The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Tobias, Amanda Jane (2010) Gastrin interactions which impact upon gastric, colonic, pancreatic and oesophageal carcinogenesis. PhD thesis, University of Nottingham.

Access from the University of Nottingham repository:
http://eprints.nottingham.ac.uk/11348/1/thesis_pdf.pdf

Copyright and reuse:

The Nottingham ePrints service makes this work by researchers of the University of Nottingham available open access under the following conditions.

This article is made available under the University of Nottingham End User licence and may be reused according to the conditions of the licence. For more details see: http://eprints.nottingham.ac.uk/end_user_agreement.pdf

A note on versions:

The version presented here may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the repository url above for details on accessing the published version and note that access may require a subscription.

For more information, please contact eprints@nottingham.ac.uk

Gastrin Interactions Which Impact Upon Gastric, Colonic, Pancreatic and Oesophageal Carcinogenesis

Amanda J. Tobias BSc (Hons)

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy
June 2009

CONTENTS

Page Number

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

A
Aberrant crypts foci = ACF
Adenoma prevention with Celecoxib = APC
Adenomatous polyposis coli = APC
Adenomatous polyposis polyp prevention on Vioxx study = APPROVe
3-amino-9-ethylcarbazole = AEC
(strept) Avidin-biotin complex = ABC
Azoxyymethane = AOM
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine = PhIP

B
Barrett's oesophagus = BE
Blood vessel density = BVD
Body mass index = BMI
Bromodeoxyuridine = BrdU

C
Celecoxib long-term arthritis safety study = CLASS
Central nervous system = CNS
Cholecystokinin = CCK
CCK-2 receptor intron 4 splice variant = CCK-2Ri4sv
Columnar lined oesophagus = CLO
Cyclooygenase = COX
COX-2 specific inhibitor = coxib
Cytotoxin associated gene A = cagA

D
Deleted in pancreatic cancer = DPC4
3,3'-Diaminobenzidine tetrahydrochloride = DAB

E
Endoplasmic reticulum = ER
Enterchromaffin-like = ECL
Epidermal growth factor = EGF

F
Fas-associated death domain = FADD
Familial adenomatous polyposis = FAP
Food and drug administration = FDA

G
Gastroesophageal reflux disease = GERD
Gastrointestinal = GI
Gastrin 17 = G17
Gastrin 34 = G34
Gastrin 17-diptheria toxoid = G17DT
Glycine extended gastrin =G-gly
Glycine extended G17 = gly-17
Glycine extended G34 = gly-34

H
Helicobacter felis= H. felis
Helicobacter pylori = H. pylori
Heparin-binding epidermal growth factor = HB-EGF
High grade dysplasia = HGD

I
IκB kinases = IKK
Inhibitor of apoptosis = IAP
Insulin-gastrin = INS-GAS
Intestinal metaplasia = IM
Intraductal papillary mucinous neoplasms = IPMNs

J

K

L
Lymph node = LN

M
Messenger RNA = mRNA
Metaplasia-dysplasia-adenocarcinoma sequence = MCS
Metalloprotinases = MMPs
Mitogen-activated protein kinase = MAPK
Multiple intestinal neoplasms = Min
Mutated in colorectal cancer = MCC

N
Narrow band imaging = NBI
None steroidal anti inflammatory drugs = NSAIDs

O
Overnight = O/N

P
Pancreatic intraepithelial neoplasia PanINs
Pathogenicity island = PAI
Permeability transition pore = PTP
Phosphate buffered saline = PBS
Phosphatidylinositol-3,4,5-triphosphate = PIP3
Phosphorylated Akt = P-Akt
Polyoma virus middle T = PyMT
Prevention of spontaneous adenomatous polyp = Pre-SAP
Prostaglandin E_2 = PGE_2
Protein kinase C = PKC
Proton pump inhibitor = PPI

Q

R
Retinoblastoma = Rb
Room temperature = RT

S
Severe combined immunodeficiency = SCID
Small interfering RNA = siRNA
Somatostatin receptor = SSTR
Sydney strain 1 = SS1

T
Thromboxane A2 = TXA2
Thymidine phosphorylase = TP
Transforming growth factor = TGF
Tumour associated macrophages = TAM
Tumour necrosis factor = TNF
TNF receptor 1 = TNFR1
TNF related apoptosis-inducing ligand receptor 1 = TRAIL-R1
TNF related ligand receptor 2 = TRAIL-R2
Tumour node metastasis system = TNM

U

V
Vacuolating cytotoxin A = VacA
Vascular endothelial growth factor-receptor 2 = VEGF-R2
Vioxx gastrointestinal outcomes research = VIGOR

W
World health organisation = WHO

X
X-linked inhibitor of apoptosis = XIAP

Y

Z

Acknowledgements

My first thanks go to my supervisor Professor Sue Watson for believing in me and keeping me going when the end seemed unreachable.

Next, I must thank the members of the Division of Pre-Clinical Oncology, both past and present who have supported me in my lab work; Phil Clarke for all things histology related, