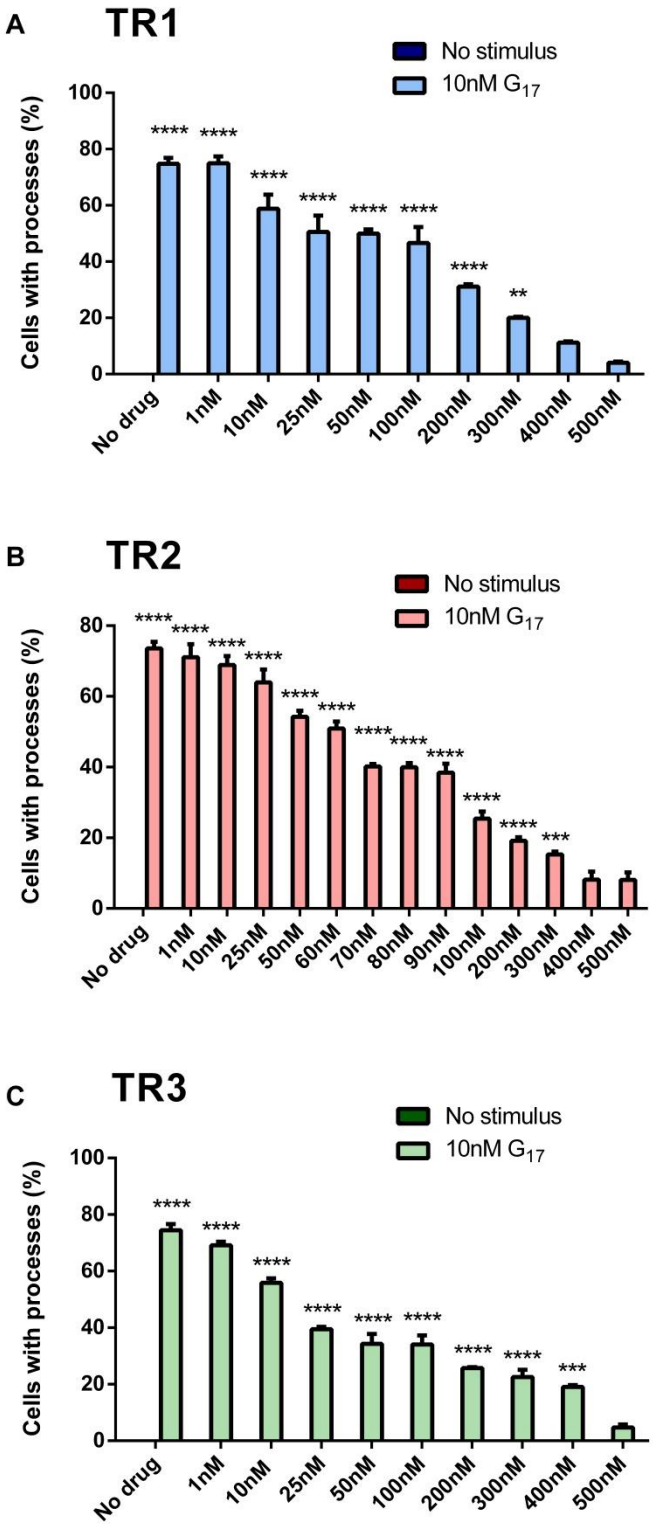

Figure 47. Branching morphogenesis in control and gastrin stimulated (10nM, 6hrs) AGS_{GR} cells with and without TR1 (A) TR2 (B) and TR3 (C) pre-treatment (N=3, n=3). Each experiment was repeated three times and values are expressed as mean percentage of total cells ± SEM. Statistical significance was determined using two way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (**P<0.01, ***P<0.001 and ****P<0.0001 vs no drug controls).

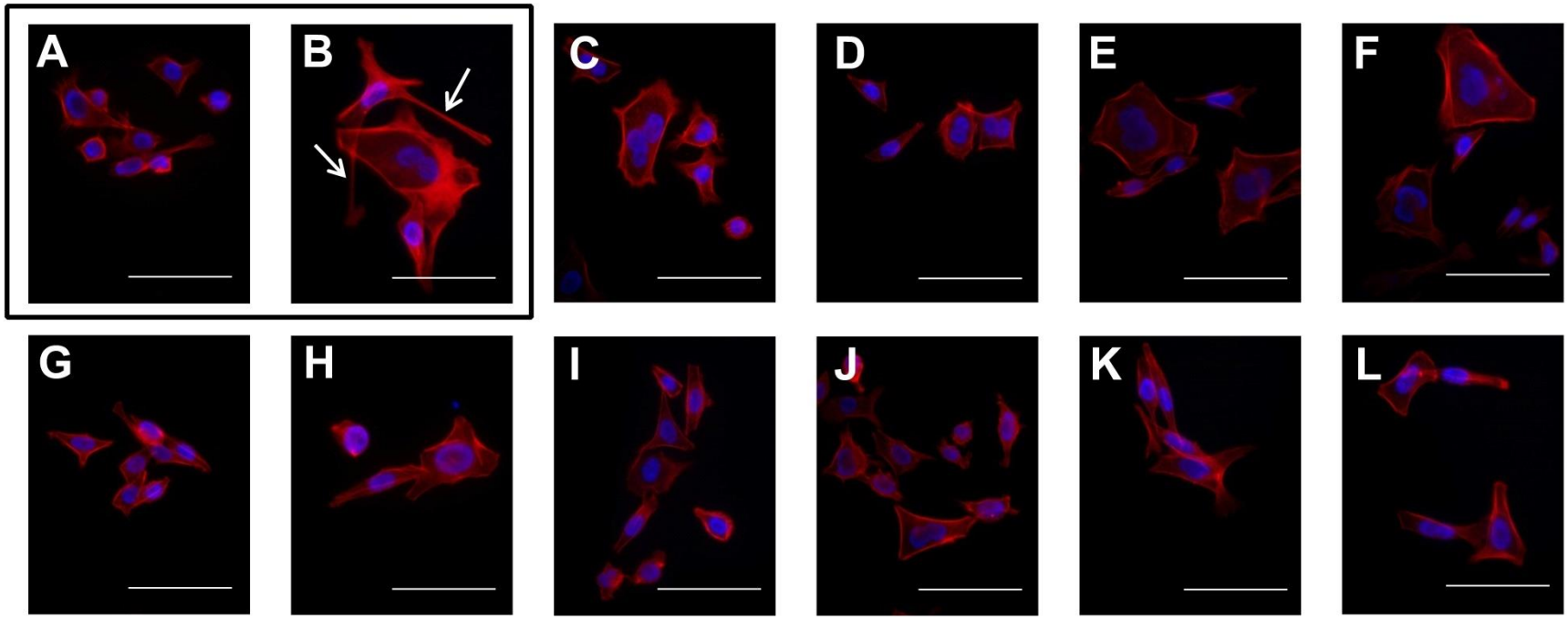

Figure 48. Phalloidin-594 staining of actin shows remodelling of the actin cytoskeleton and the extension of long processes in AGS_{GR} cells. Untreated AGS_{GR} cells (A) show no branching morphogenesis and were used as a negative control, whereas 10nM G_{17} 6hrs stimulated the formation of long processes and was used as a positive control (B). Pre-treatment with CCK2 receptor antagonists without 10nM G_{17} treatment 6hrs: netazepide (25nM, C), YM022 (10nM, E), TR1 (400nM, G), TR2 (400nM, I) and TR3 (500nM, K) did not induce the formation of processes. Pre-treatment with CCK2 receptor antagonists followed by 10nM G_{17} 6hrs showed complete inhibition of branching morphogenesis: netazepide (25nM, D), YM022 (10nM, F), TR1 (400nM, H), TR2 (400nM, J) and TR3 (500nM, L) (scale bar 50µm) (N=3, n=6).

3.3.6 The long term effects of gastrin on clonogenic survival are inhibited by compounds TR1, TR2 and TR3 in AGS_{GR} cells

Gastrin has previously been shown to inhibit clonogenic survival and etoposide cytotoxicity in AGS_{GR} cells after 6hrs treatment (Pritchard unpublished data). Therefore clonogenic assays were performed to assess whether pre-treatment with TR1, TR2, TR3, YM022 or netazepide would improve long term cell survival and reverse the inhibition of cell cytotoxicity in AGS_{GR} cells.

Pre-treatment with YM022 (25nM) or netazepide (25nM) completely reversed the inhibition of clonogenic survival induced by 10nM G_{17}. YM022 and netazepide (both 1nM) completely reversed the inhibition of etoposide-induced cytotoxicity induced by 10nM G_{17} (Figures 49 and 51) (N=3, n=3).

Improvements in cell survival were seen with all 3 compounds at concentrations of TR1 (600nM), TR2 (100nM) and TR3 (700nM). All 3 compounds at concentrations of 1nM completely reversed the inhibition of etoposide-induced cytotoxicity induced by 10nM G_{17} (Figures 50 and 51) (N=3, n=3).

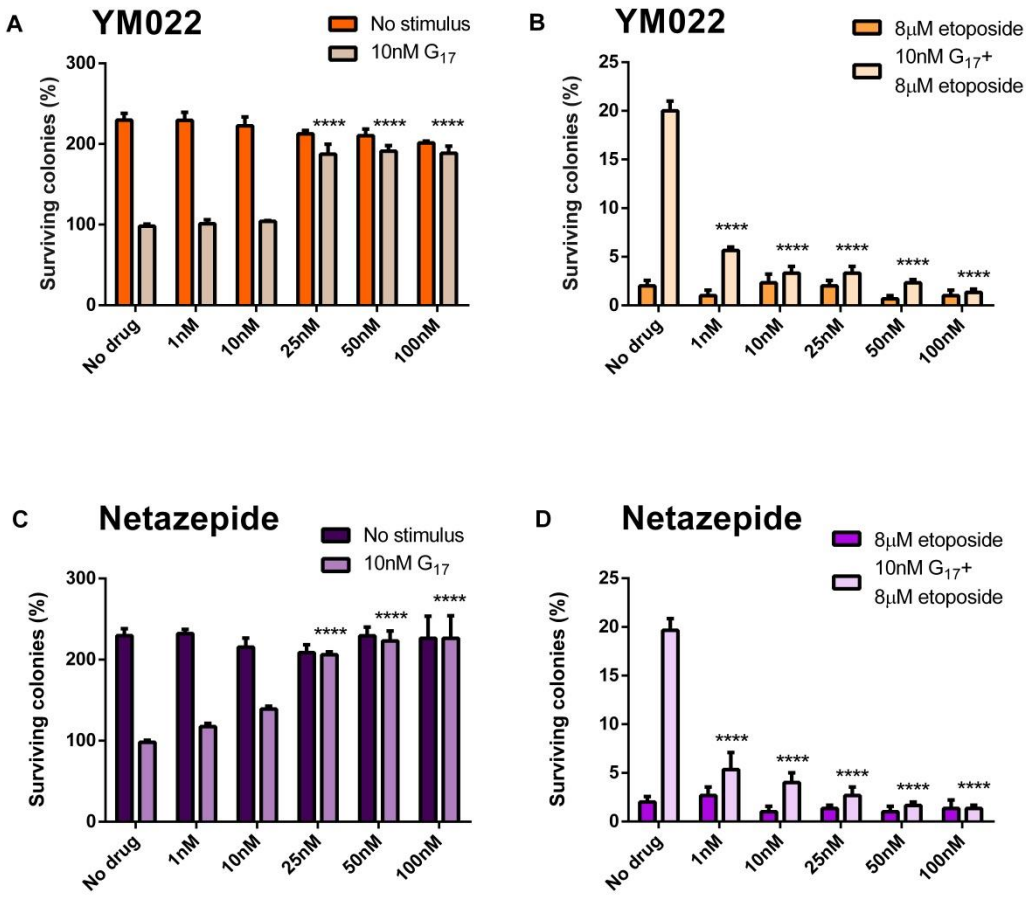

Figure 49. Clonogenic survival assays for YM022 (A, B) and netazepide (C, D) dose responses after 6hrs 10nM G_{17} treatment with and without 1hr 8μM etoposide treatment (N=3, n=3). Each experiment was repeated three times and values are expressed as mean percentage of control ± SEM. Statistical significance was determined using two way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (****P<0.0001 vs no drug controls).

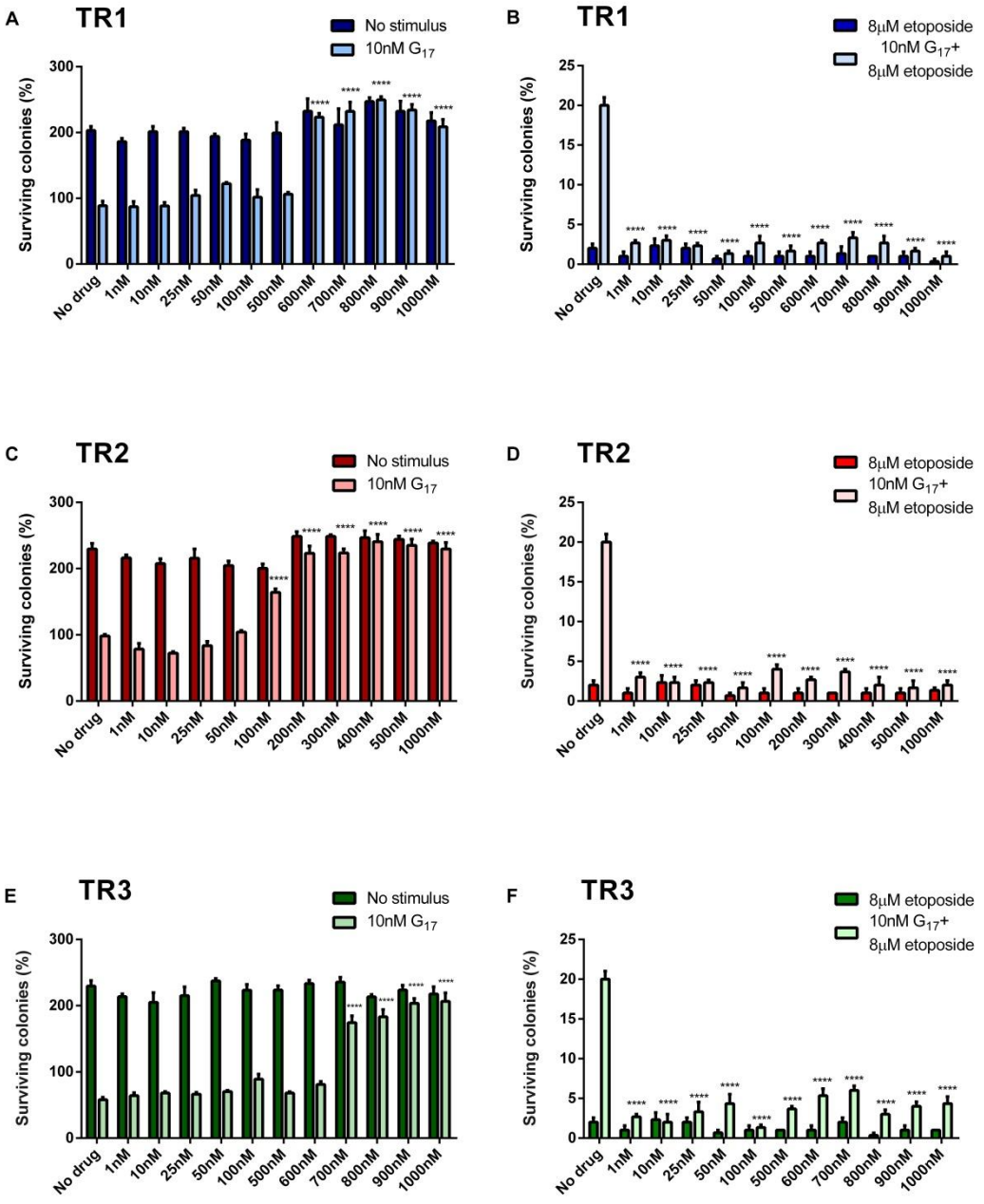

Figure 50. Clonogenic survival assays for TR1 (A, B), TR2 (C, D) and TR3 (E, F) dose responses after 6hrs 10nM G_{17} treatment with and without 1hr 8μM etoposide treatment (N=3, n=3). Each experiment was repeated three times and values are expressed as mean percentage of control ± SEM. Statistical significance was determined using two way ANOVA with Sidak *post-hoc* test and P<0.05 was considered significant (****P<0.0001 vs no drug controls).

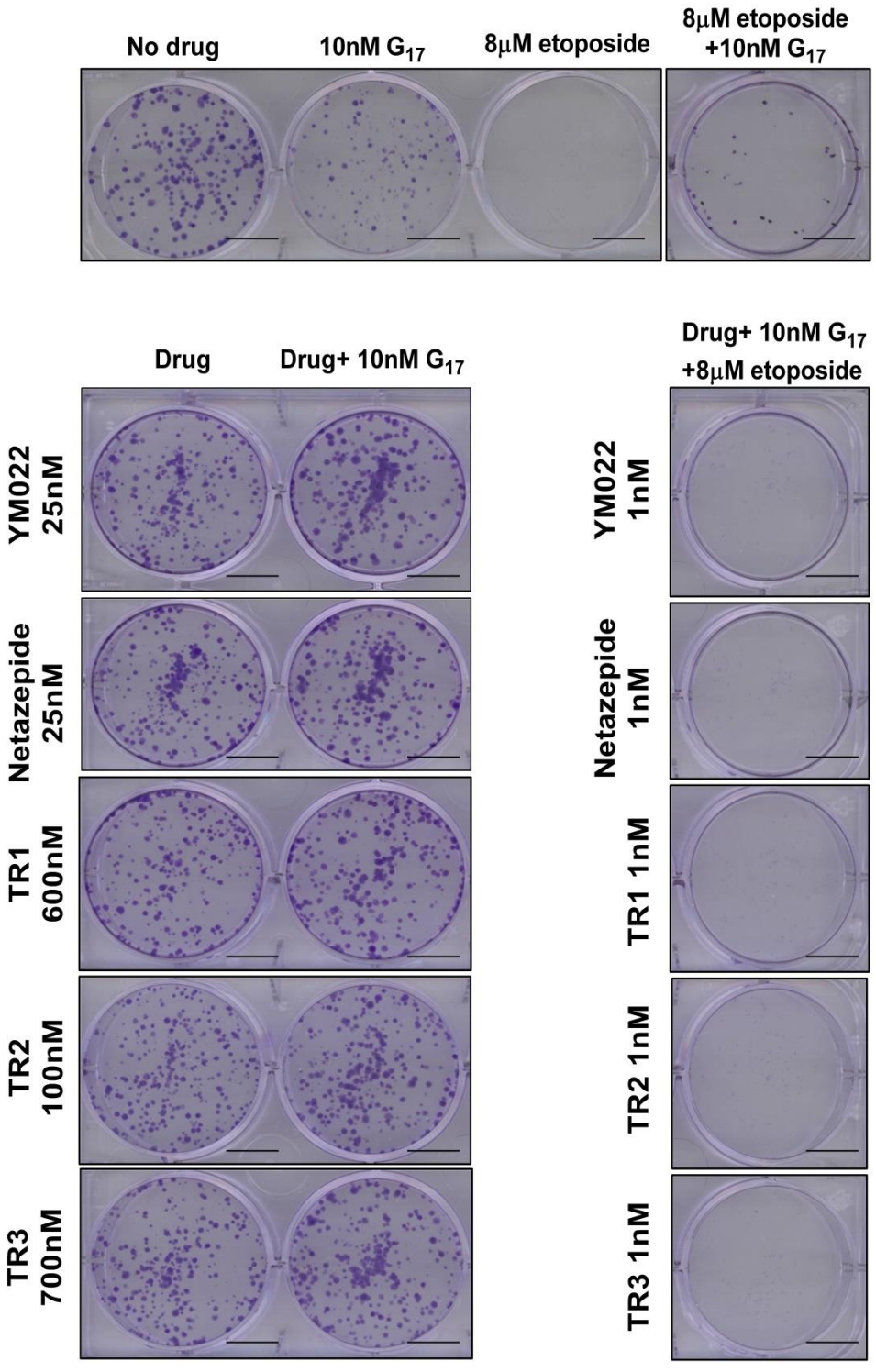

Figure 51. Clonogenic assays of AGS_{GR} cells stained with 0.3% crystal violet for the minimum concentrations of YM022, netazepide, TR1, TR2 and TR3 that completely reverse the inhibition of clonogenic survival and etoposide-induced cytotoxicity caused by 10nM G_{17} 6hrs (scale bar 1cm) (N=3, n=3).

3.3.7 The proliferative effects of gastrin are inhibited by compounds TR1, TR2 and TR3 in AR42J cells

Rat exocrine pancreatic tumour cells (AR42J) constitutively express the CCK2R and were therefore used to validate the minimum concentration at which compounds TR1, TR2 and TR3 inhibited the proliferative effects of gastrin. Using the modified thymidine analogue EdU, newly synthesised DNA is fluorescently labelled with Alexa Fluor® 594 (red). EdU incorporation assays were performed in order to assess the cellular proliferation rate in response to 0.1nM gastrin, following pre-treatment with the minimum concentrations of TR1 (500nM), TR2 (100nM) and TR3 (500nM) that inhibited the anti-proliferative response of 10nM gastrin in AGS_{GR} cells.

Untreated, DMSO (1%) and G_{17} 0.1nM treatments were used as negative, vehicle and positive controls with YM022 and netazepide (100nM) treatments used as positive inhibition controls.

Significant inhibition of the proliferative effects of 0.1nM G_{17} ($P<0.001$) were seen when cells were pre-treated with the following minimum concentrations of TR1 (500nM), TR2 (100nM) and TR3 (500nM). Treatment with TR1, TR2 and TR3 alone at these concentrations did not induce cellular toxicity as AR42J cell growth was not affected (Figure 52 and 53) (N=3, n=3).

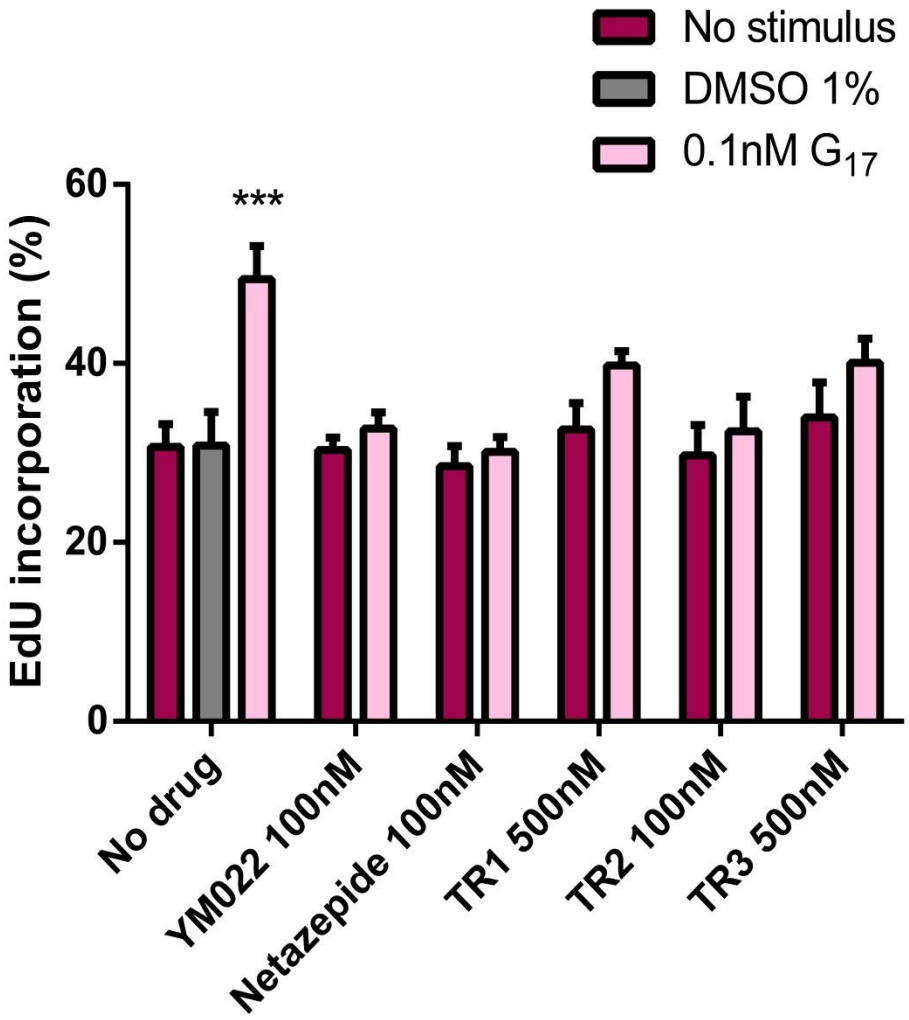

Figure 52. EdU incorporation assays for TR1 (500nM), TR2 (100nM), TR3 (500nM), YM022 (100nM) and netazepide (100nM), in AR42J cells with and without 8hrs 0.1nM G_{17} treatment (N=3, n=3). Each experiment was repeated three times and values are expressed as mean percentage of total cells per three reference fields ± SEM. Statistical significance was determined using two way ANOVA with Bonferroni *post-hoc* test and P<0.05 was considered significant (***P<0.001 vs no drug controls).

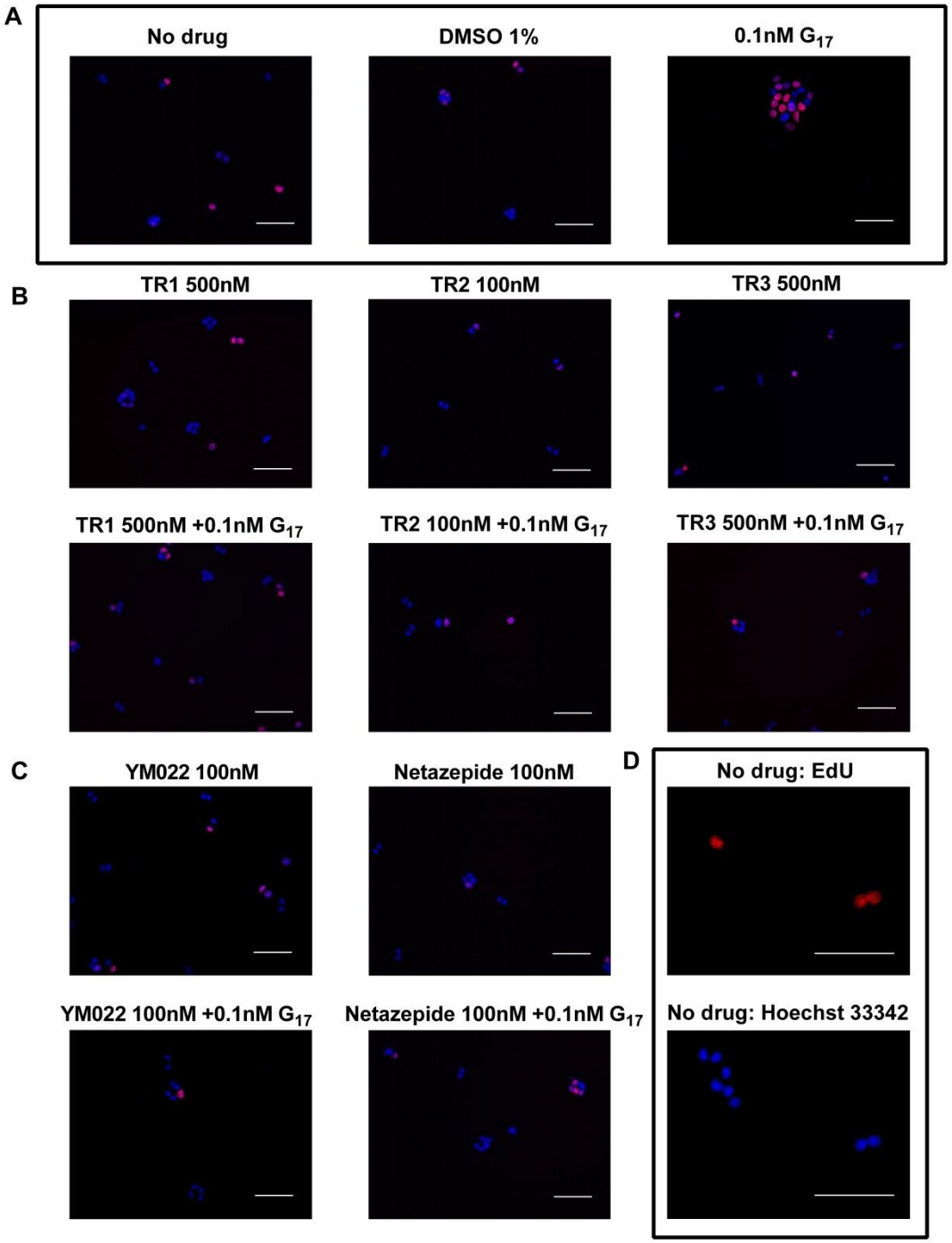

Figure 53. EdU incorporation assays for (B) TR1 (500nM), TR2 (100nM), TR3 (500nM), (C) YM022 (100nM) and netazepide (100nM), in AR42J cells with and without 8hrs 0.1nM G_{17} treatment. (A) Untreated, DMSO (1%) and 0.1nM G_{17} treatments were used as controls, mean cell number per treatment 105 ±6. (D) Proliferating cells with incorporated EdU were visualised with Alexa Fluor®594 (red) and non-proliferating cells were stained using Hoechst 33342 nuclear stain (blue), representative image of untreated cells (scale bar 200µm) (N=3, n=6).

3.3.8 Discussion

Patients with autoimmune atrophic gastritis and pernicious anaemia develop hypergastrinaemia as a consequence of reduced gastric acid secretion. Elevated gastrin concentrations have previously been shown to induce ECL cell hyperplasia which predisposes some patients to progression towards ECL cell dysplasia and type 1 gastric neuroendocrine tumour (NET) formation.

In localised type-1 gastric NETs, multiple polypoid tumours (<1cm diameter) may be successfully removed endoscopically, with an 80-100% 5-year survival rate [790]. Even in metastatic disease endoscopic resection plays an important role in minimising tumour size. However, in many cases resection is not possible because of tumour multiplicity and other therapies must be explored. Many hormonal approaches have also been investigated particularly the use of somatostatin analogues such as lanreotide and octreotide, both of which have provided good symptomatic control with evidence of tumour regression [791-793]. The development of new gene markers in conjunction with more effective therapies is expected to improve diagnosis, prognosis and response to therapy for patients with type-1 gastric NETS.

Gastrin receptor antagonists inhibit gastrin/CCK2 receptor binding and have the potential to reduce the downstream activation of biological processes such as gastric acid secretion [155], tissue remodelling and proliferation [463], particularly of ECL cells. CCK2 receptor antagonists have shown promising results in patients with type-1 gastric NETs [459, 460].

In this preclinical study, the effects of three novel gastrin receptor antagonists (TR1, TR2 and TR3) were examined and compared to the potency of the previously established compounds YM022 and netazepide, using CCK2 receptor bearing cells (AGS_{GR} and AR42J).

Gastrin has both direct and indirect influences on AGS cells dependent upon the existence of cell surface receptors, particularly CCK2R. The presence of CCK2R has been associated

with the pro- and anti-proliferative effects of G_{17}; proliferation was arrested in the G_1 phase of the cell cycle when this receptor was expressed [663]. Activation of the gastrin/CCK2R pathway leads to the production of several, growth regulator proteins including TGFα related peptides, Reg and histamine which act in a paracrine manner on CCK2 receptor non-bearing cells to stimulate cell growth [718], as determined by flow cytometry separation of Hoechst 33342 labelled AGS_{GR} cells co-cultured with AGS_{GFP} cells that only express the green fluorescent protein [663]. Alternatively, gastrin has also been shown to modulate pancreatic cell growth *in vitro* using AR42J cells [794] and *in vivo* in rats [795]. AR42J cells treated with pentagastrin showed increased DNA and protein synthesis and subsequent cell proliferation via activation of the CCK2 receptor.

Using a mouse fibroblast cell line that expresses the human CCK2 receptor (N-hCCKBR), YM022 proved to be a highly potent anti-proliferative drug with respect to binding affinity for the CCK2 receptor, inhibition of PI_3kinase production, increased intracellular calcium and DNA synthesis as assessed by the inhibition of [*methyl*-^{3}H] thymidine incorporation with and without gastrin [443]. Similarly, in enzyme immunoassays and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation studies, netazepide reduced ECL cell secretion (IC_{50} 1.2nM) and cell growth (IC_{50} 0.0013nM) *in vitro*, and was 1000 times more potent than a previous benzodiazepine derived drug L-365,260 [446].

In spontaneously hypergastrinaemic cotton rats (*Sigmodon hispidus*) that are predisposed to developing gastric neuroendocrine tumours, Kidd *et al*. also reported reduced ECL cell secretion and hyperplasia with a 60% decrease in subsequent neoplasm formation following a single, subcutaneous injection of netazepide (300µmol/kg/month) for <4 months [454].

In this study, complete inhibition of the anti-proliferative effects of gastrin was observed in SRB cell growth assays in AGS_{GR} cells and inhibition of the proliferative effects of gastrin in

AR42J cells was observed in EdU incorporation assays for all 3 compounds at concentrations of 500nM, 100nM and 500nM for TR1, TR2 and TR3 respectively. Of the three compounds, TR2 had the highest potency with complete inhibition of the effects of G_{17} being observed at a concentration that was five times lower than TR1 or TR3, which were also both less potent than YM022 and netazepide (both 60nM). However, TR2 did not have a higher potency than either YM022 or netazepide. None of the compounds tested produced cellular toxicity, as cell growth was unaffected at concentrations of TR1 (500nM), TR2 (100nM) or TR3 (500nM). These results were also confirmed using haemocytometer counts. The minimum concentration needed for complete inhibition of G_{17} induced effects did not increase floating cell numbers suggesting that the compounds did not induce cell death.

It has been established that cell migration is essential for gastric maintenance and wound healing. However, the ability of aberrant cells to metastasise defines the aggressive potential of a tumour and migration to secondary locations decreases the effectiveness of a treatment. Gastrin has been shown to increase cell migration in scratch wound assays of AGS_{GR} cells, in direct response to CCK2 receptor stimulation and this is partially mediated by PKC, MAPK and EGF-R pathways, and by matrix metalloproteinases in a paracrine manner [672]. The EGF-R pathway is initiated by the production of prostaglandins by gastric epithelial cells, primarily PGE_2 in response to inflammation-induced Cox-2 expression [796].

Intraperitoneal pre-treatment with YM022 (10, 30 and 100mg/kg) has been shown to dose dependently abolish gastrin induced Cox-2 overexpression and reduce PGE_2 expression [797], limiting cell migration in rats which had been injected with gastrin (100µg/kg). In the same way, the levels of epidermal growth factors (Reg I, HB-EGF, TGFα and amphiregulin) were significantly reduced in hypergastrinaemic INS-GAS mice, following weekly netazepide (40mg/kg) subcutaneous injections [451].

We found that pre-treatment of AGS_{GR} cells with TR1 200nM, TR2 70nM or TR3 900nM completely inhibited gastrin-stimulated cell migration, without influencing cell migration when treated alone at concentrations between 1nM-1μM. Whilst TR1 and TR3 had a lower potency when compared with YM022 and netazepide (100nM), TR2 was 30% more potent that both of its predecessors.

Hypergastrinaemia can evoke considerable tissue remodelling in the gastric epithelium. It has previously been reported that heregulin (neuregulin) in gastric epithelial cells [798], and transforming growth factor (TGF)-α in rat gastric mucus cells [799] promote the formation of branching morphogenesis (long tube-like projections) as part of the EGF-R pathway. CCK2 receptor inhibition using YM022 (1μmol/kg/hr via osmotic pumps) or netazepide (300μmol/kg via subcutaneous injection) reduced both ECL cell density and completely reversed the transformed cellular morphology (gastric processes) [800]. These data suggest that whilst gastrin is essential for the structural integrity of gastric ECL cells, under hypergastrinaemic conditions CCK2 receptor antagonism may provide an additional layer of mucosal protection against the formation of gastric neuroendocrine tumours.

Branching morphogenesis (the formation of long, cellular processes) was completely inhibited by TR1, TR2 and TR3 (all at concentrations of 400nM), with no structural changes being detected following treatment with any of the three compounds alone (1nM-500nM). This time, all three drugs were less potent than YM022 and netazepide, which both inhibited structural remodelling at 100nM. Gastrin stimulates multiple downstream signalling pathways of the CCK2 receptor including MAPK, PKC and PI3K. It has previously been suggested that branching morphogenesis occurs in a PKC-dependent, PI3K independent manner in AGS_{GR} cells however the full PKC dependent mechanism still remains unclear. Further work is therefore needed to assess TR1, TR2 and TR3 binding

affinity for the CCK2 receptor and their subsequent inhibition of downstream signalling pathways that may be associated with branching morphogenesis.

Clonogenic survival assays determine a cell's ability to retain long term reproducibility and chemosensitivity. In this study AGS_{GR} cells treated with 10nM G_{17} for 6hrs showed inhibition of cell growth by ~60%. This inhibition was completely reversed by all 3 compounds at concentrations of TR1 600nM, TR2 200nM and TR3 700nM. Whilst TR2 was the most potent of the 3 compounds, it was less potent that YM022 and netazepide (both 25nM). In AGS_{GR} cells treated with 10nM G_{17} for 6hrs followed by 1hr 8μM etoposide (a known cytotoxic drug), chemotherapy-induced cell death was reduced ~2-fold. This inhibition was completely reversed by all three drugs as well as YM022 and netazepide at low concentrations of 1nM. Although this suggests that all three compounds are equally as potent as YM022 and netazepide at increasing AGS_{GR} cell chemosensitivity, lower concentrations of all three compounds and YM022 and netazepide will need to be assessed to elucidate an appropriate dose-response.

These studies suggest that the CCK2 receptor selective compounds TR1, TR2 and TR3 inhibit gastrin mediated effects *in vitro*, in order of highest to lowest potency (TR2>TR1>TR3). Although complete mechanistic understanding of CCK2 receptor activation remains unclear and there is an abundance of previously assessed CCK2 receptor antagonists that have been unsuccessful, the potential increase in oral bioavailability of TR1, TR2 and TR3 compared with previously synthesised compounds may increase the potency and therapeutic utility of these compounds, particularly TR2. However, selectivity for the CCK2 receptor will need to be explored for potential idiosyncratic effects and cellular toxicology will need to be thoroughly elucidated. *In vivo* translation of these findings in both hypergastrinaemic and malignant animal models will also be required before undergoing phase I clinical trials in healthy volunteers. However, TR2 has the

potential to be further developed as a treatment for hypergastrinaemia associated conditions such as type-1 gastric NETs.

4 Discussion

4.1 Summary of major findings and conceptual advances

The initial aim of this study was to identify miRNAs that were differentially expressed in response to gastrin. As gastrin has been shown to stimulate multiple pathways involved in the initiation and progression of gastric tumours via the CCK2 receptor, further understanding of these regulatory mechanisms may provide therapeutic insights into the processes involved in gastric tumourigenesis. miRNAs are non-coding RNAs that regulate ~30% of the human genome and have previously been shown to be dysregulated both *in vitro* and *in vivo* during *H. pylori* infection, a known cause of both hypergastrinaemia and gastric cancer. However the specific dysregulation of miRNA profiles by gastrin remains unclear.

I demonstrated that gastrin significantly altered the expression of six miRNAs from a panel of 84 of the most abundant and best characterised miRNAs (according to miRbase version 19) [801-804]. Of the six differentially expressed miRNAs, three were upregulated and three were downregulated; however only two of these proved to be significant, miR-376c (5.2 fold) and miR-222 (2.3 fold). Due to the relatively low abundance of miR-376c in both the untreated and gastrin treated samples, miR-222 was chosen as a potential 'hit' and was further investigated *in vitro* and *in vivo*. miR-222 was upregulated in response to gastrin following CCK2R activation via the PKC and PI3K pathways in AGS_{GR} cells. Increased miR-222 expression was also demonstrated in hypergastrinaemic INS-GAS mice, and this increased further following *H. felis* infection. Additionally, miR-222 was upregulated in eight patients who had hypergastrinaemia (>400pM) and type-1 gastric NETs at baseline. This expression significantly reduced when patients were taking 50mg netazepide (CCK2R antagonist) daily and returned to baseline levels after cessation of treatment. Gastrin-induced miR-222 overexpression reduced p27 expression, a cell cycle regulatory protein, and this resulted in cellular phenotypical changes that have previously been associated with gastric neoplasia i.e. migration and structural remodelling.

Other researchers within our research group at the University of Liverpool had previously demonstrated that PAPPA2 mRNA expression was increased in the same eight hypergastrinaemic patients as previously described, and also that this decreased whilst the patients were taking 50mg netazepide daily and returned to baseline after cessation of treatment. Further investigation showed that PAPPA2 (mRNA and protein) expressions were increased in response to gastrin treatment of the AGS_{GR} cell line *in vitro.* Gastrin-induced PAPPA2 overexpression also significantly increased cellular phenotypical changes such as migration and the extension of long processes that are associated with gastric tumourigenesis.

In vitro, I investigated whether three novel CCK2R antagonists (TR1, TR2 and TR3) reduced/reversed gastrin-stimulated premalignant phenotypical changes including cell growth, structural remodelling, migration and clonogenic survival. All three compounds significantly inhibited gastrin-induced cellular phenomena in two cell lines with compound TR2 having the highest potency. In all cell based assays TR2 showed equal or slightly reduced potency compared to the previously developed CCK2R antagonists, YM022 and netazepide. Clinical application for CCK2R antagonists has to date been poor due to low oral bioavailability. Therefore pending further investigation, TR2 may provide promise as a novel CCK2R antagonist for treating gastrin-related disorders.

4.2 Strengths and limitations of the current study

4.2.1 Transformed cell lines

Gastric cancer cell lines derived from human gastric tumours have made major contributions to our understanding of gastric cancer development and cell biology. Transformed cell lines allow experiments to be repeated reproducibly, grow rapidly and are relatively easy to maintain compared with other experimental techniques. This permits

investigations of the responses of a specific cell type to various well-defined experimental conditions such as drug treatments and growth factors.

In this study human gastric adenocarcinoma cells (AGS) that have been stably transfected with the human CCK2R (AGS_{GR}) were used to investigate gastrin-stimulated cellular responses. However, AGS cells do not normally express the CCK2R and transformed AGS_{GR} cells have a higher level of CCK2R expression than normal gastric ECL cells. Therefore, this cell line may not fully reflect the responses of physiological ECL cell populations to gastrin. Cell lines that natively express the CCK2R such as rat pancreatic tumour cells (AR42J) may more accurately represent physiological responses to gastrin and could be used to confirm our findings in AGS_{GR} cells. Primary cell cultures derived from the gastric mucosa may also provide a better understanding of gastrin-induced cell responses within the stomach. The isolation and subsequent culturing of heterogeneous populations of normal gastric epithelial cells would more closely mimic the properties of the cells *in vivo.* However, primary gastric cells have less reproducibility than immortalised cell lines and subculturing has been shown to alter cellular characteristics and gene expressions over time [805]. Each passage selects for subpopulations of cells that are more capable of proliferating *ex-vivo* such as stromal fibroblasts, which alters the physiological cell populations and may skew sequential results.

3-dimensional (3D) pluripotent stem-cell derived gastric organoids [806] maintain the native architecture of the multiple differentiated cell types within the stomach and some of these would have physiological levels of CCK2R expression. Cells within these gastroids form complex cell-cell and cell-matrix interactions which more accurately mimic the *in vivo* environment of the originating tissue and this would allow the investigation of pathological mechanisms involved with aberrant CCK2R activation. However, the tumour microenvironment is complex and involves surrounding components such as stromal cells

and immune cells [807], which all contribute to the process of carcinogenesis. Thus even experiments involving epithelial gastroids may not be fully indicative of the events which occur *in vivo*.

4.2.2 INS-GAS mouse model

Animal models of gastric cancer have been shown to provide reproducible methods for investigating disease mechanisms and treatments for malignancy, in ways that are not possible in humans. The physiology and anatomy of the mouse stomach is relatively similar to humans, however structural and size differences are still present. There is also a high degree of similarity between the human and murine genomes (~85%) [808]. Genetically modified hypergastrinaemic INS-GAS mice were therefore utilised to model human gastrin-associated cellular changes.

Transgenic INS-GAS mice are genetically modified to overexpress the human gastrin gene and therefore have high circulating gastrin concentrations. Initially, young INS-GAS mice have increased parietal and ECL cell numbers which decrease over time leading to gastric atrophy. The majority of INS-GAS mice spontaneously develop gastric dysplasia and invasive gastric adenocarcinoma after ~20 months of age [112]. In humans, atrophic gastritis is driven either by long-term *H. pylori* infection or by autoimmune mechanisms and hypergastrinaemia is secondary to gastric hypoacidity. Patients with hypergastrinaemia have an increased risk of developing both gastric adenocarcinomas and gastric NETs (depending upon the original aetiology) [809-812], however INS-GAS mice have only been shown to develop gastric adenocarcinomas. Female Japanese cotton rats (*Sigmodon hispidus*) are hypergastrinaemic wild-type rodents that have been shown to spontaneously develop gastric NETs after ~8 months of age [366-368]. Additionally, the African *Mastomys natalensis* rodent (Z strain) has been shown to develop gastric adenocarcinomas or gastric NETs after 18-24 months of age, the latter being observed more frequently [369, 813]. Since

gastrin is a cofactor for the development of both gastric adenocarcinomas and gastric neuroendocrine tumours, the use of Japanese cotton rats or *Mastomys natalensis* would help to determine the effects of hypergastrinaemia on the development of gastric premalignant phenotypes with neuroendocrine characteristics that mimic human carcinogenesis. Unfortunately, these animals were not available at the University of Liverpool so I was not able to conduct any such studies during this PhD.

4.2.3 *Helicobacter felis* infection model

It is well established that *H. pylori* infection can contribute to the development of gastric cancer in humans and hypergastrinaemia may contribute in some cases [614]. However, many *H. pylori* strains do not colonise various mouse strains [814, 815]. *H. felis* has been shown to readily colonise the murine stomach and was therefore used as a model of bacteria-induced gastric neoplasia in this study. *H. felis* has been shown to cause murine gastric mucosal inflammation with premalignant cellular phenotypes that are morphologically similar to human *H. pylori* pathogenesis [816]. However, *H. felis* strains do not contain *cag*PAI or *vacA* genes, the most extensively characterised bacterial virulence factors that are strongly associated with gastric pathology in humans, and this has resulted in some criticism when used to model *H. pylori*-induced conditions.

In this study, I demonstrated that six week *H. felis* infection further induced miR-222 expression in three month old hypergastrinaemic INS-GAS mice compared with uninfected INS-GAS mice. Bacterial infection (*H. felis*) in this model has been shown to accelerate the gastric atrophy, metaplasia, dysplasia and gastric adenocarcinoma sequence associated with human pathology [817]. However, previous studies have shown that wild-type mice of the same genetic background (FVB/N) maintained under similar conditions did not develop the same *H. felis*-induced gastric pathology [112]. Therefore, it would be useful to assess miR-222 expression profiles in wild-type FVB/N mice following *H. felis* infection. It would also

be interesting to assess longer time-points in *H. felis* infected mouse models (up to two years) as extended periods of infection in wild-type and INS-GAS mice have previously been shown to cause gastric lesions similar to those observed in *H. pylori* infected humans [818, 819].

H. pylori strains that can successfully colonise the murine stomach have recently been developed such as G1.1, TN2 SS1 and B1287, of which the best characterised is the Sydney strain of *H. pylori* (SS1) [387]. The mouse-adapted *H. pylori* strain SS1 (*cagA*$^+$/*vacA*$^+$) efficiently and rapidly colonises multiple mouse strains and can persist for long periods of time [820]. After 8 months infection with *H. pylori* SS1, chronic atrophic gastritis and secondary hypergastrinaemia were observed in C57BL/6 mice and these increased in severity with the extent of colonisation [387]. This is comparable to the progressive destruction of the corpus mucosa observed in human *H. pylori* infection. Similarly, Mongolian gerbils infected with *H. pylori* (G1.1 [821], TN2 [822] or B1287 [823] strains) develop hypergastrinaemia and severe inflammation and this can result in the development of gastric NETs and less frequently, gastric adenocarcinoma [824, 825]. Therefore, these rodent adapted *H. pylori* strains may be useful for future investigations as they best resemble human pathogenic *H. pylori* and closely mimic the premalignant phenotypical changes observed within the gastric mucosa in humans.

4.2.4 Human samples

For this study, samples were taken from a small number of patients from a single hospital. They had hypergastrinaemia and type-1 gastric NETs (n=8) and were on a daily regimen of 50mg netazepide; they underwent intensive monitoring for more than one year. This strategy has both advantages and disadvantages. Small sample size studies are relatively quick to conduct with respect to enrolling patients, so that a particular research question can be addressed relatively quickly. The investigation of a new hypothesis in a small

sample size is therefore cost effective for the initial determination of an area of focus. However the interpretation of results may not accurately reflect the whole population and therefore further confirmatory studies are needed involving a larger cohort of patients who have differences in geographical location, age, race and sex. A placebo arm was also not included in the initial open label study and this will be required in any subsequent trials. Further investigation of the specificity of our observations also means that patients with different sources of hypergastrinaemia including PPI use, autoimmune atrophic gastritis, active *H. pylori* infection or Zollinger-Ellison syndrome should now be assessed. Biomarker expression profiles that can distinguish between sources of hypergastrinaemia may be of clinical value for the diagnosis and treatment of gastrin-related conditions.

4.2.5 Investigation of miRNAs

Several miRNAs have previously been shown to be clustered within a locus and are transcribed simultaneously with the host gene, forming monocistronic, dicistronic or polycistronic primary miRNA (pri-miRNA) transcripts [471, 557]. Therefore, an increase in gene transcription may increase the expression of more than one miRNA. miR-222 forms a cluster with miR-221 and is cleaved from the same dicistronic pri-miRNA. An increase in miR-222 transcription may therefore also increase miR-221 transcription. miR-221 has previously been shown to exert independent biological effects that may also contribute to carcinogenesis via targeting cell cycle inhibitor proteins in prostate [826], hepatocellular [827, 828], colorectal [829] and breast [830] cancers . The miRNA PCR array initially used in this study contained miR-222 and miR-221 primers separately. Therefore we were able to determine whether gastrin increased the transcription of both miRNAs within the cluster or each one individually. However, separate primer assays for miR-221 should also be used to confirm whether the observed cellular responses arose specifically as a result of gastrin-induced miR-222 overexpression.

It has previously been suggested that one miRNA may sometimes regulate multiple targets and *vice versa* one mRNA can be targeted by numerous miRNAs [689, 831-834]. This adds an additional layer of functional complexity and makes miRNAs potent regulators of several biological processes. The initial miScript™ miRNA PCR array used in this study only contained 84 of the most abundant and best characterised miRNAs according to miRbase version 19 [801-804] and may therefore not have included all of the miRNAs that are affected by gastrin. Therefore, the use of whole genome miRNA deep sequencing (miR-seq) could elucidate all miRNA expression changes that may occur in association with gastrin. This might identify additional specific miRNA patterns/profiles that are associated with gastrin-related conditions. However, miR-seq is an expensive and time-consuming technique that would not have been possible for this particular study.

4.3 Potential translational impact

The main causes of hypergastrinaemia are gastric atrophy and the use of PPIs; the former occurs most frequently after chronic *H. pylori* infection. Gastric atrophy is currently the highest (independent) known risk factor for the development of gastric tumours [218, 293, 835, 836] and the trophic effects of increased circulating gastrin concentrations may contribute in some cases.

Parallel assays that detect the serum concentrations of human pepsinogens (pepsinogen I and/or pepsinogen I: pepsinogen II ratios) and gastrin have been used to indicate the extent of atrophic gastritis [837-839]. Altered expressions of this panel of biomarkers reflect changes in gastric morphology and the physiological function of the mucosa. However, serum pepsinogen testing has variable specificity and has been associated with false-positive results [840] for the detection of premalignant lesions such as atrophy and dysplasia. There is also no standard cut-off value for PGI or PGI: PGII ratios [841-844] meaning that it is difficult to make accurate comparisons between studies. Specific biomarkers for gastrin-

induced cellular responses, particularly those that are highly associated with an increased risk of gastric neoplasia have not yet been established. This study has identified miR-222 and PAPPA2 as gastrin-regulated genes which are overexpressed in hypergastrinaemic conditions. This overexpression dysregulates several biological processes including increased gastric cellular remodelling and migration, which are strongly associated with malignant phenotypes. Therefore, miR-222 and PAPPA2 are promising candidate novel serum biomarkers for the detection of gastrin-associated clinical conditions such as type-1 gastric NETs.

Previously developed gastrin/CCK2 receptor antagonists have been shown to block the downstream signalling associated with CCK2R activation. However to date clinical application has proven disappointing due to low oral bioavailability, potency and/or selectivity. Recently, two studies have reported tumour regression in patients with hypergastrinaemia and type-1 gastric NETs who have taken the CCK2R antagonist, netazepide [459, 460]. These data indicate the potential for netazepide and related compounds to be utilised for the treatment of gastrin-associated malignant conditions. This thesis has reported that three novel CCK2R antagonist drugs which are structurally similar to netazepide significantly inhibited gastrin-induced cellular changes which have previously been associated with gastric pathologies including type-1 gastric NETs. Although the most potent of the three novel compounds was TR2, it was still equal to or less potent than YM022 and netazepide. However, if it shown to have improved bioavailability and CCK2R selectivity *in vivo*, TR2 may still prove to be a new CCK2R antagonist drug that is suitable for clinical evaluation in humans.

4.4 Future research directions

Three potential future research directions have been highlighted during this study. Further research is required to evaluate the clinical potential of miR-222 and PAPPA2 as non-invasive biomarkers of gastrin-related conditions and additional experiments are also necessary to assess whether TR2 is suitable to be developed as a novel treatment for gastrin-induced conditions.

In **Chapter 3.1**, further profiling of miR-222 expression in a larger cohort of patients with other causes of hypergastrinaemia i.e. PPI use, *H. pylori* infection and type-2 gastric NETs with associated ZES would determine whether miR-222 expression patterns can distinguish between different sources of hypergastrinaemia. If distinct miR-222 expression patterns are associated with different sources of hypergastrinaemia this would increase the utility of this biomarker in various gastrin-related conditions, thus increasing the potential for clinical application.

As gastrin-CCK2R binding has been shown to activate several downstream pathways including PI3K, MAPK and PKC pathways, further investigation of other miR-222 targets that may be involved in the regulation of these pathways would also be beneficial to understand the mechanisms involved in the development of gastrin-related pathologies. miR-222 has been shown to downregulate the tumour suppressor proteins TIMP3 and PTEN, which are involved in regulating tissue remodelling and cell cycle progression in non-squamous cell lung carcinoma (NSCLC) and hepatocellular carcinoma (HCC) [845]. It would therefore be interesting to investigate the expression of these target proteins in gastric tissues under hypergastrinaemic conditions.

As previously mentioned, miR-222 is transcribed as a dicistronic primary transcript with miR-221. Thus miR-221 expression may also be increased upon CCK2R activation. In NSCLC and HCC, miR-221 and miR-222 show similar pattern of expression and miR-221 has also

been shown to target TIMP3 and PTEN [845]. Further investigation of miR-221 expression would indicate whether dysregulation of several gastric physiological cell processes may be responsible for the malignant phenotypes associated with hypergastrinaemia. Alternatively, next-generation sequencing could identify the expression patterns of all gastrin-regulated miRNAs. This would provide a more accurate 'miRNA signature' and would potentially increase biomarker(s) specificity for particular gastrin-induced morphological changes within the stomach.

In **Chapter 3.2** experiments were only conducted *in vitro* and follow up studies should therefore include *in vivo* application. Transgenic mouse models should be used to confirm that altered PAPPA2 expression is caused by gastrin. For example, PAPPA2 expression should be assessed in hypergastrinaemic INS-GAS mice and compared with that in wild-type FVB/N mice, with and without *H. felis* infection for short and long periods of time. PAPPA2 deficient mice [846, 847] and wild-type mice on the same genetic background could also be used to further explore whether gastric morphological changes occur as a result of gastrin-induced overexpression of PAPPA2.

As previous studies have suggested that PAPPA2 can be detected in the blood [4, 781, 848], PAPPA2 enzyme-linked immunosorbent assays (ELISAs) could be employed to measure the circulating concentrations of PAPPA2 protein in patients with hypergastrinaemia and type-1 gastric NETs compared with healthy controls. Immunohistochemistry would additionally show which cells within the stomach express PAPPA2 in health and disease. It would additionally be useful to investigate PAPPA2 expression in different hypergastrinaemic conditions such as PPI usage, *H. pylori* infection and type-2 gastric NETs with associated ZES in order to explore the specificity of PAPPA2 as a biomarker for gastrin-related conditions.

In **Chapter 3.3**, TR2 was the most potent of the three novel compounds tested *in vitro*. Further examination of gastrin-regulated gene expression (such as miR-222, PAPPA2, Mcl-1 [180], PAI-1 [185], PAI-2 [173] and MMP7 [190]) in human immortalised cell lines and primary cells by qPCR and western blot, at a range of doses of TR2, would now be helpful. Evaluating the effects of TR2 in gastric corpus organoids derived from hypergastrinaemic mice and humans, compared with wild-type mice and healthy humans, would also be helpful to assess the effects of TR2 on gastrin-induced gene expressions in a 3D model that more closely mimics the *in vivo* gastric environment. Both short and long-term *in vivo* studies in hypergastrinaemic mice are also needed to determine whether TR-2A (the pro-drug of TR2) affects gastric morphology, gastric acid secretion or gastrin-related gene expression. These experiments are required before future human clinical trials of TR2 can be contemplated.

4.5 Conclusions

Gastrin altered the expression of miRNAs which are involved in the physiological maintenance of the gastric mucosa, specifically miR-222. *In vitro* changes were validated in hypergastrinaemic murine models (INS-GAS) and human clinical samples obtained from patients with hypergastrinaemia and type-1 gastric NETs. Increased expression of miR-222, in response to CCK2R activation by gastrin, inhibited the production of proteins such as p27 that control biological processes which may contribute to gastric premalignant morphological changes. Similarly, gastrin increased the expression of the matrix remodelling protein PAPPA2 *in vitro* and this was shown to increase cell migration and tissue remodelling; prerequisites for gastric carcinogenesis. These data suggest that both miR-222 and PAPPA2 are gastrin-related genes which may be further developed as serum biomarkers of gastrin-associated gastric pathologies.

In vitro, treatment with the novel CCK2R antagonists, TR1, TR2 or TR3 significantly reversed gastrin-induced cellular phenomena including dysregulated growth, increased migration, clonogenic survival and increased structural remodelling, with TR2 proving the most potent of the three tested compounds. Although TR2 is equal to or less potent than previously developed CCK2R antagonists *in vitro*, it is still a promising drug candidate for the treatment of gastrin-related conditions *in vivo* as it may have an increased oral bioavailability which would support its further clinical development.

5 References

1. Schubert ML, Peura DA. Control of gastric acid secretion in health and disease. Gastroenterology 2008;134:1842-60.

2. Schlossberg LaZ, G. The Gastrointestinal Tract. The Johns Hopkins Atlas of Human Functional Anatomy. 4 ed. Baltimore and London: The John Hopkins University Press Ltd, 1997.

3. Chao CH, MR. GAST (gastrin). Atlas of Genetics and Cytogenetics in Oncology and Haematology 2011;15:921-927.

4. Moore AR, Parsons BN, Boyce MJ, et al. Sa1824 Identification of Potential Biomarkers of Netazepide (YF476) Treatment of Type I Gastric Neuroendocrine Tumours. Gastroenterology 2015;148:S-342.

5. Omasits U, Ahrens CH, Müller S, et al. Protter: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics 2013;30:884-886.

6. Bundgaard JR, Rehfeld JF. Posttranslational processing of progastrin. Cellular Peptide Hormone Synthesis and Secretory Pathways: Springer, 2010:207-220.

7. Lodish HF, Zhou B, Liu G, et al. Micromanagement of the immune system by microRNAs. Nat Rev Immunol 2008;8:120-130.

8. Semple G, Ryder H, Rooker DP, et al. (3 R)-N-(1-(tert-Butylcarbonylmethyl)-2, 3-dihydro-2-oxo-5-(2-pyridyl)-1 H-1, 4-benzodiazepin-3-yl)-N'-(3-(methylamino) phenyl) urea (YF476): A Potent and Orally Active Gastrin/CCK-B Antagonist. Journal of medicinal chemistry 1997;40:331-341.

9. Schubert ML. Functional anatomy and physiology of gastric secretion. Current opinion in gastroenterology 2015;31:479-485.

10. Conteduca V, Sansonno D, Lauletta G, et al. H. pylori infection and gastric cancer: state of the art (review). International journal of oncology 2013;42:5-18.

11. Wiborg O, Berglund L, Boel E, et al. Structure of a human gastrin gene. Proceedings of the National Academy of Sciences 1984;81:1067-1069.

12. Wank SA. Cholecystokinin receptors. American Journal of Physiology - Gastrointestinal and Liver Physiology 1995;269:G628-G646.

13. Winter J, Jung S, Keller S, et al. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 2009;11:228-234.

14. Monack DM, Mueller A, Falkow S. Persistent bacterial infections: the interface of the pathogen and the host immune system. Nat Rev Micro 2004;2:747-765.

15. Dockray GJ. Topical review. Gastrin and gastric epithelial physiology. J Physiol 1999;518 (Pt 2):315-24.

16. Wadhwa R, Song S, Lee J-S, et al. Gastric cancer-molecular and clinical dimensions. Nat Rev Clin Oncol 2013;10:643-655.

17. Babkin B, P. Die aussere Sekretion der Verdauungsdrusen. Berlin: Springer, 1928.

18. Bayliss WM, Starling EH. On the Causation of the So-Called 'Peripheral Reflex Secretion' of the Pancreas. (Preliminary Communication.). Proceedings of the Royal Society of London 1901;69:352-353.

19. Edkins JS. On the Chemical Mechanism of Gastric Secretion. Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character 1905;76:376.

20. Edkins JS. The chemical mechanism of gastric secretion. The Journal of Physiology 1906;34:133-144.

21. Komarov SA. Gastrin. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.) 1938;38:514-516.

22. Grossman MI. Gastrin--reminiscence and speculation. The American journal of digestive diseases 1966;11:90-96.

23. Cushny AR, Edmunds, C. W., & Gunn, J. A. Text-book of pharmacology and therapeutics. 11th ed. Philadelphia: Lea & Febiger, 1936:410-453.

24. Popielski L. Die Sekretionstätigkeit der Bauchspeicheldrüse unter dem Einfluss von Salzsäure und Darmextrakt (des sogenannten Sekretins). Archiv für die gesamte Physiologie des Menschen und der Tiere 1907;120:451-491.

25. Neale AV, Klumpp TG. The Action Of Histamine On The Pancreas. The Journal of Clinical Investigation 1930;9:197-208.

26. Gregory RA, & Tracy, H. J. The constitution and properties of two gastrins extracted from hog antral mucosa: The isolation of two gastrins from hog antral mucosa. Gut 1964:103-117.

27. Wu J, Li X. Regulation of gastric acid secretion. Chinese Journal of Gastroenterology 2012;17:179-182.

28. Fritsch WP, Hausamen TU, Scholten T. [Gastrointestinal hormones. I. Hormones of the gastrin group]. Z Gastroenterol 1977;15:264-76.

29. Ito R, Sato K, Helmer T, et al. Structural analysis of the gene encoding human gastrin: the large intron contains an Alu sequence. Proc Natl Acad Sci U S A 1984;81:4662-6.

30. Lund T, Geurts van Kessel AHM, Dixon JE, et al. The genes for human gastrin and cholecystokinin are located on different chromosomes. Human Genetics 1986;73:77-80.

31. Desmond H, Pauwels S, Varro A, et al. Isolation and characterization of the intact gastrin precursor from a gastrinoma. FEBS letters 1987;210:185-188.

32. Daugherty D, Yamada T. Posttranslational processing of gastrin. Physiol Rev 1989;69:482-502.

33. Varro A, Dockray GJ. Post-translational processing of progastrin: Inhibition of cleavage, phosphorylation and sulphation by brefeldin A. Biochemical Journal 1993;295:813-819.

34. Dickinson C, Sawada M, Guo Y, et al. Specificity of prohormone convertase endoproteolysis of progastrin in AtT-20 cells. Journal of Clinical Investigation 1995;96:1425.

35. Muller L, Lindberg I. The cell biology of the prohormone convertases PCI and PC2. Progress in nucleic acid research and molecular biology 1999;63:69-108.

36. Zhou A, Webb G, Zhu X, et al. Proteolytic processing in the secretory pathway. Journal of Biological Chemistry 1999;274:20745-20748.

37. Smith KA, Patel O, Lachal S, et al. Production, Secretion, and Biological Activity of the C-Terminal Flanking Peptide of Human Progastrin. Gastroenterology 2006;131:1463-1474.

38. Fricker LD. Carboxypeptidase E. Annual review of physiology 1988;50:309-321.

39. Udupi V, Gomez P, Song L, et al. Effect of Carboxypeptidase E Deficiency on Progastrin Processing and Gastrin Messenger Ribonucleic Acid Expression in Mice with the fat Mutation 1. Endocrinology 1997;138:1959-1963.

40. Rehfeld J, Zhu X, Norrbom C, et al. Prohormone convertases 1/3 and 2 together orchestrate the site-specific cleavages of progastrin to release gastrin-34 and gastrin-17. Biochem. J 2008;415:35-43.

41. Rehfeld JF, Lindberg I, Friis-Hansen L. Progastrin processing differs in 7B2 and PC2 knockout animals: a role for 7B2 independent of action on PC2. FEBS letters 2002;510:89-93.

42. Varro Ae, Voronina S, Dockray G. Pathways of processing of the gastrin precursor in rat antral mucosa. Journal of Clinical Investigation 1995;95:1642.

43. Cuttitta F. Peptide amidation: signature of bioactivity. The Anatomical Record 1993;236:87-95.

44. Merkler DJ. C-terminal amidated peptides: production by the in vitro enzymatic amidation of glycine-extended peptides and the importance of the amide to bioactivity. Enzyme and microbial technology 1994;16:450-456.

45. Malstrom J, Stadil F, Rehfeld JF. Gastrins in tissue. Concentration and component pattern in gastric, duodenal, and jejunal mucosa of normal human subjects and patients with duodenal ulcer. Gastroenterology 1976;70:697-703.

46. Eipper BA, Quon AS, Mains RE, et al. The Catalytic Core of Peptidylglycine. alpha.-Hydroxylating Monooxygenase: Investigation by Site-Directed Mutagenesis, Cu X-ray Absorption Spectroscopy, and Electron Paramagnetic Resonance. Biochemistry 1995;34:2857-2865.

47. Kulathila R, Merkler KA, Merkler DJ. Enzymatic formation of C-terminal amides. Natural product reports 1999;16:145-154.

48. Freeman JC, Villafranca JJ, Merkler DJ. Redox cycling of enzyme-bound copper during peptide amidation. Journal of the American Chemical Society 1993;115:4923-4924.

49. Young SD, Tamburini PP. Enzymatic peptidyl. alpha.-amidation proceeds through formation of an. alpha.-hydroxyglycine intermediate. Journal of the American Chemical Society 1989;111:1933-1934.

50. Tajima M, Iida T, Yoshida S, et al. The reaction product of peptidylglycine alpha-amidating enzyme is a hydroxyl derivative at alpha-carbon of the carboxyl-terminal glycine. Journal of Biological Chemistry 1990;265:9602-9605.

51. Takahashi K, Okamoto H, Seino H, et al. Peptidylglycine α-amidating reaction: evidence for a two-step mechanism involving a stable intermediate at neutral pH. Biochemical and biophysical research communications 1990;169:524-530.

52. Katopodis AG, Ping D, May SW. A novel enzyme from bovine neurointermediate pituitary catalyzes dealkylation of. alpha.-hydroxyglycine derivatives, thereby functioning sequentially with peptidylglycine. alpha.-amidating monooxygenase in peptide amidation. Biochemistry 1990;29:6115-6120.

53. Katopodis AG, Ping D, Smith CE, et al. Functional and structural characterization of peptidylamidoglycolate lyase, the enzyme catalyzing the second step in peptide amidation. Biochemistry 1991;30:6189-6194.

54. Suzuki K, Shimoi H, Iwasaki Y, et al. Elucidation of amidating reaction mechanism by frog amidating enzyme, peptidylglycine alpha-hydroxylating monooxygenase, expressed in insect cell culture. The EMBO journal 1990;9:4259.

55. Rehfeld JF. Processing of precursors of gastroenteropancreatic hormones: diagnostic significance. J Mol Med 1998;76:338-45.

56. Brand SJ, Fuller PJ. Differential gastrin gene expression in rat gastrointestinal tract and pancreas during neonatal development. Journal of Biological Chemistry 1988;263:5341-5347.

57. Larsson L. Peptide secretory pathways in GI tract: cytochemical contributions to regulatory physiology of the gut. American Journal of Physiology-Gastrointestinal and Liver Physiology 1980;239:G237-G246.

58. Hilaire R, Jones AL. Epidermal growth factor: its biologic and metabolic effects with emphasis on the hepatocyte. Hepatology 1982;2:601S-613S.

59. Gregory H, Walsh S, Hopkins CR. The identification of urogastrone in serum, saliva, and gastric juice. Gastroenterology 1979;77:313-318.

60. Godley JM, Brand SJ. Regulation of the gastrin promoter by epidermal growth factor and neuropeptides. Proceedings of the National Academy of Sciences 1989;86:3036-3040.

61. Kadonaga JT, Jones KA, Tjian R. Promoter-specific activation of RNA polymerase II transcription by Sp1. Trends in Biochemical Sciences 1986;11:20-23.

62. Imagawa M, Chiu, R. & Karin, M. . Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell 1987;51:251-260.

63. Merchant JL, Demediuk B, Brand SJ. A GC-rich element confers epidermal growth factor responsiveness to transcription from the gastrin promoter. Mol Cell Biol 1991;11:2686-96.

64. Walsh JH, Grossman MI. Gastrin (first of two parts). The New England journal of medicine 1975;292:1324.

65. Uvnäs-Wallensten K, Efendic S, Johansson C, et al. Effect of intraluminal pH on the release of somatostatin and gastrin into antral, bulbar and ileal pouches of conscious dogs. Acta physiologica Scandinavica 1980;110:391-400.

66. Bachwich D, Merchant J, Brand SJ. Identification of a cis-regulatory element mediating somatostatin inhibition of epidermal growth factor-stimulated gastrin gene transcription. Molecular Endocrinology 1992;6:1175-1184.

67. Karnik PS, Monahan SJ, Wolfe MM. Inhibition of gastrin gene expression by somatostatin. Journal of Clinical Investigation 1989;83:367.

68. Karnik PS, Wolfe MM. Somatostatin stimulates gastrin mRNA turnover in dog antral mucosa. Journal of Biological Chemistry 1990;265:2550-2555.

69. Brand SJ, Stone D. Reciprocal regulation of antral gastrin and somatostatin gene expression by omeprazole-induced achlorhydria. J Clin Invest 1988;82:1059-66.

70. Bate G, Varro A, Dimaline R, et al. Control of preprogastrin messenger RNA translation by gastric acid in the rat. Gastroenterology 1996;111:1224-1229.

71. Varro A, Henry J, Vaillant C, et al. Discrimination between temperature-and brefeldin A-sensitive steps in the sulfation, phosphorylation, and cleavage of progastrin and its derivatives. Journal of Biological Chemistry 1994;269:20764-20770.

72. Dockray GJ, Varro A, Dimaline R. Gastric endocrine cells: gene expression, processing, and targeting of active products. Physiological reviews 1996;76:767-798.

73. Richardson BC, Mercier JC. The Primary Structure of the Ovine β-Caseins. European Journal of Biochemistry 1979;99:285-298.

74. Moore A, Boulton AP, Heid HW, et al. Purification and tissue-specific expression of casein kinase from the lactating guinea-pig mammary gland. European Journal of Biochemistry 1985;152:729-737.

75. Meggio F, Boulton AP, Marchiori F, et al. Substrate-specificity determinants for a membrane-bound casein kinase of lactating mammary gland. European Journal of Biochemistry 1988;177:281-284.

76. Hortin G, Folz R, Gordon JI, et al. Characterization of sites of tyrosine sulfation in proteins and criteria for predicting their occurrence. Biochemical and biophysical research communications 1986;141:326-333.

77. Huttner WB. Tyrosine sulfation and the secretory pathway. Annual Review of Physiology 1988;50:363-376.

78. Gregory H, Hardy P, Jones D, et al. The Antral Hormone Gastrin. Structure of Gastrin. Nature 1964;204:931.

79. Mutt V, Jorpes J. Structure of porcine cholecystokinin-pancreozymin. European Journal of Biochemistry 1968;6:156-162.

80. Bundgaard J, Vuust J, Rehfeld J. Tyrosine O-sulfation promotes proteolytic processing of progastrin. The EMBO journal 1995;14:3073.

81. Bundgaard JR, Rehfeld JF. Distinct linkage between post-translational processing and differential secretion of progastrin derivatives in endocrine cells. Journal of Biological Chemistry 2008;283:4014-4021.

82. Hilsted L, Rehfeld JF. Alpha-carboxyamidation of antral progastrin. Relation to other post-translational modifications. Journal of Biological Chemistry 1987;262:16953-16957.

83. Mellman I, Fuchs R, Helenius A. Acidification of the endocytic and exocytic pathways. Annu Rev Biochem 1986;55:663-700.

84. Schuldiner S, Shirvan A, Linial M. Vesicular neurotransmitter transporters: from bacteria to humans. Physiol Rev 1995;75:369-92.

85. Knoth J, Zallakian M, Njus D. Stoichiometry of hydrogen ion-linked dopamine transport in chromaffin granule ghosts. Biochemistry 1981;20:6625-6629.

86. Johnson RG. Accumulation of Biological Amines into Chromaffin Granules: A Model for Hormone and Neurotransmitter Transport Physiol Rev 1988;68:233-292.

87. Merickel A, Edwards R. Transport of histamine by vesicular monoamine transporter-2. Neuropharmacology 1995;34:1543-1547.

88. Peter D, Jimenez J, Liu Y, et al. The chromaffin granule and synaptic vesicle amine transporters differ in substrate recognition and sensitivity to inhibitors. Journal of Biological Chemistry 1994;269:7231-7237.

89. Pearse AG. The cytochemistry and ultrastructure of polypeptide hormone-producing cells of the APUD series and the embryologic, physiologic and pathologic implications of the concept. J Histochem Cytochem 1969;17:303-13.

90. Thiele EA, Eipper BA. Effect of secretagogues on components of the secretory system in AtT-20 cells. Endocrinology 1990;126:809-17.

91. Dickinson CJ, Marino L, Yamada T. Inhibition of the alpha-amidation of gastrin: effects on gastric acid secretion. Am J Physiol 1990;258:G810-4.

92. Glembotski CC. The alpha-amidation of alpha-melanocyte stimulating hormone in intermediate pituitary requires ascorbic acid. J Biol Chem 1984;259:13041-8.

93. Fenger M, Hilsted L. Influence of ascorbic acid on in vivo amidation of alpha-melanocyte stimulating hormone in guinea pig pituitary. Acta Endocrinol (Copenh) 1988;118:119-24.

94. Wu S, Sumii K, Walsh J. Studies of Regulation of Gastrin Synthesis and Post-translational Processing by Molecular Biology Approachesa. Annals of the New York Academy of Sciences 1990;597:17-27.

95. Macro JA, Dimaline R, Dockray GJ. Identification and expression of prohormone-converting enzymes in the rat stomach. American Journal of Physiology-Gastrointestinal and Liver Physiology 1996;270:G87-G93.

96. Macro J, Bate G, Varro A, et al. Regulation by gastric acid of the processing of progastrin-derived peptides in rat antral mucosa. The Journal of physiology 1997;502:409-419.

97. Blair E, Greenwell J, Grund E, et al. Gastrin response to meals of different composition in normal subjects. Gut 1975;16:776-773.

98. Korman M, Soveny C, Hansky J. Effect of food on serum gastrin evaluated by radioimmunoassay. Gut 1971;12:619-624.

99. Richardson C, Walsh J, Hicks M, et al. Studies on the mechanisms of food-stimulated gastric acid secretion in normal human subjects. Journal of Clinical Investigation 1976;58:623.

100. Ray JM, Squires PE, Curtis SB, et al. Expression of the calcium-sensing receptor on human antral gastrin cells in culture. Journal of Clinical Investigation 1997;99:2328.

101. Bruce JI, Yang X, Ferguson CJ, et al. Molecular and functional identification of a Ca2+ (polyvalent cation)-sensing receptor in rat pancreas. Journal of Biological Chemistry 1999;274:20561-20568.

102. Chattopadhyay N, Légrádi G, Bai M, et al. Calcium-sensing receptor in the rat hippocampus: a developmental study. Developmental brain research 1997;100:13-21.

103. Conigrave AD, Quinn SJ, Brown EM. l-Amino acid sensing by the extracellular Ca2+-sensing receptor. Proceedings of the National Academy of Sciences 2000;97:4814-4819.

104. Geibel JP, Wagner CA, Caroppo R, et al. The stomach divalent ion-sensing receptor scar is a modulator of gastric acid secretion. Journal of Biological Chemistry 2001;276:39549-39552.

105. Buchan AMJ, Squires PE, Ring M, et al. Mechanism of action of the calcium-sensing receptor in human antral gastrin cells. Gastroenterology 2001;120:1128-1139.

106. Schubert M, Bitar K, Makhlouf GM. Regulation of gastrin and somatostatin secretion by cholinergic and noncholinergic intramural neurons. American Journal of Physiology-Gastrointestinal and Liver Physiology 1982;243:G442-G447.

107. Schubert ML, Hightower J, Coy DH, et al. Regulation of acid secretion by bombesin/GRP neurons of the gastric fundus. Am J Physiol 1991;260:G156-60.

108. Schubert M, Coy D, Makhlouf G. Peptone stimulates gastrin secretion from the stomach by activating bombesin/GRP and cholinergic neurons. American Journal of Physiology-Gastrointestinal and Liver Physiology 1992;262:G685-G689.

109. Schubert ML, Makhlouf GM. Neural, hormonal, and paracrine regulation of gastrin and acid secretion. Yale J Biol Med 1992;65:553-60; discussion 621-3.

110. Schubert ML, Makhlouf GM. Gastrin secretion induced by distention is mediated by gastric cholinergic and vasoactive intestinal peptide neurons in rats. Gastroenterology 1993;104:834-9.

111. Håkanson R, Oscarson J, Sundler F. Gastrin and the trophic control of gastric mucosa. Scand J Gastroenterol Suppl 1986;118:18-30.

112. Wang TC, Dangler CA, Chen D, et al. Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer. Gastroenterology 2000;118:36-47.

113. Straus E, Yalow RS. Studies on the distribution and degradation of heptadecapeptide, big, and big big gastrin. Gastroenterology 1974;66:936-943.

114. Boniface J, Picone D, Schebalin M, et al. Clearance rate, half-life, and secretory potency of human gastrin-17-I in different species. Gastroenterology 1976;71:291-4.

115. Ganguli P, Elder J, Smith I, et al. The half life (T and one-half) of synthetic human gastrin I in man. The British journal of surgery 1970;57:848.

116. Schrumpf E, Semb L, Vold H. Metabolic clearance and disappearance rates of synthetic human gastrin in man. Scandinavian journal of gastroenterology 1973;8:731.

117. Paterson AC, Baldwin GS, Shulkes A. Metabolism of recombinant progastrin in sheep. American Journal of Physiology-Endocrinology and Metabolism 2002;283:E449-E456.

118. Walsh J, Isenberg J, Ansfield J, et al. Clearance and acid-stimulating action of human big and little gastrins in duodenal ulcer subjects. Journal of Clinical Investigation 1976;57:1125.

119. Pauwels S, Najdovski T, Dimaline R, et al. Degradation of human gastrin and CCK by endopeptides 24.11: differential behaviour of the sulphated and unsulphated peptides. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 1989;996:82-88.

120. Clendinnen BG, Davidson WD, Reeder DD, et al. Renal uptake and excretion of gastrin in the dog. Surg Gynecol Obstet 1971;132:1039-43.

121. Newton W, Jaffe B. The fate of intravenously administered radiolabeled gastrin. Surgery 1971;69:34-40.

122. Dent R, Hirsch H, James J, et al. Hypergastrinemia in patients with acute renal failure. Surgical forum 1971;23:312-313.

123. Baoheng D. Hypergastrinemia in Chronic Renal Failure. Tianjin Medical Journal 1981;1:001.

124. Schjönsby H, Willassen Y. Renal extraction of endogenous gastrin in patients with normal renal function. Scandinavian journal of gastroenterology 1976;12:205-207.

125. Petersen B, Henriksen J, Rehfeld J, et al. Removal of endogenous gastrin in man. Scandinavian journal of gastroenterology 1981;16:727-730.

126. Thompson JC, Reeder DD, Davidson WD, et al. Effect of hepatic transit of gastrin, pentagastrin, and histamine measured by gastric secretion and by assay of hepatic vein blood. Annals of surgery 1969;170:493.

127. Dencker H, Hakanson R, Liedberg G, et al. Gastrin in portal and peripheral venous blood after feeding in man. Gut 1973;14:856-60.

128. Doyle JW, Wolfe MM, McGuigan JE. Hepatic clearance of gastrin and cholecystokinin peptides. Gastroenterology 1984;87:60-68.

129. Wyllie JH, Stagg BH, Temperley JM. Inactivation of pentagastrin by the liver. Br J Surg 1974;61:22-6.

130. Fourmy D, Gigoux V, Reubi JC. Gastrin in gastrointestinal diseases. Gastroenterology 2011;141:814-818.

131. Saito A, Sankaran H, Goldfine ID, et al. Cholecystokinin receptors in the brain: characterization and distribution. Science 1980;208:1155-1156.

132. Chandra R, Liddle RA. Cholecystokinin. Current Opinion in Endocrinology, Diabetes and Obesity 2007;14:63-67.

133. Fan W, Ellacott KL, Halatchev IG, et al. Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. Nature neuroscience 2004;7:335-336.

134. Whited KL, Thao D, Lloyd KK, et al. Targeted disruption of the murine CCK1 receptor gene reduces intestinal lipid-induced feedback inhibition of gastric function. American Journal of Physiology-Gastrointestinal and Liver Physiology 2006;291:G156-G162.

135. Hill D, Campbell N, Shaw T, et al. Autoradiographic localization and biochemical characterization of peripheral type CCK receptors in rat CNS using highly selective nonpeptide CCK antagonists. The Journal of neuroscience 1987;7:2967-2976.

136. Honda T, Wada E, Battey JF, et al. Differential gene expression of CCK A and CCK B receptors in the rat brain. Molecular and Cellular Neuroscience 1993;4:143-154.

137. Kopin AS, Lee Y-M, McBride EW, et al. Expression cloning and characterization of the canine parietal cell gastrin receptor. Proceedings of the National Academy of Sciences 1992;89:3605-3609.

138. Friis-Hansen L, Sundler F, Li Y, et al. Impaired gastric acid secretion in gastrin-deficient mice. American Journal of Physiology-Gastrointestinal and Liver Physiology 1998;274:G561-G568.

139. Rehfeld JF. Accurate measurement of cholecystokinin in plasma. Clinical Chemistry 1998;44:991-1001.

140. Rehfeld J, Hansen H, Larsson L, et al. Gastrin and cholecystokinin in pituitary neurons. Proceedings of the National Academy of Sciences 1984;81:1902-1905.

141. Liddle R. Cholecystokinin. Gut peptides: biochemistry and physiology 1994:175-216.

142. Yu H-G, Tong S-L, Ding Y-M, et al. Enhanced expression of cholecystokinin-2 receptor promotes the progression of colon cancer through activation of focal adhesion kinase. International Journal of Cancer 2006;119:2724-2732.

143. Ashurst HL, Varro A, Dimaline R. Regulation of mammalian gastrin/CCK receptor (CCK2R) expression in vitro and in vivo. Exp Physiol 2008;93:223-36.

144. Magnan R, Masri B, Escrieut C, et al. Regulation of membrane cholecystokinin-2 receptor by agonists enables classification of partial agonists as biased agonists. Journal of Biological Chemistry 2011;286:6707-6719.

145. Hanyaloglu AC, Zastrow Mv. Regulation of GPCRs by endocytic membrane trafficking and its potential implications. Annu. Rev. Pharmacol. Toxicol. 2008;48:537-568.

146. Traub LM. Tickets to ride: selecting cargo for clathrin-regulated internalization. Nature reviews Molecular cell biology 2009;10:583-596.

147. Gurevich VV, Gurevich EV. The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. Pharmacology & therapeutics 2006;110:465-502.

148. Vickery RG, von Zastrow M. Distinct dynamin-dependent and-independent mechanisms target structurally homologous dopamine receptors to different endocytic membranes. The Journal of cell biology 1999;144:31-43.

149. Go WY, Holicky EL, Hadac EM, et al. Identification of a domain in the carboxy terminus of CCK receptor that affects its intracellular trafficking. American Journal of Physiology - Gastrointestinal and Liver Physiology 1998;275:G56-G62.

150. Martinsen TC, Bergh K, Waldum HL. Gastric Juice: A Barrier Against Infectious Diseases. Basic & Clinical Pharmacology & Toxicology 2005;96:94-102.

151. Mahadevan V. Anatomy of the stomach. Surgery (Oxford) 2014;32:571-574.

152. Håkanson R, Sundler F. Regulation of gastric endocrine cell proliferation. Gastrin. New York: Raven Press, Ltd 1993:307-318.

153. Simonsson M, Eriksson S, Håkanson R, et al. Endocrine cells in the human oxyntic mucosa: a histochemical study. Scandinavian journal of gastroenterology 1988;23:1089-1099.

154. Karam SM. New insights into the stem cells and the precursors of the gastric epithelium. Nutrition 1995;11:607-13.

155. Sandvik AK, Waldum HL. CCK-B (gastrin) receptor regulates gastric histamine release and acid secretion. Am J Physiol 1991;260:G925-G928.

156. Andersson K, Chen D, Mattsson H, et al. Physiological significance of ECL-cell histamine. The Yale journal of biology and medicine 1998;71:183.

157. Raptis S, Dollinger H, Von Berger L, et al. Effects of somatostatin on gastric secretion and gastrin release in man. Digestion 1975;13:15-26.

158. Moyer MP, Armstrong A, Aust JB, et al. Effects of gastrin, glutamine, and somatostatin on the in vitro growth of normal and malignant human gastric mucosal cells. Archives of Surgery 1986;121:285-288.

159. Nagata A, Ito M, Iwata N, et al. G protein-coupled cholecystokinin-B/gastrin receptors are responsible for physiological cell growth of the stomach mucosa in vivo. Proceedings of the National Academy of Sciences 1996;93:11825-11830.

160. Hollande F, Imdahl A, Mantamadiotis T, et al. Glycine-extended gastrin acts as an autocrine growth factor in a nontransformed colon cell line. Gastroenterology 1997;113:1576-1588.

161. Koh TJ, Dockray GJ, Varro A, et al. Overexpression of glycine-extended gastrin in transgenic mice results in increased colonic proliferation. Journal of Clinical Investigation 1999;103:1119.

162. Tielemans Y, Willems G, Sundler F, et al. Self-replication of enterochromaffin-like cells in the mouse stomach. Digestion 1990;45:138-46.

163. Modlin IM, Lawton GP, Miu K, et al. Pathophysiology of the fundic enterochromaffin-like (ECL) cell and gastric carcinoid tumours. Ann R Coll Surg Engl 1996;78:133-8.

164. Rindi G, Luinetti O, Cornaggia M, et al. Three subtypes of gastric argyrophil carcinoid and the gastric neuroendocrine carcinoma: a clinicopathologic study. Gastroenterology 1993;104:994-1006.

165. Kinoshita Y, Ishihara S. Mechanism of gastric mucosal proliferation induced by gastrin. Journal of Gastroenterology and Hepatology 2000;15:7-11.

166. Terazono K, Yamamoto H, Takasawa S, et al. A novel gene activated in regenerating islets. J Biol Chem 1988;263:2111-4.

167. Watanabe T, Yonemura Y, Yonekura H, et al. Pancreatic beta-cell replication and amelioration of surgical diabetes by Reg protein. Proc Natl Acad Sci U S A 1994;91:3589-92.

168. Watanabe T, Yonekura H, Terazono K, et al. Complete nucleotide sequence of human reg gene and its expression in normal and tumoral tissues. The reg protein, pancreatic stone protein, and pancreatic thread protein are one and the same product of the gene. J Biol Chem 1990;265:7432-9.

169. Fukui H, Kinoshita Y, Maekawa T, et al. Regenerating gene protein may mediate gastric mucosal proliferation induced by hypergastrinemia in rats. Gastroenterology 1998;115:1483-93.

170. Jain RN, Brunkan CS, Chew CS, et al. Gene expression profiling of gastrin target genes in parietal cells. Physiological genomics 2006;24:124-132.

171. Zavros Y, Rieder G, Ferguson A, et al. Genetic or chemical hypochlorhydria is associated with inflammation that modulates parietal and G-cell populations in mice. Gastroenterology 2002;122:119-133.

172. Hinkle KL, Bane GC, Jazayeri A, et al. Enhanced calcium signaling and acid secretion in parietal cells isolated from gastrin-deficient mice. American Journal of Physiology-Gastrointestinal and Liver Physiology 2003;284:G145-G153.

173. Varro A, Hemers E, Archer D, et al. Identification of plasminogen activator inhibitor-2 as a gastrin-regulated gene: Role of Rho GTPase and menin. Gastroenterology 2002;123:271-80.

174. Kidd M, Tang LH, Modlin IM, et al. Gastrin-mediated alterations in gastric epithelial apoptosis and proliferation in a mastomys rodent model of gastric neoplasia. Digestion 2000;62:143-51.

175. Przemeck SM, Varro A, Berry D, et al. Hypergastrinemia increases gastric epithelial susceptibility to apoptosis. Regul Pept 2008;146:147-56.

176. Cui G, Takaishi S, Ai W, et al. Gastrin-induced apoptosis contributes to carcinogenesis in the stomach. Lab Invest 2006;86:1037-51.

177. Varro A, Noble PJ, Pritchard DM, et al. Helicobacter pylori induces plasminogen activator inhibitor 2 in gastric epithelial cells through nuclear factor-kappaB and RhoA: implications for invasion and apoptosis. Cancer Res 2004;64:1695-702.

178. Sun WH, Zhu F, Chen GS, et al. Blockade of cholecystokinin-2 receptor and cyclooxygenase-2 synergistically induces cell apoptosis, and inhibits the proliferation of human gastric cancer cells in vitro. Cancer Lett 2008;263:302-11.

179. Ramamoorthy S, Stepan V, Todisco A. Intracellular mechanisms mediating the anti-apoptotic action of gastrin. Biochem Biophys Res Commun 2004;323:44-8.

180. Pritchard DM, Berry D, Przemeck SM, et al. Gastrin increases mcl-1 expression in type I gastric carcinoid tumors and a gastric epithelial cell line that expresses the CCK-2 receptor. Am J Physiol Gastrointest Liver Physiol 2008;295:G798-805.

181. Todisco A, Ramamoorthy S, Witham T, et al. Molecular mechanisms for the antiapoptotic action of gastrin. Am J Physiol Gastrointest Liver Physiol 2001;280:G298-307.

182. Miyazaki Y, Shinomura Y, Tsutsui S, et al. Gastrin induces heparin-binding epidermal growth factor–like growth factor in rat gastric epithelial cells transfected with gastrin receptor. Gastroenterology 1999;116:78-89.

183. Chen CN, Hsieh FJ, Cheng YM, et al. Expression of inducible nitric oxide synthase and cyclooxygenase-2 in angiogenesis and clinical outcome of human gastric cancer. J Surg Oncol 2006;94:226-33.

184. Clarke PA, Dickson JH, Harris JC, et al. Gastrin Enhances the Angiogenic Potential of Endothelial Cells via Modulation of Heparin-Binding Epidermal-Like Growth Factor. Cancer Research 2006;66:3504-3512.

185. Norsett KG, Steele I, Duval C, et al. Gastrin stimulates expression of plasminogen activator inhibitor-1 in gastric epithelial cells. Am J Physiol Gastrointest Liver Physiol 2011;301:G446-53.

186. Varro A, Hemers E, Archer D, et al. Identification of plasminogen activator inhibitor-2 as a gastrin-regulated gene: Role of Rho GTPase and menin. Gastroenterology 2002;123:271-280.

187. Tatsuguchi A, Fukuda Y, Ishizaki M, et al. Localization of matrix metalloproteinases and tissue inhibitor of metalloproteinases-2 in normal human and rabbit stomachs. Digestion 1999;60:246-254.

188. Kumar JD, Steele I, Moore AR, et al. Gastrin stimulates MMP-1 expression in gastric epithelial cells: putative role in gastric epithelial cell migration. Am J Physiol Gastrointest Liver Physiol 2015;309:G78-86.

189. Wroblewski LE, Pritchard DM, Carter S, et al. Gastrin-stimulated gastric epithelial cell invasion: the role and mechanism of increased matrix metalloproteinase 9 expression. Biochem J 2002;365:873-9.

190. Wroblewski LE, Noble PJ, Pagliocca A, et al. Stimulation of MMP-7 (matrilysin) by Helicobacter pylori in human gastric epithelial cells: role in epithelial cell migration. J Cell Sci 2003;116:3017-26.

191. Varro A, Kenny S, Hemers E, et al. Increased gastric expression of MMP-7 in hypergastrinemia and significance for epithelial-mesenchymal signaling. Am J Physiol Gastrointest Liver Physiol 2007;292:11.

192. Guarino M, Rubino B, Ballabio G. The role of epithelial-mesenchymal transition in cancer pathology. Pathology 2007;39:305-18.

193. Lacourse KA, Friis-Hansen L, Rehfeld JF, et al. Disturbed progastrin processing in carboxypeptidase E-deficient fat mice. FEBS Letters 1997;416:45-50.

194. Stepan VM, Krametter DF, Matsushima M, et al. Glycine-extended gastrin regulates HEK cell growth. Am J Physiol 1999;277:R572-81.

195. Ramanathan V, Jin G, Westphalen CB, et al. P53 gene mutation increases progastrin dependent colonic proliferation and colon cancer formation in mice. Cancer Invest 2012;30:275-86.

196. Aly A, Shulkes A, Baldwin GS. Short term infusion of glycine-extended gastrin(17) stimulates both proliferation and formation of aberrant crypt foci in rat colonic mucosa. Int J Cancer 2001;94:307-13.

197. Wang TC, Koh TJ, Varro A, et al. Processing and proliferative effects of human progastrin in transgenic mice. J Clin Invest 1996;98:1918-29.

198. Ottewell PD, Watson AJ, Wang TC, et al. Progastrin stimulates murine colonic epithelial mitosis after DNA damage. Gastroenterology 2003;124:1348-1357.

199. Patel O, Marshall KM, Bramante G, et al. The C-terminal flanking peptide (CTFP) of progastrin inhibits apoptosis via a PI3-kinase-dependent pathway. Regulatory Peptides 2010;165:224-231.

200. Marshall KM, Patel O, Bramante G, et al. The C-terminal flanking peptide of progastrin induces gastric cell apoptosis and stimulates colonic cell division in vivo. Peptides 2013;46:83-93.

201. Zollinger RM, Ellison EH. Primary peptic ulcerations of the jejunum associated with islet cell tumors of the pancreas. Annals of surgery 1955;142:709.

202. Roy PK, Venzon DJ, Feigenbaum KM, et al. Gastric secretion in Zollinger-Ellison syndrome: correlation with clinical expression, tumor extent and role in diagnosis—A prospective NIH study of 235 patients and a review of 984 cases in the literature. Medicine 2001;80:189-222.

203. Arnold R. Diagnosis and differential diagnosis of hypergastrinemia. Wiener Klinische Wochenschrift 2007;119:564-569.

204. Annibale B, Rindi G, D'ambra G, et al. Antral gastrin cell hyperfunction and Helicobacter pylori infection. Alimentary pharmacology & therapeutics 1996;10:607-615.

205. Cowley D, Dymock I, Boyes B, et al. Zollinger-Ellison syndrome type 1: clinical and pathological correlations of a case. Gut 1973;14:25-29.

206. Taylor I, Calam J, Rotter J, et al. Family studies of hypergastrinemic, hyperpepsinogenemic I duodenal ulcer. Annals of internal medicine 1981;95:421-425.

207. Evans JC, Reeder DD, Thompson JC. Inactivation of gastrin and pentagastrin by tissue slices. Proc Soc Exp Biol Med 1973;143:168-70.

208. Thompson J, Reeder D, Davidson W, et al. Studies on the metabolism of gastrin, In Nobel Symposium, 1973.

209. Del Mazo J, McGuigan JE. Degradation of gastrin by gastric mucosal cells. The Journal of laboratory and clinical medicine 1976;88:292-300.

210. Hansky J. Clinical aspects of gastrin physiology. The Medical clinics of North America 1974;58:1217-1230.

211. Shepherd A, Stewart W, Wormsley K. Peptic ulceration in chronic renal failure. The Lancet 1973;301:1357-1359.

212. Shepherd A, Stewart W, Thjodleifsson B, et al. Further studies of gastric hypersecretion in chronic renal failure. BMJ 1974;1:96-98.

213. Falcao HA, Wesdorp RI, Fischer JE. Gastrin levels and gastric acid secretion in anephric patients and in patients with chronic and acute renal failure. Journal of Surgical Research 1975;18:107-111.

214. Arnold R, Creutzfeldt C, Creutzfeldt W, et al. Findings in the remaining antrum after Billroth-II operations (excluded antrum)--a contribution to the differential diagnosis of recurrent ulcers with hypergastrinemia. Verhandlungen der Deutschen Gesellschaft fur Innere Medizin 1975;82:1002-1006.

215. Cleator I, Holubitsky I, Harrison R. Anastomotic ulceration. Annals of surgery 1974;179:339.

216. Webster MW, Barnes EL, Stremple JF. Serum gastrin levels in the differential diagnosis of recurrent peptic ulceration due to retained gastric antrum. The American Journal of Surgery 1978;135:248-252.

217. Marques-Silva L, Areia M, Elvas L, et al. Prevalence of gastric precancerous conditions: a systematic review and meta-analysis. European journal of gastroenterology & hepatology 2014;26:378-387.

218. Ohata H, Kitauchi S, Yoshimura N, et al. Progression of chronic atrophic gastritis associated with Helicobacter pylori infection increases risk of gastric cancer. Int J Cancer 2004;109:138-43.

219. Kuipers EJ, Pérez-Pérez GI, Meuwissen SG, et al. Helicobacter pylori and atrophic gastritis: importance of the cagA status. Journal of the National Cancer Institute 1995;87:1777-1780.

220. Carmel R. Prevalence of undiagnosed pernicious anemia in the elderly. Archives of Internal Medicine 1996;156:1097-1100.

221. Taylor K, Roitt I, Doniach D, et al. Autoimmune phenomena in pernicious anaemia: gastric antibodies. BMJ 1962;2:1347-1352.

222. Irvine W. Gastric antibodies studied by fluorescence microscopy. Quarterly journal of experimental physiology and cognate medical sciences 1963;48:427-438.

223. Thompson JC, Fender H, Watson L, et al. The effects on gastrin and gastric secretion of five current operations for duodenal ulcer. Annals of surgery 1976;183:599.

224. Becker HD, Reeder DD, Thompson JC. Effect of truncal vagotomy with pyloroplasty or with antrectomy on food-stimulated gastrin values in patients with duodenal ulcer. Surgery 1973;74:580-586.

225. Stern DHW, J. H. . Gastrin Release in Postoperative Ulcer Patients: Evidence for Release of Duodenal Gastrin. Gastroenterology 1973;64:363-369.

226. Walsh J, Grossman M. Circulating gastrin in peptic ulcer disease. The Mount Sinai journal of medicine, New York 1973;40:374.

227. Dale HH, Laidlaw PP. The physiological action of beta-iminazolylethylamine. J Physiol 1910;41:318-44.

228. Black JW, Duncan W, Durant CJ, et al. Definition and antagonism of histamine H2-receptors. Nature 1972;236:385-390.

229. Arrang J-M, Garbarg M, Lancelo J-C, et al. Highly potent and selective ligands for histamine H3-receptors. Nature 1987;327:117-123.

230. Hofstra CL, Desai PJ, Thurmond RL, et al. Histamine H4 receptor mediates chemotaxis and calcium mobilization of mast cells. Journal of Pharmacology and Experimental Therapeutics 2003;305:1212-1221.

231. Tougas G, Armstrong D. Efficacy of H2 receptor antagonists in the treatment of gastroesophageal reflux disease and its symptoms. Canadian journal of gastroenterology 1997;11:51-54.

232. Brimblecombe R, Duncan W, Durant G, et al. Characterization and development of cimetidine as a histamine H2-receptor antagonist. Gastroenterology 1978;74:339-347.

233. Burland W, Duncan W, Hesselbo T, et al. Pharmacological evaluation of cimetidine, a new histamine H2-receptor antagonist, in healthy man. British journal of clinical pharmacology 1975;2:481-486.

234. Sewing K, Hagie L, Ippoliti A, et al. Effect of one-month treatment with cimetidine on gastric secretion and serum gastrin and pepsinogen levels. Gastroenterology 1978;74:276-279.

235. Spence R, Celestin L, McCormick D, et al. Effect Of One Years Treatment With Cimetidine On Fasting And Oxo-Stimulated Serum Gastrin In Duodenal-Ulcer Patients. Gut 1977;18:949.

236. Forrest J, Fettes M, McLoughlin G, et al. Effect of long-term cimetidine on gastric acid secretion, serum gastrin, and gastric emptying. Gut 1979;20:404-407.

237. Cheli R, Giacosa A, Molinari F, et al. Parietal cell mass after short and long term cimetidine treatment of duodenal ulcer. Scandinavian journal of gastroenterology. Supplement 1978;54:45-47.

238. Emmanouilidis A, Nicolopoulou-Stamati P, Manousos O. Effects of Therapeutic Doses of Cimetidine and Oxmetidine on the Parietal Cell Population, Gastric Mucosal Glycoproteins and Surface Gastric Epithelium, in Duodenal Ulcer Patients. Journal of International Medical Research 1982;10:113-117.

239. Willems G, Lehy T. Radioautographic and quantitative studies on parietal and peptic cell kinetics in the mouse. A selective effect of gastrin on parietal cell proliferation. Gastroenterology 1975;69:416-426.

240. Crean G, Daniel D, Leslie G, et al. The effects of prolonged administration of large doses of cimetidine on the gastric mucosa of rats. Cimetidine. C Wastell, P Lance (eds). Edinburgh, Churchill Livingstone 1978:191-205.

241. Lodish HF, Berk A, Zipursky SL, et al. Molecular cell biology: Citeseer, 2000.

242. Clissold SP, Campoli-Richards DM. Omeprazole. Drugs 1986;32:15-47.

243. Brunner G, Creutzfeldt W, Harke U, et al. Efficacy and safety of long term treatment with omeprazole in patients with acid related diseases resistant to ranitidine. Can J Gastroenterol 1989;3:72-76.

244. Lanzon-Miller S, Pounder R, Hamilton M, et al. Twenty-four-hour intragastric acidity and plasma gastrin concentration before and during treatment with either ranitidine or omeprazole. Alimentary pharmacology & therapeutics 1987;1:239-251.

245. Warrington S, Baisley K, Boyce M, et al. Effects of rabeprazole, 20 mg, or esomeprazole, 20 mg, on 24-h intragastric pH and serum gastrin in healthy subjects. Alimentary pharmacology & therapeutics 2002;16:1301-1307.

246. Whiteside M, Mollin D, Coghill N, et al. The absorption of radioactive vitamin B12 and the secretion of hydrochloric acid in patients with atrophic gastritis. Gut 1964;5:385-399.

247. Wintrobe M, Lee G, Boggs D, et al. Megaloblastic and nonmegaloblastic macrocytic anemias. Clinical hematology. 8th ed. Philadelphia: Lea & Febiger 1981:559-604.

248. Zittoun J. Biermer's disease. La Revue du praticien 2001;51:1542-1546.

249. Meecham J, Jones EW. Addison's disease and Addisonian anaemia. The Lancet 1967;289:535-538.

250. Toh B-H, van Driel IR, Gleeson PA. Pernicious anemia. N Engl J Med 1997;337:1441-1448.

251. D'Elios MM, Appelmelk BJ, Amedei A, et al. Gastric autoimmunity: the role of Helicobacter pylori and molecular mimicry. Trends in molecular medicine 2004;10:316-323.

252. Marcolongo R, Bayeli P, Montagnani M. Gastrointestinal involvement in rheumatoid arthritis: a biopsy study. The Journal of rheumatology 1978;6:163-173.

253. Toh BH GP, Whittingham S & van Driel IR. Autoimmune gastritis and pernicious anemia. St. Louis, MO: Academic Press, 1998.

254. Ma J-Y, Borch K, Mårdh S. Human gastric H, K-adenosine triphosphatase β-subunit is a major autoantigen in atrophic corpus gastritis: expression of the recombinant

human glycoprotein in insect cells. Scandinavian journal of gastroenterology 1994;29:790-794.

255. Claeys D, Faller G, Appelmelk BJ, et al. The gastric H+, K+-ATPase is a major autoantigen in chronic Helicobacter pylori gastritis with body mucosa atrophy. Gastroenterology 1998;115:340-347.

256. Amedei A, Bergman MP, Appelmelk BJ, et al. Molecular mimicry between Helicobacter pylori antigens and H+, K+–adenosine triphosphatase in human gastric autoimmunity. The Journal of experimental medicine 2003;198:1147-1156.

257. D'Elios MM, Amedei A, Azzurri A, et al. Molecular specificity and functional properties of autoreactive T-cell response in human gastric autoimmunity. International reviews of immunology 2005;24:111-122.

258. Annibale B, Lahner E, Bordi C, et al. Role of Helicobacter pylori infection in pernicious anaemia. Digestive and Liver Disease 2000;32:756-762.

259. Varis O, Valle J, Siurala M. Is Helicobacter pylori Involved in the Pathogenesis of the Gastritis Characteristic of Pernicious Anaemia?: Comparison between Pernicious Anaemia Relatives and Duodenal Ulcer Relatives. Scandinavian journal of gastroenterology 1993;28:705-708.

260. Pokorny G, Karacsony G, Lonovics J, et al. Types of atrophic gastritis in patients with primary Sjögren's syndrome. Annals of the rheumatic diseases 1991;50:97-100.

261. Rooney J, Vince J, Kennedy A, et al. Hypergastrinaemia in rheumatoid arthritis: disease or iatrogenesis. British medical journal 1973;2:752-753.

262. Taglieri G, Piga M, Ibba G, et al. Serum gastrin in rheumatoid arthritis. Revue du rhumatisme et des maladies osteo-articulaires 1982;49:533-535.

263. Lake-Bakaar G, Quadros E, Beidas S, et al. Gastric secretory failure in patients with the acquired immunodeficiency syndrome (AIDS). Annals of internal medicine 1988;109:502-504.

264. Welage LS, Carver PL, Revankar S, et al. Alterations in gastric acidity in patients infected with human immunodeficiency virus. Clinical infectious diseases 1995;21:1431-1438.

265. Fabris P, Bozzola L, Benedetti P, et al. H. pylori Infection in HIV-Positive Patients (A Serohistological Study). Digestive diseases and sciences 1997;42:289-292.

266. Hvid-Jensen F, Pedersen L, Drewes AM, et al. Incidence of adenocarcinoma among patients with Barrett's esophagus. New England Journal of Medicine 2011;365:1375-1383.

267. Cameron A. Barrett's esophagus: does the incidence of adenocarcinoma matter? The American journal of gastroenterology 1997;92:193.

268. Sampliner RE, Gastroenterology PPCotACo. Practice guidelines on the diagnosis, surveillance, and therapy of Barrett's esophagus. The American journal of gastroenterology 1998;93:1028-1032.

269. Eisen G, Sandler R, Murray S, et al. The relationship between gastroesophageal reflux disease and its complications with Barrett's esophagus. The American journal of gastroenterology 1997;92:27-31.

270. Lagergren J, Bergström R, Lindgren A, et al. Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. New England journal of medicine 1999;340:825-831.

271. Spechler SJ, Lee E, Ahnen D, et al. Long-term outcome of medical and surgical therapies for gastroesophageal reflux disease: follow-up of a randomized controlled trial. Jama 2001;285:2331-2338.

272. Iwao T, Toyonaga A, Kuboyama S-i, et al. Effects of omeprazole and lansoprazole on fasting and postprandial serum gastrin and serum pepsinogen A and C. Hepato-gastroenterology 1994;42:677-682.

273. Wang JS, Varro A, Lightdale CJ, et al. Elevated serum gastrin is associated with a history of advanced neoplasia in Barrett's esophagus. Am J Gastroenterol 2010;105:1039-45.

274. Green DA, Mlynarczyk CM, Vaccaro BJ, et al. Correlation between serum gastrin and cellular proliferation in Barrett's esophagus. Therapeutic advances in gastroenterology 2010:1756283X10392444.

275. Wilson KT, Fu S, Ramanujam KS, et al. Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. Cancer Research 1998;58:2929-2934.

276. Haigh CR, Attwood SE, Thompson DG, et al. Gastrin induces proliferation in Barrett's metaplasia through activation of the CCK 2 receptor. Gastroenterology 2003;124:615-625.

277. Abdalla SI, Lao-Sirieix P, Novelli MR, et al. Gastrin-induced cyclooxygenase-2 expression in Barrett's carcinogenesis. Clinical cancer research 2004;10:4784-4792.

278. Correa P. Helicobacter pylori and gastric carcinogenesis. The American journal of surgical pathology 1994;19:S37-43.

279. van den Brink GR, Hardwick JC, Tytgat GN, et al. Sonic hedgehog regulates gastric gland morphogenesis in man and mouse. Gastroenterology 2001;121:317-28.

280. Xiao C, Ogle SA, Schumacher MA, et al. Loss of parietal cell expression of Sonic hedgehog induces hypergastrinemia and hyperproliferation of surface mucous cells. Gastroenterology 2010;138:550-561. e8.

281. van den Brink GR, Hardwick JC, Nielsen C, et al. Sonic hedgehog expression correlates with fundic gland differentiation in the adult gastrointestinal tract. Gut 2002;51:628-633.

282. Zavros Y, Orr MA, Xiao C, et al. Sonic hedgehog is associated with H+-K+-ATPase-containing membranes in gastric parietal cells and secreted with histamine stimulation. American Journal of Physiology-Gastrointestinal and Liver Physiology 2008;295:G99-G111.

283. Wakelin SJ, Forsythe JL, Garden OJ, et al. Commercially available recombinant sonic hedgehog up-regulates Ptc and modulates the cytokine and chemokine expression of human macrophages: an effect mediated by endotoxin contamination? Immunobiology 2008;213:25-38.

284. Lowrey JA, Stewart GA, Lindey S, et al. Sonic hedgehog promotes cell cycle progression in activated peripheral CD4+ T lymphocytes. The Journal of Immunology 2002;169:1869-1875.

285. Stewart GA, Lowrey JA, Wakelin SJ, et al. Sonic hedgehog signaling modulates activation of and cytokine production by human peripheral CD4+ T cells. The Journal of Immunology 2002;169:5451-5457.

286. Shiotani A, Iishi H, Uedo N, et al. Evidence that loss of sonic hedgehog is an indicator of Helicobater pylori-induced atrophic gastritis progressing to gastric cancer. Am J Gastroenterol 2005;100:581-7.

287. Ramalho-Santos M, Melton DA, McMahon AP. Hedgehog signals regulate multiple aspects of gastrointestinal development. Development 2000;127:2763-72.

288. Stemmermann GN. Intestinal metaplasia of the stomach. A status report. Cancer 1994;74:556-564.

289. Nozaki K, Ogawa M, Williams JA, et al. A molecular signature of gastric metaplasia arising in response to acute parietal cell loss. Gastroenterology 2008;134:511-522.

290. Schmidt PH, Lee JR, Joshi V, et al. Identification of a metaplastic cell lineage associated with human gastric adenocarcinoma. Laboratory investigation; a journal of technical methods and pathology 1999;79:639-646.

291. Morson B. Carcinoma arising from areas of intestinal metaplasia in the gastric mucosa. British journal of cancer 1955;9:377.

292. Correa P. A human model of gastric carcinogenesis. Cancer research 1988;48:3554-3560.

293. Filipe MI, Muñoz N, Matko I, et al. Intestinal metaplasia types and the risk of gastric cancer: a cohort study in Slovenia. International journal of cancer 1994;57:324-329.

294. Xia HH, Kalantar JS, Talley NJ, et al. Antral-type mucosa in the gastric incisura, body, and fundus (antralization): a link between Helicobacter pylori infection and intestinal metaplasia? Am J Gastroenterol 2000;95:114-21.

295. Yoshizawa N, Takenaka Y, Yamaguchi H, et al. Emergence of spasmolytic polypeptide-expressing metaplasia in Mongolian gerbils infected with Helicobacter pylori. Lab Invest 2007;87:1265-76.

296. El-Zimaity HM, Ramchatesingh J, Saeed MA, et al. Gastric intestinal metaplasia: subtypes and natural history. J Clin Pathol 2001;54:679-83.

297. Rokkas T, Pistiolas D, Sechopoulos P, et al. The long-term impact of Helicobacter pylori eradication on gastric histology: a systematic review and meta-analysis. Helicobacter 2007;12 Suppl 2:32-8.

298. Kong YJ, Yi HG, Dai JC, et al. Histological changes of gastric mucosa after Helicobacter pylori eradication: a systematic review and meta-analysis. World J Gastroenterol 2014;20:5903-11.

299. Rugge M, Farinati F, Baffa R, et al. Gastric epithelial dysplasia in the natural history of gastric cancer: a multicenter prospective follow-up study. Gastroenterology 1994;107:1288-1296.

300. Lauwers G, Riddell R. Gastric epithelial dysplasia. Gut 1999;45:784-784.

301. Kloppel G. Oberndorfer and his successors: from carcinoid to neuroendocrine carcinoma. Endocr Pathol 2007;18:141-4.

302. Gilligan CJ, Lawton GP, Tang LH, et al. Gastric carcinoid tumors: the biology and therapy of an enigmatic and controversial lesion. Am J Gastroenterol 1995;90:338-52.

303. Solcia E, Rindi G, Silini E, et al. Enterochromaffin-like (ECL) cells and their growths: relationships to gastrin, reduced acid secretion and gastritis. Baillieres Clin Gastroenterol 1993;7:149-65.

304. Rindi G, Azzoni C, Rosa SL, et al. ECL cell tumor and poorly differentiated endocrine carcinoma of the stomach: prognostic evaluation by pathological analysis. Gastroenterology 1999;116:532-542.

305. Hofler H, Stier A, Schusdziarra V, et al. Classification of neuroendocrine tumors of the gastrointestinal tract and pancreas and its therapeutic relevance. Chirurg 1997;68:107-15.

306. Bordi C, Azzoni C, D'Adda T, et al. Neuroendocrine tumors of the stomach. Pathogenesis, classification and clinical aspects. Pathologe 1997;18:313-21.

307. Modlin IM, Lye KD, Kidd M. A 50-year analysis of 562 gastric carcinoids: small tumor or larger problem? Am J Gastroenterol 2004;99:23-32.

308. Klöppel G. Tumour biology and histopathology of neuroendocrine tumours. Best Practice & Research Clinical Endocrinology & Metabolism 2007;21:15-31.

309. Debelenko LV, Emmert-Buck MR, Zhuang Z, et al. The multiple endocrine neoplasia type I gene locus is involved in the pathogenesis of type II gastric carcinoids. Gastroenterology 1997;113:773-81.

310. Carling T. Multiple endocrine neoplasia syndrome: genetic basis for clinical management. Current opinion in oncology 2005;17:7-12.

311. Lemos MC, Thakker RV. Multiple endocrine neoplasia type 1 (MEN1): analysis of 1336 mutations reported in the first decade following identification of the gene. Human mutation 2008;29:22-32.

312. Agarwal SK, Kester MB, Debelenko LV, et al. Germline mutations of the MEN1 gene in familial multiple endocrine neoplasia type 1 and related states. Human molecular genetics 1997;6:1169-1175.

313. Harding B, Lemos MC, Reed AA, et al. Multiple endocrine neoplasia type 1 knockout mice develop parathyroid, pancreatic, pituitary and adrenal tumours with hypercalcaemia, hypophosphataemia and hypercorticosteronaemia. Endocrine-related cancer 2009;16:1313-1327.

314. Crabtree JS, Scacheri PC, Ward JM, et al. A mouse model of multiple endocrine neoplasia, type 1, develops multiple endocrine tumors. Proceedings of the National Academy of Sciences 2001;98:1118-1123.

315. Bertolino P, Tong W-M, Galendo D, et al. Heterozygous Men1 mutant mice develop a range of endocrine tumors mimicking multiple endocrine neoplasia type 1. Molecular endocrinology 2003;17:1880-1892.

316. Moses RE, Frank BB, Leavitt M, et al. The syndrome of type A chronic atrophic gastritis, pernicious anemia, and multiple gastric carcinoids. Journal of clinical gastroenterology 1986;8:61-65.

317. Spoelstra-de Man A, Wagenaar SS, van der Sluys Veer A, et al. Relationship between pernicious anaemia and gastric neuroendocrine cell disorders. The Netherlands journal of medicine 2000;56:56-62.

318. Bordi C, D'Adda T, Azzoni C, et al. Neuroendocrine proliferation in the gastric mucosa: biological behaviour and management. Verhandlungen der Deutschen Gesellschaft fur Pathologie 1996;81:103-110.

319. Cadiot G, Laurent-Puig P, Thuille B, et al. Is the multiple endocrine neoplasia type 1 gene a suppressor for fundic argyrophil tumors in the Zollinger-Ellison syndrome? Gastroenterology 1993;105:579.

320. Wotherspoon A, Doglioni C, Isaacson P. Low-grade gastric B-cell lymphoma of mucosa-associated lymphoid tissue (MALT): a multifocal disease. Histopathology 1992;20:29-34.

321. Parsonnet J, Hansen S, Rodriguez L, et al. Helicobacter pylori infection and gastric lymphoma. New England Journal of Medicine 1994;330:1267-1271.

322. Konturek P, Konturek S, Starzyska T, et al. Helicobacter pylori–gastrin link in MALT lymphoma. Alimentary pharmacology & therapeutics 2000;14:1311-1318.

323. Lamarque D, Levy M, Chaumette M-T, et al. Frequent and rapid progression of atrophy and intestinal metaplasia in gastric mucosa of patients with MALT lymphoma. The American journal of gastroenterology 2006;101:1886-1893.

324. Isaacson P, Spencer J. Malignant lymphoma of mucosa-associated lymphoid tissue. Histopathology 1987;11:445-462.

325. Isaacson PG. Extranodal lymphomas. Histopathology 1995;27:97-97.

326. Bayerdörffer E, Rudolph B, Neubauer A, et al. Regression of primary gastric lymphoma of mucosa-associated lymphoid tissue type after cure of Helicobacter pylori infection. The Lancet 1995;345:1591-1594.

327. Fischbach W, Goebeler-Kolve ME, Dragosics B, et al. Long term outcome of patients with gastric marginal zone B cell lymphoma of mucosa associated lymphoid tissue (MALT) following exclusive Helicobacter pylori eradication therapy: experience from a large prospective series. Gut 2004;53:34-7.

328. Chan JK, Ng CS, Isaacson PG. Relationship between high-grade lymphoma and low-grade B-cell mucosa-associated lymphoid tissue lymphoma (MALToma) of the stomach. Am J Pathol 1990;136:1153-64.

329. de Jong D, Boot H, van Heerde P, et al. Histological grading in gastric lymphoma: pretreatment criteria and clinical relevance. Gastroenterology 1997;112:1466-74.

330. Cogliatti SB, Schmid U, Schumacher U, et al. Primary B-cell gastric lymphoma: a clinicopathological study of 145 patients. Gastroenterology 1991;101:1159-70.

331. Isaacson PG, Spencer J. The biology of low grade MALT lymphoma. J Clin Pathol 1995;48:395-7.

332. CRUK. Pancreatic cancer statistics. Cancer statistics. Volume 2015. Cancer Research UK, 2012.

333. Reber HA, Johnson F, Deveney K, et al. Trophic effects of gastrin on the exocrine pancreas in rats. Journal of Surgical Research 1977;22:554-560.

334. Dembinski AB, Johnson LR. Stimulation of Pancreatic Growth by Secretin, Caerulein, and Pentagastrin*. Endocrinology 1980;106:323-328.

335. Chen D, Nylander AG, Norlen P, et al. Gastrin does not stimulate growth of the rat pancreas. Scand J Gastroenterol 1996;31:404-10.

336. Zhou W, Povoski SP, Bell RH. Characterization of cholecystokinin receptors and messenger RNA expression in rat pancreas: evidence for expression of cholecystokinin-A receptors but not cholecystokinin-B (gastrin) receptors. Journal of Surgical Research 1995;58:281-289.

337. Zhou W, Povoski SP, Longnecker DS, et al. Novel expression of gastrin (cholecystokinin-B) receptors in azaserine-induced rat pancreatic carcinoma: receptor determination and characterization. Cancer research 1992;52:6905-6911.

338. Todisco A, Takeuchi Y, Urumov A, et al. Molecular mechanisms for the growth factor action of gastrin. American Journal of Physiology-Gastrointestinal and Liver Physiology 1997;273:G891-G898.

339. Smith JP, Shih A, Wu Y, et al. Gastrin regulates growth of human pancreatic cancer in a tonic and autocrine fashion. The American journal of physiology 1996;270:R1078-R1084.

340. Smith JP, Fantaskey A, Liu G, et al. Identification of gastrin as a growth peptide in human pancreatic cancer. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 1995;268:R135-R141.

341. Goetze JP, Nielsen FC, Burcharth F, et al. Closing the gastrin loop in pancreatic carcinoma. Cancer 2000;88:2487-2494.

342. Caplin M, Savage K, Khan K, et al. Expression and processing of gastrin in pancreatic adenocarcinoma. British journal of surgery 2000;87:1035-1040.

343. Blackmore M, Hirst BH. Autocrine stimulation of growth of AR4-2J rat pancreatic tumour cells by gastrin. Br J Cancer 1992;66:32-8.

344. Raderer M, Wrba F, Kornek G, et al. Association between Helicobacter pylori infection and pancreatic cancer. Oncology 1998;55:16-19.

345. Stolzenberg-Solomon RZ, Blaser MJ, Limburg PJ, et al. Helicobacter pylori seropositivity as a risk factor for pancreatic cancer. Journal of the National Cancer Institute 2001;93:937-941.

346. Ferrand A, Wang TC. Gastrin and cancer: A review. Cancer Letters 2006;238:15-29.

347. Stepan VM, Sawada M, Todisco A, et al. Glycine-extended gastrin exerts growth-promoting effects on human colon cancer cells. Mol Med 1999;5:147-59.

348. Duckworth CA, Clyde D, Worthley DL, et al. Progastrin-induced secretion of insulin-like growth factor 2 from colonic myofibroblasts stimulates colonic epithelial proliferation in mice. Gastroenterology 2013;145:197-208. e3.

349. Howell GM, Ziober BL, Humphrey LE, et al. Regulation of autocrine gastrin expression by the TGF alpha autocrine loop. J Cell Physiol 1995;162:256-65.

350. Hoosein NM, Kiener PA, Curry RC, et al. Evidence for autocrine growth stimulation of cultured colon tumor cells by a gastrin/cholecystokinin-like peptide. Exp Cell Res 1990;186:15-21.

351. Singh P, Velasco M, Given R, et al. Progastrin expression predisposes mice to colon carcinomas and adenomas in response to a chemical carcinogen. Gastroenterology 2000;119:162-71.

352. Cobb S, Wood T, Ceci J, et al. Intestinal expression of mutant and wild-type progastrin significantly increases colon carcinogenesis in response to azoxymethane in transgenic mice. Cancer 2004;100:1311-23.

353. Ciccotosto GD, McLeish A, Hardy KJ, et al. Expression, processing, and secretion of gastrin in patients with colorectal carcinoma. Gastroenterology 1995;109:1142-53.

354. Siddheshwar R, Gray J, Kelly S. Plasma levels of progastrin but not amidated gastrin or glycine extended gastrin are elevated in patients with colorectal carcinoma. Gut 2001;48:47-52.

355. Pannequin J, Bonnans C, Delaunay N, et al. The wnt target jagged-1 mediates the activation of notch signaling by progastrin in human colorectal cancer cells. Cancer research 2009;69:6065-6073.

356. Umar S, Sarkar S, Wang Y, et al. Functional cross-talk between β-catenin and NFκB signaling pathways in colonic crypts of mice in response to progastrin. Journal of Biological Chemistry 2009;284:22274-22284.

357. Ferrand A, Sandrin MS, Shulkes A, et al. Expression of gastrin precursors by CD133-positive colorectal cancer cells is crucial for tumour growth. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 2009;1793:477-488.

358. Galiatsatos P, Foulkes WD. Familial adenomatous polyposis. The American journal of gastroenterology 2006;101:385-398.

359. Campbell W, Spence R, Parks T. Familial adenomatous polyposis. British journal of surgery 1994;81:1722-1733.

360. Watson SA, Smith AM. Hypergastrinemia promotes adenoma progression in the APCMin−/+ mouse model of familial adenomatous polyposis. Cancer research 2001;61:625-631.

361. Wang TC, Dockray GJ. Lessons from genetically engineered animal models. I. Physiological studies with gastrin in transgenic mice. Am J Physiol 1999;277:G6-11.

362. Orbuch M, Venzon DJ, Lubensky IA, et al. Prolonged hypergastrinemia does not increase the frequency of colonic neoplasia in patients with Zollinger-Ellison syndrome. Dig Dis Sci 1996;41:604-13.

363. Chinwalla AT, Cook LL, Delehaunty KD, et al. Initial sequencing and comparative analysis of the mouse genome. Nature 2002;420:520-562.

364. King M-C, Wilson A. Evolution at two levels in humans and chimpanzees. Essential Readings in Evolutionary Biology 2014;188:301.

365. Olson MV. When less is more: gene loss as an engine of evolutionary change. The American Journal of Human Genetics 1999;64:18-23.

366. Kawase S, Ishikura H. Female-predominant occurrence of spontaneous gastric adenocarcinoma in cotton rats. Laboratory animal science 1995;45:244-248.

367. Waldum HL, Rørvik H, Falkmer S, et al. Neuroendocrine (ECL cell) differentiation of spontaneous gastric carcinomas of cotton rats (Sigmodon hispidus). Comparative Medicine 1999;49:241-247.

368. Cui G, Qvigstad G, Falkmer S, et al. Spontaneous ECLomas in cotton rats (Sigmodon hispidus): tumours occurring in hypoacidic/hypergastrinaemic animals with normal parietal cells. Carcinogenesis 2000;21:23-27.

369. Kumazawa H, Takagi H, Sudo K, et al. Adenocarcinoma and carcinoid developing spontaneously in the stomach of mutant strains of Mastomys natalensis. Virchows Archiv A 1989;416:141-151.

370. Nilsson O, Wängberg B, Johansson L, et al. Praomys (Mastomys) natalensis: a model for gastric carcinoid formation. The Yale journal of biology and medicine 1992;65:741.

371. Saito T, Inokuchi K, Takayama S, et al. Sequential morphological changes in N-methyl-N'-nitro-N-nitrosoguanidine carcinogenesis in the glandular stomach of rats. Journal of the National Cancer Institute 1970;44:769-783.

372. Sugimura T, Fujimura S. Tumour production in glandular stomach of rat by N-methyl-N'-nitro-N-nitrosoguanidine. 1967.

373. Tatematsu M, Ogawa K, Hoshiya T, et al. Induction of Adenocarcinomas in the Glandular Stomach of BALB/c Mice Treated with N-Methyl-N-nitrosourea. Japanese journal of cancer research 1992;83:915-918.

374. Marshall B, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. The Lancet 1984;323:1311-1315.

375. IARC. Anonymous Live flukes and Helicobacter pylori. IARC Monogr Eval Carcinog Risks Hum 1994;61:1-241.

376. Karita M, Kouchiyama T, Okita K, et al. New small animal model for human gastric Helicobacter pylori infection: success in both nude and euthymic mice. The American journal of gastroenterology 1991;86:1596-1603.

377. Karita M, Li Q, Cantero D, et al. Establishment of a small animal model for human Helicobacter pylori infection using germ-free mouse. The American journal of gastroenterology 1994;89:208-213.

378. Marchetti M, Arico B, Burroni D, et al. Development of a mouse model of Helicobacter pylori infection that mimics human disease. Science 1995;267:1655-1658.

379. Blanchard TG, Czinn SJ, Maurer R, et al. Urease-specific monoclonal antibodies prevent Helicobacter felis infection in mice. Infection and immunity 1995;63:1394-1399.

380. Blanchard TG, Czinn SJ, Nedrud JG, et al. Helicobacter-associated gastritis in SCID mice. Infection and immunity 1995;63:1113-1115.

381. Chen M, Lee A, Hazell S, et al. Immunisation against gastric infection with Helicobacter species: first step in the prophylaxis of gastric cancer? Zentralblatt für Bakteriologie 1993;280:155-165.

382. Dick-Hegedus E, Lee A. Use of a mouse model to examine anti-Helicobacter pylori agents. Scandinavian journal of gastroenterology 1991;26:909-915.

383. Lee A, Hazell SL, O'Rourke J, et al. Isolation of a spiral-shaped bacterium from the cat stomach. Infection and immunity 1988;56:2843-2850.

384. Lee A, Fox JG, Otto G, et al. A small animal model of human Helicobacter pylori active chronic gastritis. Gastroenterology 1990;99:1315-23.

385. Sakagami T, Dixon M, O'Rourke J, et al. Atrophic gastric changes in both Helicobacter felis and Helicobacter pylori infected mice are host dependent and separate from antral gastritis. Gut 1996;39:639-48.

386. Cai X, Carlson J, Stoicov C, et al. Helicobacter felis eradication restores normal architecture and inhibits gastric cancer progression in C57BL/6 mice. Gastroenterology 2005;128:1937-52.

387. Lee A, O'Rourke J, De Ungria MC, et al. A standardized mouse model of Helicobacter pylori infection: introducing the Sydney strain. Gastroenterology 1997;112:1386-1397.

388. Fanning E, Knippers R. Structure and function of simian virus 40 large tumor antigen. Annual review of biochemistry 1992;61:55-85.

389. Tooze J. Molecular Biology of Tumor Viruses, Part 2, DNA Tumor Viruses, revised. Cold Spring Harbor Press, Cold Spring Harbor, NY 1981;7:632-641.

390. Li Q, Karam SM, Gordon JI. Simian virus 40 T antigen-induced amplification of pre-parietal cells in transgenic mice. Effects on other gastric epithelial cell lineages and evidence for a p53-independent apoptotic mechanism that operates in a committed progenitor. Journal of Biological Chemistry 1995;270:15777-15788.

391. Wang TC, Bonner-Weir S, Oates PS, et al. Pancreatic gastrin stimulates islet differentiation of transforming growth factor alpha-induced ductular precursor cells. J Clin Invest 1993;92:1349-56.

392. Lee C-W, Rickman B, Rogers AB, et al. Helicobacter pylori Eradication Prevents Progression of Gastric Cancer in Hypergastrinemic INS-GAS Mice. Cancer Research 2008;68:3540-3548.

393. Koh TJ, Goldenring JR, Ito S, et al. Gastrin deficiency results in altered gastric differentiation and decreased colonic proliferation in mice. Gastroenterology 1997;113:1015-1025.

394. Zavros Y, Eaton KA, Kang W, et al. Chronic gastritis in the hypochlorhydric gastrin-deficient mouse progresses to adenocarcinoma. Oncogene 2005;24:2354-2366.

395. Singh P, Velasco M, Given R, et al. Mice overexpressing progastrin are predisposed for developing aberrant colonic crypt foci in response to AOM. Am J Physiol Gastrointest Liver Physiol 2000;278:G390-9.

396. Wang TC, Koh TJ, Varro A, et al. Processing and proliferative effects of human progastrin in transgenic mice. Journal of Clinical Investigation 1996;98:1918.

397. Ferrand A, Bertrand C, Portolan G, et al. Signaling Pathways Associated with Colonic Mucosa Hyperproliferation in Mice Overexpressing Gastrin Precursors. Cancer Research 2005;65:2770-2777.

398. Summy JM, Gallick GE. Src family kinases in tumor progression and metastasis. Cancer and metastasis reviews 2003;22:337-358.

399. Langhans N, Rindi G, Chiu M, et al. Abnormal gastric histology and decreased acid production in cholecystokinin-B/gastrin receptor-deficient mice. Gastroenterology 1997;112:280-6.

400. Rindi G, Langhans N, Rehfeld JF, et al. Abnormal gastric morphology and function in CCK-B/gastrin receptor-deficient mice. Yale J Biol Med 1998;71:347-54.

401. Chen D, Zhao CM, Al-Haider W, et al. Differentiation of gastric ECL cells is altered in CCK(2) receptor-deficient mice. Gastroenterology 2002;123:577-85.

402. Rozengurt E, Walsh JH. Gastrin, CCK, signaling, and cancer. Annual review of physiology 2001;63:49-76.

403. Tang LH, Luque EA, Efstathiou JA, et al. Gastrin receptor expression and function during rapid transformation of the enterochromaffin-like cells in an African rodent. Regulatory peptides 1997;72:9-18.

404. Corazza GR, D'Ambro A, Grimoldi D. Double-blind study of proglumide, zolimidine and carbenoxolone in peptic ulcer and gastroduodenitis. Minerva Dietol Gastroenterol 1979;25:47-58.

405. Hahne W, Jensen R, Lemp G, et al. Proglumide and benzotript: members of a different class of cholecystokinin receptor antagonists. Proceedings of the National Academy of Sciences 1981;78:6304-6308.

406. Seva C, Scemama JL, Bastié MJ, et al. Lorglumide and loxiglumide inhibit gastrin-stimulated DNA synthesis in a rat tumoral acinar pancreatic cell line (AR42J). Cancer research 1990;50:5829-5833.

407. Revel L, Ferrari F, Makovec F, et al. Characterization of antigastrin activity in vivo of CR 2194, a new R-4-benzamido-5-oxo-pentanoic acid derivative. European journal of pharmacology 1992;216:217-224.

408. Beltinger J, Hildebrand P, Drewe J, et al. Effects of spiroglumide, a gastrin receptor antagonist, on acid secretion in humans. European journal of clinical investigation 1999;29:153-159.

409. Makovec F, Peris W, Frigerio S, et al. Structure-antigastrin activity relationships of new spiroglumide amido acid derivatives. Journal of medicinal chemistry 1996;39:135-142.

410. Rovati L, D'AMATO M, Peris W, et al. Selectivity and Potency of New Basic CCK-B Antagonists. Annals of the New York Academy of Sciences 1994;713:395-397.

411. Makovec F, D'Amato M. CCKB/gastrin receptor antagonists as potential drugs for peptic ulcer therapy. Drug discovery today 1997;2:283-293.

412. Piccolomini R, Di Bonaventura G, Picciani C, et al. In vitro activity of clarithromycin against intracellular Helicobacter pylori. Antimicrobial agents and chemotherapy 2001;45:1568-1571.

413. Horwell DC, Hughes J, Hunter JC, et al. Rationally designed "dipeptoid" analogues of CCK. alpha-Methyltryptophan derivatives as highly selective and orally active

gastrin and CCK-B antagonists with potent anxiolytic properties. J Med Chem 1991;34:404-14.

414. Hughes J, Boden P, Costall B, et al. Development of a class of selective cholecystokinin type B receptor antagonists having potent anxiolytic activity. Proc Natl Acad Sci U S A 1990;87:6728-32.

415. Hayward NJ, Harding M, Lloyd SA, et al. The effect of CCKB/gastrin antagonists on stimulated gastric acid secretion in the anaesthetized rat. Br J Pharmacol 1991;104:973-7.

416. Dethloff LA, Barr BM, Bestervelt LL. Inhibition of gastrin-stimulated cell proliferation by the CCK-B/gastrin receptor ligand CI-988. Food and Chemical Toxicology 1999;37:105-110.

417. Augelli-Szafran CE, Horwell DC, Kneen C, et al. Cholecystokinin B antagonists. Synthesis and quantitative structure-activity relationships of a series of C-terminal analogues of CI-988. Bioorg Med Chem 1996;4:1733-45.

418. Schmassmann A, Garner A, Flogerzi B, et al. Cholecystokinin type B receptor antagonist PD-136,450 is a partial secretory agonist in the stomach and a full agonist in the pancreas of the rat. Gut 1994;35:270-4.

419. Howbert J, Lobb K, Brown R, et al. A novel series of non-peptide CCK and gastrin antagonists: medicinal chemistry and electrophysiological demonstration of antagonism. Multiple Cholecystokinin Receptors: Progress Toward CNS Therapeutic Targets 1992:28-37.

420. Howbert JJ, Lobb KL, Britton TC, et al. Diphenylpyrazolidinone and benzodiazepine cholecystokinin antagonists: A case of convergent evolution in medicinal chemistry. Bioorganic & Medicinal Chemistry Letters 1993;3:875-880.

421. Ding X-Q, Håkanson R. Evaluation of the Specificity and Potency of a Series of Cholecystokinin-B/Gastrin Receptor Antagonists in vivo. Pharmacology & Toxicology 1996;79:124-130.

422. Lotti VJ, Chang RS. A new potent and selective non-peptide gastrin antagonist and brain cholecystokinin receptor (CCK-B) ligand: L-365,260. European journal of pharmacology 1989;162:273-280.

423. Bock MG, DiPardo RM, Evans BE, et al. Benzodiazepine gastrin and brain cholecystokinin receptor ligands; L-365,260. Journal of medicinal chemistry 1989;32:13-16.

424. Lindström E, Björkqvist M, Håkanson R. Pharmacological analysis of CCK2 receptor antagonists using isolated rat stomach ECL cells. British journal of pharmacology 1999;127:530-536.

425. Patel S, Smith AJ, Chapman KL, et al. Biological properties of the benzodiazepine amidine derivative L-740,093, a cholecystokinin-B/gastrin receptor antagonist with high affinity in vitro and high potency in vivo. Molecular pharmacology 1994;46:943-948.

426. Nishida A, Miyata K, Tsutsumi R, et al. Pharmacological profile of (R)-1-[2,3-dihydro-1-(2'-methylphenacyl)-2-oxo- 5-phenyl-1H-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea (YM022), a new potent and selective gastrin/cholecystokinin-B receptor antagonist, in vitro and in vivo. Journal of Pharmacology and Experimental Therapeutics 1994;269:725-731.

427. Yuki H, Nishida A, Miyake A, et al. YM022, a potent and selective gastrin/CCK-B receptor antagonist, inhibits peptone meal-induced gastric acid secretion in Heidenhain pouch dogs. Digestive diseases and sciences 1997;42:707-714.

428. Attoub S, Moizo L, Laigneau J, et al. YM022, a highly potent and selective CCKB antagonist inhibiting gastric acid secretion in the rat, the cat and isolated rabbit glands. Fundamental & clinical pharmacology 1998;12:256-262.

429. Takinami Y, Yuki H, Nishida A, et al. YF476 is a new potent and selective gastrin/cholecystokinin-B receptor antagonist in vitro and in vivo. Alimentary pharmacology & therapeutics 1997;11:113-120.

430. Miura N, Yoneta T, Ukawa H, et al. Pharmacological profiles of Z-360, a novel CCK B/gastrin (CCK 2) receptor antagonist with excellent oral potency. Gastroenterology 2001;120:A311.

431. Grabowska AM, Morris TM, McKenzie AJ, et al. Pre-clinical evaluation of a new orally-active CCK-2R antagonist, Z-360, in gastrointestinal cancer models. Regulatory Peptides 2008;146:46-57.

432. Morita H, Miura N, Hori Y, et al. Effects of Z-360, a novel CCK B/gastrin (CCK 2) receptor antagonist, on meal-induced acid secretion and experimental ulcer models in dogs and rats. Gastroenterology 2001;120:A311.

433. Allison BD, Phuong VK, McAtee LC, et al. Identification and optimization of anthranilic sulfonamides as novel, selective cholecystokinin-2 receptor antagonists. J Med Chem 2006;49:6371-90.

434. Morton MF, Barrett TD, Freedman J, et al. JNJ-26070109 [(R)4-Bromo-N-[1-(2,4-difluoro-phenyl)-ethyl]-2-(quinoxaline-5-sulfonylamino)-benzamide]: A Novel, Potent, and Selective Cholecystokinin 2 Receptor Antagonist with Good Oral Bioavailability. Journal of Pharmacology and Experimental Therapeutics 2011;338:328-336.

435. Rosen MD, Hack MD, Allison BD, et al. Discovery of potent cholecystokinin-2 receptor antagonists: elucidation of key pharmacophore elements by X-ray crystallographic and NMR conformational analysis. Bioorg Med Chem 2008;16:3917-25.

436. Watson S, Durrant L, Elston P, et al. Inhibitory Effects of the Gastrin Receptor Antagonist (L-365,260) on Gastrointestinal Tumor Cells. Cancer 1991;68:1255-1260.

437. Sethi T, Herget T, Wu SV, et al. CCKA and CCKB receptors are expressed in small cell lung cancer lines and mediate Ca2+ mobilization and clonal growth. Cancer research 1993;53:5208-5213.

438. Sethi T, Rozengurt E. Gastrin stimulates Ca2+ mobilization and clonal growth in small cell lung cancer cells. Cancer research 1992;52:6031-6035.

439. Upp JR, Singh P, Townsend CM, et al. Clinical significance of gastrin receptors in human colon cancers. Cancer research 1989;49:488-492.

440. Lignon M-F, Bernad N, Martinez J. Pharmacological characterization of type B cholecystokinin binding sites on the human JURKAT T lymphocyte cell line. Molecular pharmacology 1991;39:615-620.

441. Smith JP, Kramer ST, Demers LM. Effects of gastrin and difluoromethylornithine on growth of human colon cancer. Digestive diseases and sciences 1993;38:520-528.

442. Ishizuka J, Martinez J, Townsend Jr CM, et al. The effect of gastrin on growth of human stomach cancer cells. Annals of surgery 1992;215:528.

443. Murayama T, Matsumori Y, Iwata N, et al. Antiproliferative Effect of a Novel Cholecystokinin-B/Gastrin Receptor Antagonist, YM022. Cancer Science 1996;87:743-750.

444. Akira M, Shinobu M, Hiroyuki K. Characterization of cloned human cholecystokinin-B receptor as a gastrin receptor. Biochemical pharmacology 1994;47:1339-1343.

445. Saita Y, Yazawa H, Honma Y, et al. Characterization of YM022: its CCK B/gastrin receptor binding profile and antagonism to CCK-8-induced Ca 2+ mobilization. European Journal of Pharmacology: Molecular Pharmacology 1994;269:249-254.

446. Kidd M, Modlin I, Black J, et al. A comparison of the effects of gastrin, somatostatin and dopamine receptor ligands on rat gastric enterochromaffin-like cell secretion and proliferation. Regulatory peptides 2007;143:109-117.

447. Harris JC, Clarke PA, Awan A, et al. An Antiapoptotic Role for Gastrin and the Gastrin/CCK-2 Receptor in Barrett's Esophagus. Cancer Research 2004;64:1915-1919.

448. Lipkin M, Deschner E. Early proliferative changes in intestinal cells. Cancer research 1976;36:2665-2668.

449. Jin G, Ramanathan V, Quante M, et al. Inactivating cholecystokinin-2 receptor inhibits progastrin-dependent colonic crypt fission, proliferation, and colorectal cancer in mice. The Journal of clinical investigation 2009;119:2691.

450. Lee C-W, Rickman B, Rogers AB, et al. Combination of Sulindac and Antimicrobial Eradication of Helicobacter pylori Prevents Progression of Gastric Cancer in Hypergastrinemic INS-GAS Mice. Cancer Research 2009;69:8166-8174.

451. Takaishi S, Cui G, Frederick DM, et al. Synergistic Inhibitory Effects of Gastrin and Histamine Receptor Antagonists on Helicobacter-Induced Gastric Cancer. Gastroenterology 2005;128:1965-1983.

452. Sørdal Ø, Waldum H, Nordrum IS, et al. The Gastrin Receptor Antagonist Netazepide (YF476) Prevents Oxyntic Mucosal Inflammation Induced by Helicobacter Pylori Infection in Mongolian Gerbils. Helicobacter 2013;18:397-405.

453. Martinsen TC, Kawase S, Håkanson R, et al. Spontaneous ECL cell carcinomas in cotton rats: natural course and prevention by a gastrin receptor antagonist. Carcinogenesis 2003;24:1887-1896.

454. Kidd M, Siddique Z, Drozdov I, et al. The CCK2 receptor antagonist, YF476, inhibits Mastomys ECL cell hyperplasia and gastric carcinoid tumor development. Regulatory peptides 2010;162:52-60.

455. Barrett TD, Lagaud G, Wagaman P, et al. The cholecystokinin CCK2 receptor antagonist, JNJ-26070109, inhibits gastric acid secretion and prevents omeprazole-induced acid rebound in the rat. British Journal of Pharmacology 2012;166:1684-1693.

456. Boyce M, Warrington S, Black J. Netazepide, a gastrin/CCK2 receptor antagonist, causes dose-dependent, persistent inhibition of the responses to pentagastrin in healthy subjects. Br J Clin Pharmacol 2013;76:689-98.

457. Boyce M, David O, Darwin K, et al. Single oral doses of netazepide (YF476), a gastrin receptor antagonist, cause dose-dependent, sustained increases in gastric pH compared with placebo and ranitidine in healthy subjects. Aliment Pharmacol Ther 2012;36:181-9.

458. Boyce M, Warrington S. Effect of repeated doses of netazepide, a gastrin receptor antagonist, omeprazole and placebo on 24 h gastric acidity and gastrin in healthy subjects. Br J Clin Pharmacol 2013;76:680-8.

459. Moore AR, Boyce M, Steele IA, et al. Netazepide, a Gastrin Receptor Antagonist, Normalises Tumour Biomarkers and Causes Regression of Type 1 Gastric Neuroendocrine Tumours in a Nonrandomised Trial of Patients with Chronic Atrophic Gastritis. PLoS ONE 2013;8:e76462.

460. Fossmark R, Sørdal O, Jianu CS, et al. Treatment of gastric carcinoids type 1 with the gastrin receptor antagonist netazepide (YF476) results in regression of tumours and normalisation of serum chromogranin A. Alimentary pharmacology & therapeutics 2012;36:1067-1075.

461. Meyer T, Caplin ME, Palmer DH, et al. A phase Ib/IIa trial to evaluate the CCK2 receptor antagonist Z-360 in combination with gemcitabine in patients with advanced pancreatic cancer. European Journal of Cancer 2010;46:526-533.

462. Jensen RT. Involvement of cholecystokinin/gastrin-related peptides and their receptors in clinical gastrointestinal disorders. Pharmacol Toxicol 2002;91:333-50.

463. Burkitt MD, Varro A, Pritchard DM. Importance of gastrin in the pathogenesis and treatment of gastric tumors: World J Gastroenterol. 2009 Jan 7;15(1):1-16., 2009.

464. Lawrence B, Kidd M, Svejda B, et al. A Clinical Perspective on Gastric Neuroendocrine Neoplasia. Current Gastroenterology Reports 2011;13:101-109.

465. O'Toole D, Delle Fave G, Jensen RT. Gastric and duodenal neuroendocrine tumours. Best Practice & Research Clinical Gastroenterology 2012;26:719-735.

466. Ruszniewski P, Delle Fave G, Cadiot G, et al. Well-differentiated gastric tumors/carcinomas. Neuroendocrinology 2006;84:158-64.

467. Soll AH, Walsh JH. Regulation of gastric acid secretion. Annu Rev Physiol 1979;41:35-53.

468. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993;75:843-854.

469. Rodriguez A, Griffiths-Jones S, Ashurst JL, et al. Identification of mammalian microRNA host genes and transcription units. Genome Research 2004;14:1902-1910.

470. Lewis BP, Burge CB, Bartel DP. Conserved Seed Pairing, Often Flanked by Adenosines, Indicates that Thousands of Human Genes are MicroRNA Targets. Cell 2005;120:15-20.

471. Lee Y, Kim M, Han J, et al. MicroRNA genes are transcribed by RNA polymerase II. EMBO J 2004;23:4051-4060.

472. Borchert GM, Lanier W, Davidson BL. RNA polymerase III transcribes human microRNAs. Nature Structural and Molecular Biology 2006;13:1097-1101.

473. Krol J, Sobczak K, Wilczynska U, et al. Structural Features of MicroRNA (miRNA) Precursors and Their Relevance to miRNA Biogenesis and Small Interfering RNA/Short Hairpin RNA Design. Journal of Biological Chemistry 2004;279:42230-42239.

474. Yi R, Qin Y, Macara IG, et al. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes and Development 2003;17:3011-3016.

475. Chendrimada TP, Gregory RI, Kumaraswamy E, et al. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 2005;436:740-744.

476. Berezikov E, Guryev V, van de Belt J, et al. Phylogenetic shadowing and computational identification of human microRNA genes. cell 2005;120:21-24.

477. Fromm B, Billipp T, Peck LE, et al. A Uniform System for the Annotation of Vertebrate microRNA Genes and the Evolution of the Human microRNAome. Annu Rev Genet 2015.

478. Olsen PH, Ambros V. The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol 1999;216:671-80.

479. Seggerson K, Tang L, Moss EG. Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation. Dev Biol 2002;243:215-25.

480. Pillai RS, Bhattacharyya SN, Artus CG, et al. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 2005;309:1573-6.

481. Carroll AP, Goodall GJ, Liu B. Understanding principles of miRNA target recognition and function through integrated biological and bioinformatics approaches. Wiley Interdiscip Rev RNA 2014;5:361-79.

482. Wang X. Composition of seed sequence is a major determinant of microRNA targeting patterns. Bioinformatics 2014;30:1377-83.

483. Ohler U, Yekta S, Lim LP, et al. Patterns of flanking sequence conservation and a characteristic upstream motif for microRNA gene identification. RNA 2004;10:1309-22.

484. Majoros WH, Ohler U. Spatial preferences of microRNA targets in 3' untranslated regions. BMC Genomics 2007;8:152.

485. Llave C, Xie Z, Kasschau KD, et al. Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science 2002;297:2053-2056.

486. Yekta S, Shih I-h, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. Science 2004;304:594-596.

487. Karginov FV, Cheloufi S, Chong MMW, et al. Diverse Endonucleolytic Cleavage Sites in the Mammalian Transcriptome Depend upon MicroRNAs, Drosha, and Additional Nucleases. Molecular Cell 2010;38:781-788.

488. Liu J, Carmell MA, Rivas FV, et al. Argonaute2 is the catalytic engine of mammalian RNAi. Science 2004;305:1437-1441.

489. Meister G, Landthaler M, Patkaniowska A, et al. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Molecular cell 2004;15:185-197.

490. Mallory AC, Reinhart BJ, Jones-Rhoades MW, et al. MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5′ region. The EMBO journal 2004;23:3356-3364.

491. Guo H-S, Xie Q, Fei J-F, et al. MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for Arabidopsis lateral root development. The Plant Cell 2005;17:1376-1386.

492. Parker R, Song H. The enzymes and control of eukaryotic mRNA turnover. Nature structural & molecular biology 2004;11:121-127.

493. Orban TI, Izaurralde E. Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. RNA 2005;11:459-469.

494. Jing Q, Huang S, Guth S, et al. Involvement of microRNA in AU-rich element-mediated mRNA instability. Cell 2005;120:623-634.

495. Espel E. The role of the AU-rich elements of mRNAs in controlling translation, In Seminars in cell & developmental biology, Elsevier, 2005.

496. Vasudevan S, Steitz JA. AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. Cell 2007;128:1105-18.

497. Lin CC, Liu LZ, Addison JB, et al. A KLF4-miRNA-206 autoregulatory feedback loop can promote or inhibit protein translation depending upon cell context. Mol Cell Biol 2011;31:2513-27.

498. Truesdell SS, Mortensen RD, Seo M, et al. MicroRNA-mediated mRNA translation activation in quiescent cells and oocytes involves recruitment of a nuclear microRNP. Sci Rep 2012;2:842.

499. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. Science 2007;318:1931-4.

500. Vasudevan S, Tong Y, Steitz JA. Cell-cycle control of microRNA-mediated translation regulation. Cell Cycle 2008;7:1545-9.

501. Bhattacharyya SN, Habermacher R, Martine U, et al. Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell 2006;125:1111-24.

502. Bhattacharyya SN, Habermacher R, Martine U, et al. Stress-induced reversal of microRNA repression and mRNA P-body localization in human cells. Cold Spring Harb Symp Quant Biol 2006;71:513-21.

503. Ivan M, Harris AL, Martelli F, et al. Hypoxia response and microRNAs: no longer two separate worlds. J Cell Mol Med 2008;12:1426-31.

504. Wang B, Sun YF, Song N, et al. MicroRNAs involving in cold, wounding and salt stresses in Triticum aestivum L. Plant Physiol Biochem 2014;80:90-6.

505. Hatzoglou M, Fernandez J, Yaman I, et al. Regulation of cationic amino acid transport: the story of the CAT-1 transporter. Annu Rev Nutr 2004;24:377-99.

506. Yoon JH, Abdelmohsen K, Srikantan S, et al. LincRNA-p21 suppresses target mRNA translation. Mol Cell 2012;47:648-55.

507. Gibb EA, Vucic EA, Enfield KS, et al. Human cancer long non-coding RNA transcriptomes. PLoS One 2011;6:e25915.

508. Poliseno L, Salmena L, Zhang J, et al. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature 2010;465:1033-1038.

509. Lee Y, Jeon K, Lee JT, et al. MicroRNA maturation: stepwise processing and subcellular localization. Embo j 2002;21:4663-70.

510. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nature reviews Molecular cell biology 2009;10:126-139.

511. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nature Reviews Genetics 2010;11:597.

512. Davis-Dusenbery BN, Hata A. Mechanisms of control of microRNA biogenesis. Journal of biochemistry 2010;148:381-392.

513. Yeom K-H, Lee Y, Han J, et al. Characterization of DGCR8/Pasha, the essential cofactor for Drosha in primary miRNA processing. Nucleic acids research 2006;34:4622-4629.

514. Han J, Pedersen JS, Kwon SC, et al. Posttranscriptional crossregulation between Drosha and DGCR8. Cell 2009;136:75-84.

515. Herbert KM, Pimienta G, DeGregorio SJ, et al. Phosphorylation of DGCR8 increases its intracellular stability and induces a progrowth miRNA profile. Cell reports 2013;5:1070-1081.

516. Tang X, Li M, Tucker L, et al. Glycogen synthase kinase 3 beta (GSK3beta) phosphorylates the RNAase III enzyme drosha at S300 and S302. PloS one 2011;6:e20391.

517. Wada T, Kikuchi J, Furukawa Y. Histone deacetylase 1 enhances microRNA processing via deacetylation of DGCR8. EMBO reports 2012;13:142-149.

518. Davis BN, Hilyard AC, Lagna G, et al. SMAD proteins control DROSHA-mediated microRNA maturation. nature 2008;454:56-61.

519. Suzuki HI, Yamagata K, Sugimoto K, et al. Modulation of microRNA processing by p53. Nature 2009;460:529-533.

520. Fukuda T, Yamagata K, Fujiyama S, et al. DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. Nature cell biology 2007;9:604-611.

521. Iwasaki YW, Kiga K, Kayo H, et al. Global microRNA elevation by inducible Exportin 5 regulates cell cycle entry. Rna 2013;19:490-497.

522. Wan G, Zhang X, Langley RR, et al. DNA-damage-induced nuclear export of precursor microRNAs is regulated by the ATM-AKT pathway. Cell reports 2013;3:2100-2112.

523. Melo SA, Moutinho C, Ropero S, et al. A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. Cancer cell 2010;18:303-315.

524. Bernstein E, Caudy AA, Hammond SM, et al. Role for a bidentate ribonuclease in the initiation step of RNA interference. nature 2001;409:363-366.

525. Paroo Z, Ye X, Chen S, et al. Phosphorylation of the human microRNA-generating complex mediates MAPK/Erk signaling. Cell 2009;139:112-122.

526. Melo SA, Ropero S, Moutinho C, et al. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. Nature genetics 2009;41:365-370.

527. Lee Y, Hur I, Park SY, et al. The role of PACT in the RNA silencing pathway. The EMBO journal 2006;25:522-532.

528. Auyeung VC, Ulitsky I, McGeary SE, et al. Beyond secondary structure: primary-sequence determinants license pri-miRNA hairpins for processing. Cell 2013;152:844-858.

529. Thomson JM, Newman M, Parker JS, et al. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. Genes & development 2006;20:2202-2207.

530. Ameres SL, Zamore PD. Diversifying microRNA sequence and function. Nature reviews Molecular cell biology 2013;14:475-488.

531. Heo I, Joo C, Kim Y-K, et al. TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. Cell 2009;138:696-708.

532. Katoh T, Sakaguchi Y, Miyauchi K, et al. Selective stabilization of mammalian microRNAs by 3′ adenylation mediated by the cytoplasmic poly (A) polymerase GLD-2. Genes & development 2009;23:433-438.

533. Yang W, Chendrimada TP, Wang Q, et al. Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nature structural & molecular biology 2006;13:13-21.

534. Xhemalce B, Robson SC, Kouzarides T. Human RNA methyltransferase BCDIN3D regulates microRNA processing. Cell 2012;151:278-288.

535. Das SK, Sokhi UK, Bhutia SK, et al. Human polynucleotide phosphorylase selectively and preferentially degrades microRNA-221 in human melanoma cells. Proceedings of the National Academy of Sciences 2010;107:11948-11953.

536. Rüegger S, Grosshans H. MicroRNA turnover: when, how, and why. Trends in biochemical sciences 2012;37:436-446.

537. Libri V, Helwak A, Miesen P, et al. Murine cytomegalovirus encodes a miR-27 inhibitor disguised as a target. Proceedings of the National Academy of Sciences 2012;109:279-284.

538. Chatterjee S, Fasler M, Büssing I, et al. Target-mediated protection of endogenous microRNAs in C. elegans. Developmental cell 2011;20:388-396.

539. de Moor CH, Meijer H, Lissenden S. Mechanisms of translational control by the 3′ UTR in development and differentiation, In Seminars in cell & developmental biology, Elsevier, 2005.

540. Lai EC. Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. Nature Genetics 2002;30.

541. Lukiw WJ. Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus. Neuroreport 2007;18:297-300.

542. Iorio MV, Ferracin M, Liu C-G, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer research 2005;65:7065-7070.

543. Yang L, Belaguli N, Berger DH. MicroRNA and colorectal cancer. World journal of surgery 2009;33:638-646.

544. Liao Y-L, Tsai K-W, Lin W-c. miRNAs in gastric cancer. Gastric Carcinoma—Molecular Aspects and Current Advances. ISBN 2011:978-953.

545. Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 2009;10:704-714.

546. Zhang B, Pan X, Cobb GP, et al. microRNAs as oncogenes and tumor suppressors. Developmental biology 2007;302:1-12.

547. Calin GA, Sevignani C, Dumitru CD, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A 2004;101:2999-3004.

548. Tanzer A, Stadler PF. Molecular evolution of a microRNA cluster. Journal of molecular biology 2004;339:327-335.

549. Eis PS, Tam W, Sun L, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc Natl Acad Sci U S A 2005;102:3627-32.

550. Costinean S, Zanesi N, Pekarsky Y, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proc Natl Acad Sci U S A 2006;103:7024-9.

551. Ota A, Tagawa H, Karnan S, et al. Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. Cancer Res 2004;64:3087-95.

552. Mendell JT. miRiad roles for the miR-17-92 cluster in development and disease. Cell 2008;133:217-22.

553. Tsuchida A, Ohno S, Wu W, et al. miR-92 is a key oncogenic component of the miR-17–92 cluster in colon cancer. Cancer science 2011;102:2264-2271.

554. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 2006;103:2257-61.

555. Petrocca F, Visone R, Onelli MR, et al. E2F1-regulated microRNAs impair TGFβ-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer cell 2008;13:272-286.

556. Hossain A, Kuo MT, Saunders GF. Mir-17-5p regulates breast cancer cell proliferation by inhibiting translation of AIB1 mRNA. Molecular and cellular biology 2006;26:8191-8201.

557. Hayashita Y, Osada H, Tatematsu Y, et al. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer research 2005;65:9628-9632.

558. O'Donnell KA, Wentzel EA, Zeller KI, et al. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 2005;435:839-43.

559. Olive V, Bennett MJ, Walker JC, et al. miR-19 is a key oncogenic component of mir-17-92. Genes & Development 2009;23:2839-2849.

560. Matsumura I, Tanaka H, Kanakura Y. E2F1 and c-Myc in cell growth and death. Cell Cycle 2003;2:333-8.

561. Lewis BP, Shih IH, Jones-Rhoades MW, et al. Prediction of mammalian microRNA targets. Cell 2003;115:787-98.

562. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proceedings of the National Academy of Sciences of the United States of America 2005;102:13944-13949.

563. Sánchez-Beato M, Sánchez-Aguilera A, Piris MA. Cell cycle deregulation in B-cell lymphomas. Blood 2003;101:1220-1235.

564. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proceedings of the National Academy of Sciences 2002;99:15524-15529.

565. Pasquinelli AE, Reinhart BJ, Slack F, et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 2000;408:86-89.

566. Reinhart BJ, Slack FJ, Basson M, et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. nature 2000;403:901-906.

567. Johnson SM, Grosshans H, Shingara J, et al. RAS Is Regulated by the let-7 MicroRNA Family. Cell 2005;120:635-647.

568. Dhillon A, Hagan S, Rath O, et al. MAP kinase signalling pathways in cancer. Oncogene 2007;26:3279-3290.

569. Takamizawa J, Konishi H, Yanagisawa K, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer research 2004;64:3753-3756.

570. Yan LX, Huang XF, Shao Q, et al. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. Rna 2008;14:2348-60.

571. Lawrie CH, Chi J, Taylor S, et al. Expression of microRNAs in diffuse large B cell lymphoma is associated with immunophenotype, survival and transformation from follicular lymphoma. Journal of cellular and molecular medicine 2009;13:1248-1260.

572. Nishizawa T, Suzuki H. The role of microRNA in gastric malignancy. Int J Mol Sci 2013;14:9487-96.

573. Zhou X, Xia Y, Li L, et al. MiR-101 inhibits cell growth and tumorigenesis of Helicobacter pylori related gastric cancer by repression of SOCS2. Cancer biology & therapy 2015;16:160-169.

574. Varambally S, Cao Q, Mani R-S, et al. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. science 2008;322:1695-1699.

575. Su H, Yang J-R, Xu T, et al. MicroRNA-101, down-regulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. Cancer research 2009;69:1135-1142.

576. Li S, Fu H, Wang Y, et al. MicroRNA-101 regulates expression of the v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS) oncogene in human hepatocellular carcinoma. Hepatology 2009;49:1194-1202.

577. Friedman JM, Liang G, Liu C-C, et al. The putative tumor suppressor microRNA-101 modulates the cancer epigenome by repressing the polycomb group protein EZH2. Cancer research 2009;69:2623-2629.

578. Strillacci A, Griffoni C, Sansone P, et al. MiR-101 downregulation is involved in cyclooxygenase-2 overexpression in human colon cancer cells. Experimental cell research 2009;315:1439-1447.

579. Niwa T, Tsukamoto T, Toyoda T, et al. Inflammatory Processes Triggered by Helicobacter pylori Infection Cause Aberrant DNA Methylation in Gastric Epithelial Cells. Cancer Research 2010;70:1430-1440.

580. Ding S-Z, Goldberg JB, Hatakeyama M. Helicobacter pylori infection, oncogenic pathways and epigenetic mechanisms in gastric carcinogenesis. Future Oncology 2010;6:851-862.

581. Hayashi Y, Tsujii M, Jun W, et al. CagA mediates epigenetic regulation to attenuate let-7 expression in Helicobacter pylori-related carcinogenesis. Gut 2012.

582. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature 2006;441:424-30.

583. Zhang YM, Noto JM, Hammond CE, et al. Helicobacter pylori-induced posttranscriptional regulation of H-K-ATPase alpha-subunit gene expression by miRNA. Am J Physiol Gastrointest Liver Physiol 2014;306:G606-13.

584. Zhu Y, Jiang Q, Lou X, et al. MicroRNAs up-regulated by CagA of Helicobacter pylori induce intestinal metaplasia of gastric epithelial cells. PloS one 2012;7:e35147.

585. Shiotani A, Uedo N, Iishi H, et al. H. pylori eradication did not improve dysregulation of specific oncogenic miRNAs in intestinal metaplastic glands. Journal of gastroenterology 2012;47:988-998.

586. Zhang Z, Li Z, Gao C, et al. miR-21 plays a pivotal role in gastric cancer pathogenesis and progression. Lab Invest 2008;88:1358-66.

587. Xi Y, Nakajima G, Gavin E, et al. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. RNA 2007;13:1668-74.

588. Leite KR, Canavez JM, Reis ST, et al. miRNA analysis of prostate cancer by quantitative real time PCR: comparison between formalin-fixed paraffin embedded and fresh-frozen tissue, In Urologic Oncology: Seminars and Original Investigations, Elsevier, 2011.

589. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell research 2008;18:997-1006.

590. Ferracin M, Pedriali M, Veronese A, et al. MicroRNA profiling for the identification of cancers with unknown primary tissue-of-origin. J Pathol 2011;225:43-53.

591. du Rieu MC, Torrisani J, Selves J, et al. MicroRNA-21 is induced early in pancreatic ductal adenocarcinoma precursor lesions. Clin Chem 2010;56:603-12.

592. Calin GA, Ferracin M, Cimmino A, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. New England Journal of Medicine 2005;353:1793-1801.

593. Chiorazzi N, Rai KR, Ferrarini M. Chronic Lymphocytic Leukemia. New England Journal of Medicine 2005;352:804-815.

594. Markou A, Liang Y, Lianidou E. Prognostic, therapeutic and diagnostic potential of microRNAs in non-small cell lung cancer. Clinical Chemistry and Laboratory Medicine 2011;49:1591-1603.

595. Li X, Zhang Y, Zhang Y, et al. Survival prediction of gastric cancer by a seven-microRNA signature. Gut 2010;59:579-585.

596. Rothe F, Ignatiadis M, Chaboteaux C, et al. Global microRNA expression profiling identifies MiR-210 associated with tumor proliferation, invasion and poor clinical outcome in breast cancer. 2011.

597. Zhao JJ, Lin J, Yang H, et al. MicroRNA-221/222 negatively regulates estrogen receptor alpha and is associated with tamoxifen resistance in breast cancer. J Biol Chem 2008;283:31079-86.

598. Lei L, Huang Y, Gong W. miR-205 promotes the growth, metastasis and chemoresistance of NSCLC cells by targeting PTEN. Oncology reports 2013;30:2897-2902.

599. Xu M, Jin H, Xu C-X, et al. MiR-34c inhibits osteosarcoma metastasis and chemoresistance. Medical Oncology 2014;31:1-7.

600. Poutahidis T, Tsangaris T, Kanakoudis G, et al. Helicobacter pylori-induced gastritis in experimentally infected conventional piglets. Vet Pathol 2001;38:667-78.

601. Momtaz H, Dabiri H, Souod N, et al. Study of Helicobacter pylori genotype status in cows, sheep, goats and human beings. BMC Gastroenterol 2014;14:61.

602. Dore MP, Bilotta M, Vaira D, et al. High prevalence of Helicobacter pylori infection in shepherds. Digestive diseases and sciences 1999;44:1161-1164.

603. Al-Sulami A, Al-Edani T, Al-Abdula A. Culture method and PCR for the detection of Helicobacter pylori in drinking water in Basrah Governorate Iraq. Gastroenterology research and practice 2012;2012.

604. Moreno Y, Ferrús M. Specific detection of cultivable Helicobacter pylori cells from wastewater treatment plants. Helicobacter 2012;17:327-332.

605. Lu Y, Redlinger TE, Avitia R, et al. Isolation and genotyping of Helicobacter pylori from untreated municipal wastewater. Applied and environmental microbiology 2002;68:1436-1439.

606. Bahrami AR, Rahimi E, Ghasemian Safaei H. Detection of Helicobacter pylori in city water, dental units' water, and bottled mineral water in Isfahan, Iran. The Scientific World Journal 2013;2013.

607. Boehnke KF, Eaton KA, Valdivieso M, et al. Animal Model Reveals Potential Waterborne Transmission of Helicobacter pylori Infection. Helicobacter 2015.

608. Axon A. Review article Is Helicobacter pylori transmitted by the gastro-oral route? Alimentary pharmacology & therapeutics 1995;9:585-588.

609. Axon AT. The transmission of Helicobacter pylori: which theory fits the facts? European journal of gastroenterology & hepatology 1996;8:1-2.

610. Lee A, Fox J, Otto G, et al. Transmission of Helicobacter spp. A challenge to the dogma of faecal-oral spread. Epidemiology and infection 1991;107:99-109.

611. Blaser MJ. Ecology of Helicobacter pylori in the human stomach. Journal of Clinical Investigation 1997;100:759.

612. Xia H, Talley NJ. Natural acquisition and spontaneous elimination of Helicobacter pylori infection: clinical implications. The American journal of gastroenterology 1997;92:1780-1787.

613. Sipponen P. Helicobacter pylori gastritis—epidemiology. Journal of gastroenterology 1997;32:273-277.

614. Bhandari A, Crowe SE. Helicobacter pylori in Gastric Malignancies. Current Gastroenterology Reports 2012:1-8.

615. Corso G, Seruca R, Roviello F. Gastric cancer carcinogenesis and tumor progression. Annali Italiani di Chirurgia 2012;83:172-176.

616. D'Elia L, Galletti F, Strazzullo P. Dietary salt intake and risk of gastric cancer. Cancer Treat Res 2014;159:83-95.

617. Aditi A, Graham DY. Vitamin C, gastritis, and gastric disease: a historical review and update. Dig Dis Sci 2012;57:2504-15.

618. Na HK, Woo JH. Helicobacter pylori Induces Hypermethylation of CpG Islands Through Upregulation of DNA Methyltransferase: Possible Involvement of Reactive Oxygen/Nitrogen Species. J Cancer Prev 2014;19:259-64.

619. Viala J, Chaput C, Boneca IG, et al. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat Immunol 2004;5:1166-74.

620. Lindholm C, Quiding-Jarbrink M, Lonroth H, et al. Local cytokine response in Helicobacter pylori-infected subjects. Infect Immun 1998;66:5964-71.

621. Eaton KA, Mefford M, Thevenot T. The role of T cell subsets and cytokines in the pathogenesis of Helicobacter pylori gastritis in mice. J Immunol 2001;166:7456-61.

622. El-Omar EM. The importance of interleukin 1beta in Helicobacter pylori associated disease. Gut 2001;48:743-7.

623. El-Omar EM, Rabkin CS, Gammon MD, et al. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. Gastroenterology 2003;124:1193-201.

624. Gyulai Z, Klausz G, Tiszai A, et al. Genetic polymorphism of interleukin-8 (IL-8) is associated with Helicobacter pylori-induced duodenal ulcer. Eur Cytokine Netw 2004;15:353-8.

625. Hamajima N, Matsuo K, Suzuki T, et al. Low expression myeloperoxidase genotype negatively associated with Helicobacter pylori infection. Jpn J Cancer Res 2001;92:488-93.

626. El-Omar EM, Carrington M, Chow W-H, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 2000;404:398-402.

627. Ohyauchi M, Imatani A, Yonechi M, et al. The polymorphism interleukin 8 -251 A/T influences the susceptibility of Helicobacter pylori related gastric diseases in the Japanese population. Gut 2005;54:330-5.

628. Machado JC, Figueiredo C, Canedo P, et al. A proinflammatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. Gastroenterology 2003;125:364-71.

629. Shimizu T, Marusawa H, Matsumoto Y, et al. Accumulation of Somatic Mutations in TP53 in Gastric Epithelium With Helicobacter pylori Infection. Gastroenterology 2014;147:407-417.e3.

630. Tomb JF, White O, Kerlavage AR, et al. The complete genome sequence of the gastric pathogen Helicobacter pylori. Nature 1997;388:539-47.

631. Salama N, Guillemin K, McDaniel TK, et al. A whole-genome microarray reveals genetic diversity among Helicobacter pylori strains. Proc Natl Acad Sci U S A 2000;97:14668-73.

632. Ghose C, Perez-Perez GI, Dominguez-Bello MG, et al. East Asian genotypes of Helicobacter pylori strains in Amerindians provide evidence for its ancient human carriage. Proc Natl Acad Sci U S A 2002;99:15107-11.

633. Linz B, Balloux F, Moodley Y, et al. An African origin for the intimate association between humans and Helicobacter pylori. Nature 2007;445:915-8.

634. Hessey SJ, Spencer J, Wyatt JI, et al. Bacterial adhesion and disease activity in Helicobacter associated chronic gastritis. Gut 1990;31:134-8.

635. Ilver D, Arnqvist A, Ogren J, et al. Helicobacter pylori adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science 1998;279:373-7.

636. Mahdavi J, Sonden B, Hurtig M, et al. Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. Science 2002;297:573-8.

637. Yamaoka Y. Mechanisms of disease: Helicobacter pylori virulence factors. Nat Rev Gastroenterol Hepatol 2010;7:629-41.

638. Touati E, Michel V, Thiberge JM, et al. Chronic Helicobacter pylori infections induce gastric mutations in mice. Gastroenterology 2003;124:1408-19.

639. Kikuchi S, Crabtree JE, Forman D, et al. Association between infections with CagA-positive or -negative strains of Helicobacter pylori and risk for gastric cancer in young adults. Research Group on Prevention of Gastric Carcinoma Among Young Adults. Am J Gastroenterol 1999;94:3455-9.

640. Censini S, Lange C, Xiang Z, et al. cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci U S A 1996;93:14648-53.

641. Terradot L, Waksman G. Architecture of the Helicobacter pylori Cag-type IV secretion system. Febs j 2011;278:1213-22.

642. Baghaei K, Shokrzadeh L, Jafari F, et al. Determination of Helicobacter pylori virulence by analysis of the cag pathogenicity island isolated from Iranian patients. Dig Liver Dis 2009;41:634-8.

643. Huang JQ, Zheng GF, Sumanac K, et al. Meta-analysis of the relationship between cagA seropositivity and gastric cancer. Gastroenterology 2003;125:1636-1644.

644. Parsonnet J, Friedman G, Orentreich N, et al. Risk for gastric cancer in people with CagA positive or CagA negative Helicobacter pylori infection. Gut 1997;40:297-301.

645. Wu AH, Crabtree JE, Bernstein L, et al. Role of Helicobacter pylori CagA+ strains and risk of adenocarcinoma of the stomach and esophagus. International journal of cancer 2003;103:815-821.

646. Shimoyama T, Crabtree JE. Mucosal chemokines in Helicobacter pylori infection. J Physiol Pharmacol 1997;48:315-23.

647. Brandt S, Kwok T, Hartig R, et al. NF-κB activation and potentiation of proinflammatory responses by the Helicobacter pylori CagA protein. Proceedings of the National Academy of Sciences of the United States of America 2005;102:9300-9305.

648. Leunk R, Johnson P, David B, et al. Cytotoxic activity in broth-culture filtrates of Campylobacter pylori. Journal of medical microbiology 1988;26:93-99.

649. Telford JL, Ghiara P, Dell'Orco M, et al. Gene structure of the Helicobacter pylori cytotoxin and evidence of its key role in gastric disease. The Journal of experimental medicine 1994;179:1653-1658.

650. van Doorn LJ, Figueiredo C, Sanna R, et al. Expanding allelic diversity of Helicobacter pylori vacA. J Clin Microbiol 1998;36:2597-603.

651. Atherton JC, Cao P, Peek RM, et al. Mosaicism in Vacuolating Cytotoxin Alleles of Helicobacter pylori: Association of specific VacA types with cytotoxin production and peptic ulceration. Journal of Biological Chemistry 1995;270:17771-17777.

652. Atherton J, Peek R, Tham K, et al. Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori. Gastroenterology 1997;112:92-99.

653. Ghiara P, Marchetti M, Blaser MJ, et al. Role of the Helicobacter pylori virulence factors vacuolating cytotoxin, CagA, and urease in a mouse model of disease. Infect Immun 1995;63:4154-60.

654. Wang J, van Doorn LJ, Robinson PA, et al. Regional variation among vacA alleles of Helicobacter pylori in China. J Clin Microbiol 2003;41:1942-5.

655. Miehlke S, Kirsch C, Agha-Amiri K, et al. The Helicobacter pylori vacA s1, m1 genotype and cagA is associated with gastric carcinoma in Germany. International journal of cancer 2000;87:322-327.

656. Miehlke S, Yu J, Schuppler M, et al. Helicobacter pylori vacA, iceA, and cagA status and pattern of gastritis in patients with malignant and benign gastroduodenal disease. The American journal of gastroenterology 2001;96:1008-1013.

657. Sugimoto M, Zali MR, Yamaoka Y. The association of vacA genotypes and Helicobacter pylori-related gastroduodenal diseases in the Middle East. Eur J Clin Microbiol Infect Dis 2009;28:1227-36.

658. Argent RH, Thomas RJ, Letley DP, et al. Functional association between the Helicobacter pylori virulence factors VacA and CagA. Journal of medical microbiology 2008;57:145-150.

659. Wroblewski LE, Peek RM, Jr., Wilson KT. Helicobacter pylori and gastric cancer: factors that modulate disease risk. Clin Microbiol Rev 2010;23:713-39.

660. Unemo M, Aspholm-Hurtig M, Ilver D, et al. The sialic acid binding SabA adhesin of Helicobacter pylori is essential for nonopsonic activation of human neutrophils. J Biol Chem 2005;280:15390-7.

661. Ohno T, Sugimoto M, Nagashima A, et al. Relationship between Helicobacter pylori hopQ genotype and clinical outcome in Asian and Western populations. J Gastroenterol Hepatol 2009;24:462-8.

662. Kudo T, Nurgalieva ZZ, Conner ME, et al. Correlation between Helicobacter pylori OipA protein expression and oipA gene switch status. J Clin Microbiol 2004;42:2279-81.

663. Varro A, Noble PJ, Wroblewski LE, et al. Gastrin-cholecystokinin(B) receptor expression in AGS cells is associated with direct inhibition and indirect stimulation of cell proliferation via paracrine activation of the epidermal growth factor receptor. Gut 2002;50:827-33.

664. Burkitt MD, Pritchard DM. Review article: pathogenesis and management of gastric carcinoid tumours. Aliment.Pharmacol.Ther. 2006;24:1305-1320.

665. Modlin IM, Lye KD, Kidd M. Carcinoid tumors of the stomach. Surg.Oncol. 2003;12:153-172.

666. Murugesan SV, Steele IA, Dimaline R, et al. Correlation between a short-term intravenous octreotide suppression test and response to antrectomy in patients with type-1 gastric neuroendocrine tumours. Eur J Gastroenterol Hepatol 2012.

667. Fox JG, Rogers AB, Ihrig M, et al. Helicobacter pylori-associated gastric cancer in INS-GAS mice is gender specific. Cancer Res. 2003;63:942-950.

668. Fox JG, Wang TC, Rogers AB, et al. Host and microbial constituents influence Helicobacter pylori-induced cancer in a murine model of hypergastrinemia. Gastroenterology 2003;124:1879-1890.

669. Dockray G, Dimaline R, Varro A. Gastrin: old hormone, new functions. Pflugers Arch. 2005;449:344-355.

670. Dimaline R, Varro A. Novel roles of gastrin. J Physiol 2014;592:2951-2958.

671. Higham AD, Bishop LA, Dimaline R, et al. Mutations of RegIalpha are associated with enterochromaffin-like cell tumor development in patients with hypergastrinemia. Gastroenterology 1999;116:1310-1318.

672. Noble PJ, Wilde G, White MR, et al. Stimulation of gastrin-CCKB receptor promotes migration of gastric AGS cells via multiple paracrine pathways. Am.J.Physiol Gastrointest.Liver Physiol 2003;284:G75-G84.

673. Pagliocca A, Wroblewski LE, Ashcroft FJ, et al. Stimulation of the gastrin-cholecystokinin(B) receptor promotes branching morphogenesis in gastric AGS cells. Am.J.Physiol Gastrointest.Liver Physiol 2002;283:G292-G299.

674. Sluijter JP, van Mil A, van Vliet P, et al. MicroRNA-1 and-499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells. Arteriosclerosis, thrombosis, and vascular biology 2010;30:859-868.

675. Chen J-F, Tao Y, Li J, et al. microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. The Journal of cell biology 2010;190:867-879.

676. Bou Kheir T, Futoma-Kazmierczak E, Jacobsen A, et al. miR-449 inhibits cell proliferation and is down-regulated in gastric cancer. Mol Cancer 2011;10:29.

677. Yang X, Feng M, Jiang X, et al. miR-449a and miR-449b are direct transcriptional targets of E2F1 and negatively regulate pRb-E2F1 activity through a feedback loop by targeting CDK6 and CDC25A. Genes Dev 2009;23:2388-93.

678. Lize M, Pilarski S, Dobbelstein M. E2F1-inducible microRNA 449a/b suppresses cell proliferation and promotes apoptosis. Cell Death Differ 2010;17:452-8.

679. Crone SG, Jacobsen A, Federspiel B, et al. microRNA-146a inhibits G protein-coupled receptor-mediated activation of NF-kappaB by targeting CARD10 and COPS8 in gastric cancer. Mol Cancer 2012;11:71.

680. Jiang C, Chen X, Alattar M, et al. MicroRNAs in tumorigenesis, metastasis, diagnosis and prognosis of gastric cancer. Cancer Gene Ther 2015;22:291-301.

681. Ishiguro H, Kimura M, Takeyama H. Role of microRNAs in gastric cancer. World J Gastroenterol 2014;20:5694-9.

682. Hwang MS, Yu N, Stinson SY, et al. miR-221/222 targets adiponectin receptor 1 to promote the epithelial-to-mesenchymal transition in breast cancer. PLoS One 2013;8:e66502.

683. He H, Jazdzewski K, Li W, et al. The role of microRNA genes in papillary thyroid carcinoma. Proc Natl Acad Sci U S A 2005;102:19075-80.

684. Zhong C, Ding S, Xu Y, et al. MicroRNA-222 promotes human non-small cell lung cancer H460 growth by targeting p27. Int J Clin Exp Med 2015;8:5534-40.

685. Quintavalle C, Garofalo M, Zanca C, et al. miR-221/222 overexpession in human glioblastoma increases invasiveness by targeting the protein phosphate PTPmu. Oncogene 2012;31:858-68.

686. Zhou J, Zhou Y, Yin B, et al. 5-Fluorouracil and oxaliplatin modify the expression profiles of microRNAs in human colon cancer cells in vitro. Oncol Rep 2010;23:121-8.

687. Matsuzaki J, Suzuki H. Role of MicroRNAs-221/222 in Digestive Systems. J Clin Med 2015;4:1566-77.

688. Fu Z, Qian F, Yang X, et al. Circulating miR-222 in plasma and its potential diagnostic and prognostic value in gastric cancer. Med Oncol 2014;31:164.

689. Kim Y-K, Yu J, Han TS, et al. Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer. Nucleic acids research 2009;37:1672-1681.

690. Wang M, Zhao C, Shi H, et al. Deregulated microRNAs in gastric cancer tissue-derived mesenchymal stem cells: novel biomarkers and a mechanism for gastric cancer. Br J Cancer 2014;110:1199-210.

691. Li N, Tang B, Zhu ED, et al. Increased miR-222 in H. pylori-associated gastric cancer correlated with tumor progression by promoting cancer cell proliferation and targeting RECK. FEBS Lett 2012;586:722-8.

692. Liu W, Song N, Yao H, et al. miR-221 and miR-222 Simultaneously Target RECK and Regulate Growth and Invasion of Gastric Cancer Cells. Med Sci Monit 2015;21:2718-25.

693. Garofalo M, Quintavalle C, Romano G, et al. miR221/222 in cancer: their role in tumor progression and response to therapy. Curr Mol Med 2012;12:27-33.

694. Li N, Yu N, Wang J, et al. miR-222/VGLL4/YAP-TEAD1 regulatory loop promotes proliferation and invasion of gastric cancer cells. Am J Cancer Res 2015;5:1158-68.

695. Chun-Zhi Z, Lei H, An-Ling Z, et al. MicroRNA-221 and microRNA-222 regulate gastric carcinoma cell proliferation and radioresistance by targeting PTEN. BMC Cancer 2010;10:367.

696. Byun SW, Chang YJ, Chung IS, et al. Helicobacter pylori decreases p27 expression through the delta opioid receptor-mediated inhibition of histone acetylation within the p27 promoter. Cancer Letters 2012;326:96-104.

697. Shu-Ping L, Xue-Jun C, Ai-Hua S, et al. CagA+ H. pylori Induces Akt1 Phosphorylation and Inhibits Transcription of p21 WAF1/CIP1 and p27 KIP1 via PI3K/Akt1 Pathway. Biomedical and Environmental Sciences 2010;23:273-278.

698. Wen S, So Y, Singh K, et al. Promotion of cytoplasmic mislocalization of p27 by Helicobacter pylori in gastric cancer. Oncogene 2012;31:1771-80.

699. Eguchi H, Herschenhous N, Kuzushita N, et al. Helicobacter pylori increases proteasome-mediated degradation of p27(kip1) in gastric epithelial cells. Cancer Res 2003;63:4739-46.

700. Kuzushita N, Rogers AB, Monti NA, et al. p27kip1 deficiency confers susceptibility to gastric carcinogenesis in Helicobacter pylori-infected mice. Gastroenterology 2005;129:1544-56.

701. Yu J, Leung W, Ng E, et al. Effect of Helicobacter pylori eradication on expression of cyclin D2 and p27 in gastric intestinal metaplasia. Alimentary pharmacology & therapeutics 2001;15:1505-1511.

702. Kim SS, Meitner P, Konkin TA, et al. Altered expression of Skp2, c-Myc and p27 proteins but not mRNA after H. pylori eradication in chronic gastritis. Modern pathology 2006;19:49-58.

703. Peek RM, Jr., Wirth HP, Moss SF, et al. Helicobacter pylori alters gastric epithelial cell cycle events and gastrin secretion in Mongolian gerbils. Gastroenterology 2000;118:48-59.

704. Eguchi H, Carpentier S, Kim S, et al. P27kip1 regulates the apoptotic response of gastric epithelial cells to Helicobacter pylori. Gut 2004;53:797-804.

705. Shirin H, Sordillo EM, Kolevska TK, et al. Chronic Helicobacter pylori infection induces an apoptosis-resistant phenotype associated with decreased expression of p27kip1. Infection and immunity 2000;68:5321-5328.

706. Wander SA, Zhao D, Slingerland JM. p27: a barometer of signaling deregulation and potential predictor of response to targeted therapies. Clin Cancer Res 2011;17:12-8.

707. Detjen K, Yule D, Tseng MJ, et al. CCK-B receptors produce similar signals but have opposite growth effects in CHO and Swiss 3T3 cells. Am J Physiol 1997;273:C1449-57.

708. Detjen K, Fenrich MC, Logsdon CD. Transfected cholecystokinin receptors mediate growth inhibitory effects on human pancreatic cancer cell lines. Gastroenterology 1997;112:952-959.

709. Wank SA, Pisegna JR, De Weerth A. Brain and gastrointestinal cholecystokinin receptor family: structure and functional expression. Proceedings of the National Academy of Sciences 1992;89:8691-8695.

710. Nicke B, Detjen K, Logsdon CD. Muscarinic cholinergic receptors activate both inhibitory and stimulatory growth mechanisms in NIH3T3 cells. J Biol Chem 1999;274:21701-6.

711. Larrea MD, Hong F, Wander SA, et al. RSK1 drives p27Kip1 phosphorylation at T198 to promote RhoA inhibition and increase cell motility. Proceedings of the National Academy of Sciences 2009;106:9268-9273.

712. Overgaard MT, Boldt HB, Laursen LS, et al. Pregnancy-associated plasma protein-A2 (PAPP-A2), a novel insulin-like growth factor-binding protein-5 proteinase. Journal of Biological Chemistry 2001;276:21849-21853.

713. Liu JP, Baker J, Perkins AS, et al. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 1993;75:59-72.

714. Yi HK, Hwang PH, Yang DH, et al. Expression of the insulin-like growth factors (IGFs) and the IGF-binding proteins (IGFBPs) in human gastric cancer cells. Eur J Cancer 2001;37:2257-63.

715. Pavelic K, Kolak T, Kapitanovic S, et al. Gastric cancer: the role of insulin-like growth factor 2 (IGF 2) and its receptors (IGF 1R and M6-P/IGF 2R). J Pathol 2003;201:430-8.

716. Delle Fave G, Capurso G, Milione M, et al. Endocrine tumours of the stomach. Best Pract Res Clin Gastroenterol 2005;19:659-73.

717. Ramsay PT, Carr A. Gastric acid and digestive physiology. Surgical Clinics of North America 2011;91:977-982.

718. Dockray GJ, Varro A, Dimaline R, et al. The gastrins: Their production and biological activities. Annual review of physiology 2001;63:119-139.

719. Wolfe MM, Soll AH. The Physiology of Gastric Acid Secretion. New England Journal of Medicine 1988;319:1707-1715.

720. Fagerberg L, Hallstrom BM, Oksvold P, et al. Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. Mol Cell Proteomics 2014;13:397-406.

721. Nishizawa H, Pryor-Koishi K, Suzuki M, et al. Increased levels of pregnancy-associated plasma protein-A2 in the serum of pre-eclamptic patients. Mol Hum Reprod 2008;14:595-602.

722. Winn VD, Gormley M, Paquet AC, et al. Severe preeclampsia-related changes in gene expression at the maternal-fetal interface include sialic acid-binding immunoglobulin-like lectin-6 and pappalysin-2. Endocrinology 2009;150:452-62.

723. Buimer M, Keijser R, Jebbink JM, et al. Seven placental transcripts characterize HELLP-syndrome. Placenta 2008;29:444-53.

724. Wu X, Blanck A, Norstedt G, et al. Identification of genes with higher expression in human uterine leiomyomas than in the corresponding myometrium. Mol Hum Reprod 2002;8:246-54.

725. Suzuki A, Mimaki S, Yamane Y, et al. Identification and characterization of cancer mutations in Japanese lung adenocarcinoma without sequencing of normal tissue counterparts. PLoS One 2013;8:e73484.

726. Wagner PK, Otomo A, Christians JK. Regulation of pregnancy-associated plasma protein A2 (PAPPA2) in a human placental trophoblast cell line (BeWo). Reprod Biol Endocrinol 2011;9:48.

727. Andress DL. IGF-binding protein-5 stimulates osteoblast activity and bone accretion in ovariectomized mice. Am J Physiol Endocrinol Metab 2001;281:E283-8.

728. Constancia M, Hemberger M, Hughes J, et al. Placental-specific IGF-II is a major modulator of placental and fetal growth. Nature 2002;417:945-8.

729. Sciacca L, Costantino A, Pandini G, et al. Insulin receptor activation by IGF-II in breast cancers: evidence for a new autocrine/paracrine mechanism. Oncogene 1999;18:2471-9.

730. Kaaks R, Toniolo P, Akhmedkhanov A, et al. Serum C-peptide, insulin-like growth factor (IGF)-I, IGF-binding proteins, and colorectal cancer risk in women. J Natl Cancer Inst 2000;92:1592-600.

731. Ma J, Pollak MN, Giovannucci E, et al. Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. J Natl Cancer Inst 1999;91:620-5.

732. Stattin P, Bylund A, Rinaldi S, et al. Plasma insulin-like growth factor-I, insulin-like growth factor-binding proteins, and prostate cancer risk: a prospective study. J Natl Cancer Inst 2000;92:1910-7.

733. Chan JM, Stampfer MJ, Ma J, et al. Insulin-like growth factor-I (IGF-I) and IGF binding protein-3 as predictors of advanced-stage prostate cancer. J Natl Cancer Inst 2002;94:1099-106.

734. McCaig C, Duval C, Hemers E, et al. The role of matrix metalloproteinase-7 in redefining the gastric microenvironment in response to Helicobacter pylori. Gastroenterology 2006;130:1754-63.

735. Bebb JR, Letley DP, Thomas RJ, et al. Helicobacter pylori upregulates matrilysin (MMP-7) in epithelial cells in vivo and in vitro in a Cag dependent manner. Gut 2003;52:1408-13.

736. Hemers E, Duval C, McCaig C, et al. Insulin-like growth factor binding protein-5 is a target of matrix metalloproteinase-7: implications for epithelial-mesenchymal signaling. Cancer Res 2005;65:7363-9.

737. Amiri N, Christians JK. PAPP-A2 expression by osteoblasts is required for normal postnatal growth in mice. Growth Horm IGF Res 2015.

738. Crosley EJ, Dunk CE, Beristain AG, et al. IGFBP-4 and -5 are expressed in first-trimester villi and differentially regulate the migration of HTR-8/SVneo cells. Reprod Biol Endocrinol 2014;12:123.

739. Vallee BL, Auld DS. Zinc coordination, function, and structure of zinc enzymes and other proteins. Biochemistry 1990;29:5647-59.

740. Bode W, Gomis-Ruth FX, Stockler W. Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. FEBS Lett 1993;331:134-40.

741. Boldt HB, Kjaer-Sorensen K, Overgaard MT, et al. The Lin12-notch repeats of pregnancy-associated plasma protein-A bind calcium and determine its proteolytic specificity. J Biol Chem 2004;279:38525-31.

742. Laursen LS, Overgaard MT, Weyer K, et al. Cell surface targeting of pregnancy-associated plasma protein A proteolytic activity. Reversible adhesion is mediated by two neighboring short consensus repeats. J Biol Chem 2002;277:47225-34.

743. Weyer K, Overgaard MT, Laursen LS, et al. Cell surface adhesion of pregnancy-associated plasma protein-A is mediated by four clusters of basic residues located in its third and fourth CCP module. Eur J Biochem 2004;271:1525-35.

744. Boldt HB, Overgaard MT, Laursen LS, et al. Mutational analysis of the proteolytic domain of pregnancy-associated plasma protein-A (PAPP-A): classification as a metzincin. Biochem. J. 2001;358:359-367.

745. Oxvig C, Overgaard, M.T., and Sottrup-Jensen, L. . Handbook of Proteolytic Enzymes. London: Academic Press, 2004.

746. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. Nature reviews Molecular cell biology 2007;8:221-233.

747. Laviola L, Natalicchio A, Giorgino F. The IGF-I signaling pathway. Curr Pharm Des 2007;13:663-9.

748. Valentinis B, Baserga R. IGF-I receptor signalling in transformation and differentiation. Molecular Pathology 2001;54:133-137.

749. Li H, Adachi Y, Yamamoto H, et al. Insulin-like growth factor-I receptor blockade reduces tumor angiogenesis and enhances the effects of bevacizumab for a human gastric cancer cell line, MKN45. Cancer 2011;117:3135-47.

750. Khan J, Bittner ML, Saal LH, et al. cDNA microarrays detect activation of a myogenic transcription program by the PAX3-FKHR fusion oncogene. Proceedings of the National Academy of Sciences 1999;96:13264-13269.

751. Miyake H, Pollak M, Gleave ME. Castration-induced up-regulation of insulin-like growth factor binding protein-5 potentiates insulin-like growth factor-I activity and accelerates progression to androgen independence in prostate cancer models. Cancer research 2000;60:3058-3064.

752. Tsibris JC, Segars J, Coppola D, et al. Insights from gene arrays on the development and growth regulation of uterine leiomyomata. Fertility and sterility 2002;78:114-121.

753. Crawford HC, Krishna US, Israel DA, et al. Helicobacter pylori strain-selective induction of matrix metalloproteinase-7 in vitro and within gastric mucosa. Gastroenterology 2003;125:1125-1136.

754. Dockray GJ. Gastrin. Best Practice & Research Clinical Endocrinology & Metabolism 2004;18:555-568.

755. Adachi Y, Itoh F, Yamamoto H, et al. Matrix metalloproteinase matrilysin (MMP-7) participates in the progression of human gastric and esophageal cancers. International journal of oncology 1998;13:1031-1036.

756. McDonnell S, Navre M, Coffey RJ, et al. Expression and localization of the matrix metalloproteinase pump-1 (MMP-7) in human gastric and colon carcinomas. Molecular carcinogenesis 1991;4:527-533.

757. Yamamoto H, Horiuchi S, Adachi Y, et al. Expression of ets-related transcriptional factor E1AF is associated with tumor progression and over-expression of matrilysin in human gastric cancer. Carcinogenesis 2004;25:325-332.

758. Fernandez-Catalan C, Bode W, Huber R, et al. Crystal structure of the complex formed by the membrane type 1-matrix metalloproteinase with the tissue inhibitor of metalloproteinases-2, the soluble progelatinase A receptor. The EMBO Journal 1998;17:5238-5248.

759. Gomis-Ruth F-X, Maskos K, Betz M, et al. Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1. NATURE 1997;389:77.

760. Rapti M, Atkinson SJ, Lee MH, et al. The isolated N-terminal domains of TIMP-1 and TIMP-3 are insufficient for ADAM10 inhibition. Biochem J 2008;411:433-9.

761. Nagase H, Murphy G. Tailoring TIMPs for selective metalloproteinase inhibition. The Cancer Degradome: Springer, 2008:787-810.

762. Bodger K, Ahmed S, Pazmany L, et al. Altered gastric corpus expression of tissue inhibitors of metalloproteinases in human and murine Helicobacter infection. J Clin Pathol 2008;61:72-8.

763. Baker AH, Zaltsman AB, George SJ, et al. Divergent effects of tissue inhibitor of metalloproteinase-1,-2, or-3 overexpression on rat vascular smooth muscle cell

invasion, proliferation, and death in vitro. TIMP-3 promotes apoptosis. Journal of Clinical Investigation 1998;101:1478.

764. Stetler-Stevenson WG. Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. Sci Signal 2008;1:re6.

765. Yan X, Baxter RC, Firth SM. Involvement of pregnancy-associated plasma protein-A2 in insulin-like growth factor (IGF) binding protein-5 proteolysis during pregnancy: a potential mechanism for increasing IGF bioavailability. The Journal of Clinical Endocrinology & Metabolism 2010;95:1412-1420.

766. Tripathi G, Salih DAM, Drozd AC, et al. IGF-independent effects of insulin-like growth factor binding protein-5 (Igfbp5) in vivo. The FASEB Journal 2009;23:2616-2626.

767. Loughran G, Huigsloot M, Kiely PA, et al. Gene expression profiles in cells transformed by overexpression of the IGF-I receptor. Oncogene 2005;24:6185-6193.

768. Samani AA, Yakar S, LeRoith D, et al. The role of the IGF system in cancer growth and metastasis: overview and recent insights. Endocrine reviews 2007;28:20-47.

769. Belfiore A, Frasca F, Pandini G, et al. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. Endocrine reviews 2009;30:586-623.

770. Sell C, Dumenil G, Deveaud C, et al. Effect of a null mutation of the insulin-like growth factor I receptor gene on growth and transformation of mouse embryo fibroblasts. Molecular and cellular biology 1994;14:3604-3612.

771. Jones R, Campbell C, Wood G, et al. Reversibility and recurrence of IGF-IR-induced mammary tumors. Oncogene 2009;28:2152-2162.

772. Wu Y, Yakar S, Zhao L, et al. Circulating insulin-like growth factor-I levels regulate colon cancer growth and metastasis. Cancer research 2002;62:1030-1035.

773. Wu Y, Cui K, Miyoshi K, et al. Reduced circulating insulin-like growth factor I levels delay the onset of chemically and genetically induced mammary tumors. Cancer Research 2003;63:4384-4388.

774. Rossant J, Cross JC. Placental development: lessons from mouse mutants. Nature Reviews Genetics 2001;2:538-548.

775. Lala P, Hamilton G. Growth factors, proteases and protease inhibitors in the maternal—fetal dialogue. Placenta 1996;17:545-555.

776. Salamonsen LA. Role of proteases in implantation. Reviews of Reproduction 1999;4:11-22.

777. Smith GC, Stenhouse EJ, Crossley JA, et al. Early pregnancy levels of pregnancy-associated plasma protein a and the risk of intrauterine growth restriction, premature birth, preeclampsia, and stillbirth. The Journal of Clinical Endocrinology & Metabolism 2002;87:1762-1767.

778. Kwik M, Morris J. Association between first trimester maternal serum pregnancy associated plasma protein-A and adverse pregnancy outcome. Australian and New Zealand Journal of Obstetrics and Gynaecology 2003;43:438-442.

779. Dugoff L, Hobbins JC, Malone FD, et al. First-trimester maternal serum PAPP-A and free-beta subunit human chorionic gonadotropin concentrations and nuchal translucency are associated with obstetric complications: a population-based screening study (the FASTER Trial). American journal of obstetrics and gynecology 2004;191:1446-1451.

780. Spencer K, Cowans N, Nicolaides K. Low levels of maternal serum PAPP-A in the first trimester and the risk of pre-eclampsia. Prenatal diagnosis 2008;28:7-10.

781. Christians JK, Hoeflich A, Keightley PD. PAPPA2, an Enzyme That Cleaves an Insulin-Like Growth-Factor-Binding Protein, Is a Candidate Gene for a Quantitative Trait Locus Affecting Body Size in Mice. Genetics 2006;173:1547-1553.

782. Milner JM, Cawston TE. Matrix metalloproteinase knockout studies and the potential use of matrix metalloproteinase inhibitors in the rheumatic diseases. Curr Drug Targets Inflamm Allergy 2005;4:363-75.

783. Drummond AH, Beckett P, Brown PD, et al. Preclinical and Clinical Studies of MMP Inhibitors in Cancer. Annals of the New York Academy of Sciences 1999;878:228-235.

784. Munnangi S, Gross SJ, Madankumar R, et al. Pregnancy associated plasma protein-A2: a novel biomarker for Down syndrome. Placenta 2014;35:900-6.

785. Selvik LKM, Fjeldbo CS, Flatberg A, et al. The duration of gastrin treatment affects global gene expression and molecular responses involved in ER stress and anti-apoptosis. BMC Genomics 2013;14:429.

786. Håkanson R, Sundler F. Trophic Effects of Gastrin. Scandinavian Journal of Gastroenterology 1991;26:130-136.

787. Carrasco M, Hernanz A, De La Fuente M. Effect of cholecystokinin and gastrin on human peripheral blood lymphocyte functions, implication of cyclic AMP and interleukin 2. Regulatory peptides 1997;70:135-142.

788. Abdalla E. Gastrin is a modulator of lymphocyte apoptosis. Khartoum Medical Journal 2010;3:377-380.

789. Fino KK, Matters GL, McGovern CO, et al. Downregulation of the CCK-B receptor in pancreatic cancer cells blocks proliferation and promotes apoptosis. Am J Physiol Gastrointest Liver Physiol 2012;302:G1244-52.

790. Uygun A, Kadayifci A, Polat Z, et al. Long-term results of endoscopic resection for type I gastric neuroendocrine tumors. J Surg Oncol 2014;109:71-4.

791. Ferraro G, Annibale B, Marignani M, et al. Effectiveness of octreotide in controlling fasting hypergastrinemia and related enterochromaffin-like cell growth. J Clin Endocrinol Metab 1996;81:677-83.

792. Grozinsky-Glasberg S, Kaltsas G, Gur C, et al. Long-acting somatostatin analogues are an effective treatment for type 1 gastric carcinoid tumours. Eur J Endocrinol 2008;159:475-82.

793. Jianu CS, Fossmark R, Syversen U, et al. Five-year follow-up of patients treated for 1 year with octreotide long-acting release for enterochromaffin-like cell carcinoids. Scand J Gastroenterol 2011;46:456-63.

794. Scemama JL, Fourmy D, Zahidi A, et al. Characterisation of gastrin receptors on a rat pancreatic acinar cell line (AR42J). A possible model for studying gastrin mediated cell growth and proliferation. Gut 1987;28 Suppl:233-6.

795. Wang RN, Rehfeld JF, Nielsen FC, et al. Expression of gastrin and transforming growth factor-alpha during duct to islet cell differentiation in the pancreas of duct-ligated adult rats. Diabetologia 1997;40:887-93.

796. Buchanan FG, Wang D, Bargiacchi F, et al. Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. Journal of Biological Chemistry 2003;278:35451-35457.

797. Komori M, Tsuji S, Sun W-H, et al. Gastrin enhances gastric mucosal integrity through cyclooxygenase-2 upregulation in rats. American Journal of Physiology-Gastrointestinal and Liver Physiology 2002;283:G1368-G1378.

798. Chausovsky A, Tsarfaty I, Kam Z, et al. Morphogenetic effects of neuregulin (neu differentiation factor) in cultured epithelial cells. Molecular biology of the cell 1998;9:3195-3209.

799. Sawaoka H, Tsuji S, Gunawan E, et al. Involvement of cyclooxygenase-2 in proliferation and morphogenesis induced by transforming growth factor α in gastric epithelial cells. Prostaglandins, leukotrienes and essential fatty acids 1999;61:315-322.

800. Chen D, Zhao C-M, Norlén P, et al. Effect of cholecystokinin-2 receptor blockade on rat stomach ECL cells. Cell and Tissue Research 2000;299:81-95.

801. Griffiths-Jones S. The microRNA Registry. Nucleic Acids Research 2004;32:D109-D111.

802. Griffiths-Jones S, Grocock RJ, van Dongen S, et al. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Research 2006;34:D140-D144.

803. Griffiths-Jones S, Saini HK, van Dongen S, et al. miRBase: tools for microRNA genomics. Nucleic Acids Research 2008;36:D154-D158.

804. Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Research 2011;39:D152-D157.

805. Stacey G. Primary Cell Cultures and Immortal Cell Lines. eLS 2001.

806. McCracken KW, Catá EM, Crawford CM, et al. Modelling human development and disease in pluripotent stem-cell-derived gastric organoids. Nature 2014.

807. Hanahan D, Weinberg RA. The hallmarks of cancer. cell 2000;100:57-70.

808. Waterston RH, Lindblad-Toh K, Birney E, et al. Initial sequencing and comparative analysis of the mouse genome. Nature 2002;420:520-62.

809. Rakic S, Hinder RA, Adanja G, et al. Elevated serum gastrin levels in patients with gastric cancer. Journal of surgical oncology 1991;47:79-81.

810. Helander HF, Poorkhalkali N. Parietal cell density during gastric ulcer healing in the rat. Scandinavian journal of gastroenterology 2004;39:20-26.

811. Watanabe T, Tada M, Nagai H, et al. Helicobacter pylori infection induces gastric cancer in Mongolian gerbils. Gastroenterology 1998;115:642-648.

812. Modlin IM, Kidd M, Farhadi J. Bayliss and Starling and the nascence of endocrinology. Regulatory peptides 2000;93:109-123.

813. Koga T, Takahashi K, Sato K, et al. The effect of colonisation by Helicobacter pylori in Praomys (Mastomys) natalensis on the incidence of carcinoids. Journal of medical microbiology 2002;51:777-785.

814. Ehlers S, Warrelmann M, Hahn H. In search of an animal model for experimental Campylobacter pylori infection: administration of Campylobacter pylori to rodents. Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. Series A: Medical Microbiology, Infectious Diseases, Virology, Parasitology 1988;268:341-346.

815. Cantorna MT, Balish E. Inability of human clinical strains of Helicobacter pylori to colonize the alimentary tract of germfree rodents. Canadian journal of microbiology 1990;36:237-241.

816. Arnold IC, Zigova Z, Holden M, et al. Comparative whole genome sequence analysis of the carcinogenic bacterial model pathogen Helicobacter felis. Genome Biol Evol 2011;3:302-8.

817. Correa P. Helicobacter pylori infection and gastric cancer. Cancer Epidemiology Biomarkers & Prevention 2003;12:238s-241s.

818. Stoicov C, Saffari R, Cai X, et al. Molecular biology of gastric cancer: Helicobacter infection and gastric adenocarcinoma: bacterial and host factors responsible for altered growth signaling. Gene 2004;341:1-17.

819. Houghton J, Wang TC. Helicobacter pylori and gastric cancer: a new paradigm for inflammation-associated epithelial cancers. Gastroenterology 2005;128:1567-1578.

820. Wang X, Willen R, Svensson M, et al. Two-year follow-up of Helicobacter pylori infection in C57BL/6 and Balb/cA mice. Apmis 2003;111:514-522.

821. Wirth H-P, Beins MH, Yang M, et al. Experimental infection of Mongolian gerbils with wild-type and mutant Helicobacter pylori strains. Infection and immunity 1998;66:4856-4866.

822. Ogura K, Maeda S, Nakao M, et al. Virulence factors of Helicobacter pylori responsible for gastric diseases in Mongolian gerbil. The Journal of experimental medicine 2000;192:1601-1610.

823. Israel DA, Salama N, Arnold CN, et al. Helicobacter pylori strain-specific differences in genetic content, identified by microarray, influence host inflammatory responses. Journal of Clinical Investigation 2001;107:611.

824. Honda S, Fujioka T, Tokieda M, et al. Development of Helicobacter pylori-induced Gastric Carcinoma in Mongolian Gerbils. Cancer Research 1998;58:4255-4259.

825. Hirayama F, Takagi S, Iwao E, et al. Development of poorly differentiated adenocarcinoma and carcinoid due to long-term Helicobacter pylori colonization in Mongolian gerbils. J Gastroenterol 1999;34:450-4.

826. Galardi S, Mercatelli N, Giorda E, et al. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. Journal of Biological Chemistry 2007;282:23716-23724.

827. Fornari F, Gramantieri L, Ferracin M, et al. MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. Oncogene 2008;27:5651-5661.

828. Pineau P, Volinia S, McJunkin K, et al. miR-221 overexpression contributes to liver tumorigenesis. Proceedings of the National Academy of Sciences 2010;107:264-269.

829. Pu Xx, Huang Gl, Guo Hq, et al. Circulating miR-221 directly amplified from plasma is a potential diagnostic and prognostic marker of colorectal cancer and is correlated with p53 expression. Journal of gastroenterology and hepatology 2010;25:1674-1680.

830. Stinson S, Lackner MR, Adai AT, et al. TRPS1 targeting by miR-221/222 promotes the epithelial-to-mesenchymal transition in breast cancer. Science Signaling 2011;4:ra41-ra41.

831. Bonci D, Coppola V, Musumeci M, et al. The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nat Med 2008;14:1271-7.

832. Creighton CJ, Hernandez-Herrera A, Jacobsen A, et al. Integrated analyses of microRNAs demonstrate their widespread influence on gene expression in high-grade serous ovarian carcinoma. PLoS One 2012;7:e34546.

833. Mavrakis KJ, Van Der Meulen J, Wolfe AL, et al. A cooperative microRNA-tumor suppressor gene network in acute T-cell lymphoblastic leukemia (T-ALL). Nat Genet 2011;43:673-8.

834. Ooi CH, Oh HK, Wang HZ, et al. A densely interconnected genome-wide network of microRNAs and oncogenic pathways revealed using gene expression signatures. PLoS Genet 2011;7:e1002415.

835. Correa P, Haenszel W, Cuello C, et al. Gastric precancerous process in a high risk population: cohort follow-up. Cancer Res 1990;50:4737-40.

836. Sipponen P, Kekki M, Haapakoski J, et al. Gastric cancer risk in chronic atrophic gastritis: statistical calculations of cross-sectional data. Int J Cancer 1985;35:173-7.

837. Varis K, Isokoski M. Screening of type A gastritis. Ann Clin Res 1981;13:133-8.

838. Sipponen P, Valle J, Varis K, et al. Fasting levels of serum gastrin in different functional and morphologic states of the antrofundal mucosa. An analysis of 860 subjects. Scand J Gastroenterol 1990;25:513-9.

839. Varis K, Kekki M, Harkonen M, et al. Serum pepsinogen I and serum gastrin in the screening of atrophic pangastritis with high risk of gastric cancer. Scand J Gastroenterol Suppl 1991;186:117-23.

840. Dinis-Ribeiro M, Yamaki G, Miki K, et al. Meta-analysis on the validity of pepsinogen test for gastric carcinoma, dysplasia or chronic atrophic gastritis screening. J Med Screen 2004;11:141-7.

841. Ley C, Mohar A, Guarner J, et al. Screening markers for chronic atrophic gastritis in Chiapas, Mexico. Cancer Epidemiology Biomarkers & Prevention 2001;10:107-112.

842. Bodger K, Wyatt J, Heatley R. Serologic screening before endoscopy: the value of Helicobacter pylori serology, serum recognition of the CagA and VacA proteins, and serum pepsinogen I. Scandinavian journal of gastroenterology 1999;34:856-863.

843. Kimura Y, Fujii T, Hamamoto K, et al. Evaluation of serum pepsinogen I and II of patients with gastric cancer. Kaku igaku. The Japanese journal of nuclear medicine 1989;26:1127-1133.

844. Zhang L, Blot WJ, You W, et al. Helicobacter pylori antibodies in relation to precancerous gastric lesions in a high-risk Chinese population. Cancer Epidemiology Biomarkers & Prevention 1996;5:627-630.

845. Garofalo M, Di Leva G, Romano G, et al. miR-221&222 Regulate TRAIL Resistance and Enhance Tumorigenicity through PTEN and TIMP3 Downregulation. Cancer Cell 2009;16:498-509.

846. Conover CA, Boldt HB, Bale LK, et al. Pregnancy-associated plasma protein-A2 (PAPP-A2): tissue expression and biological consequences of gene knockout in mice. Endocrinology 2011;152:2837-2844.

847. Christians JK, De Zwaan DR, Fung S. Pregnancy associated plasma protein A2 (PAPP-A2) affects bone size and shape and contributes to natural variation in postnatal growth in mice. PloS one 2013;8:e56260.

848. Macintire K, Tuohey L, Ye L, et al. PAPPA2 is increased in severe early onset pre-eclampsia and upregulated with hypoxia. Reproduction, Fertility and Development 2014;26:351-357.

6 Supplementary data

Table 2. Mature miRNA expression patterns of all 84 mature miRNAs arrayed the pathway-focused miScript miRNA PCR array for AGS_{GR} cells treated with 10nM G_{17} 24hrs compared with untreated controls.

	Human mature miRNA	Fold change	P value
*‡	miR-376c-3p	0.010	5.18
***‡**	**miR-222-3p**	**<0.000**	**2.34**
‡	miR-141-3p	0.163	2.11
	miR-32-5p	0.943	1.84
*	miR-22-3p	0.026	1.82
*	miR-142-3p	0.029	1.81
*	miR-27a-3p	0.002	1.80
	miR-130a-3p	0.150	1.60
	miR-210-3p	0.513	1.57
*	miR-29a-3p	0.002	1.47
	miR-124-3p	0.409	1.37
*	miR-29c-3p	0.001	1.36
*	miR-140-3p	0.044	1.34
	miR-424-5p	0.403	1.32
	miR-101-3p	0.432	1.30
	miR-146a-5p	0.415	1.29
	miR-144-3p	0.417	1.28
	miR-196b-5p	0.417	1.27
	miR-185-5p	0.102	1.26
	miR-143-3p	0.737	1.26
	let-7c-5p	0.262	1.25
	miR-150-5p	0.428	1.22
	miR-302c-3p	0.166	1.20
	miR-23a-3p	0.585	1.18
	let-7b-5p	0.459	1.17
	miR-155-5p	0.423	1.15
	miR-24-3p	0.502	1.12
	let-7d-5p	0.552	1.10
	miR-186-5p	0.462	1.08
	miR-223-3p	0.425	1.08
	miR-126-3p	0.425	1.07
	miR-122-5p	0.425	1.07
	miR-19b-3p	0.520	1.06
	miR-19a-3p	0.551	1.06
	miR-28-5p	0.783	1.05
	miR-16-5p	0.658	1.04
	miR-125a-5p	0.708	1.03
	miR-181a-5p	0.969	1.03
	miR-15a-5p	0.881	1.00
	miR-195-5p	0.855	0.98
	miR-125b-5p	0.890	0.97
	let-7a-5p	0.848	0.97
	miR-9-5p	0.560	0.97

Human mature miRNA	Fold change	P value
miR-191-5p	0.917	0.95
let-7e-5p	0.829	0.95
miR-106b-5p	0.760	0.93
miR-302a-3p	0.672	0.93
miR-425-5p	0.644	0.93
miR-200c-3p	0.959	0.93
miR-30b-5p	0.697	0.92
let-7i-5p	0.507	0.92
miR-142-5p	0.710	0.90
miR-30a-5p	0.598	0.90
miR-27b-3p	0.817	0.90
miR-302b-3p	0.709	0.88
miR-20a-5p	0.384	0.88
miR-181b-5p	0.454	0.87
miR-151a-5p	0.562	0.86
miR-93-5p	0.187	0.86
miR-21-5p	0.964	0.85
miR-15b-5p	0.787	0.85
let-7f-5p	0.848	0.83
miR-99a-5p	0.852	0.83
miR-92a-3p	0.504	0.83
miR-30d-5p	0.390	0.82
miR-26a-5p	0.728	0.81
miR-18a-5p	0.163	0.81
miR-23b-3p	0.610	0.81
miR-17-5p	0.171	0.80
miR-320a	0.251	0.78
miR-194-5p	0.250	0.74
miR-374a-5p	0.472	0.74
miR-423-5p	0.288	0.72
let-7g-5p	0.338	0.72
miR-128-3p	0.207	0.72
miR-103a-3p	0.813	0.70
miR-26b-5p	0.617	0.69
miR-7-5p	0.401	0.68
miR-100-5p	0.418	0.60
miR-30e-5p	0.191	0.51
miR-30c-5p	0.385	0.47
ǂ miR-96-5p	0.738	0.27
ǂ miR-29b-3p	0.669	0.22
ǂ miR-25-3p	0.089	0.07

* Statistically significant versus untreated control samples (P<0.05 was considered significant)

ǂ Fold regulation beyond the 2-fold boundary relative to untreated control samples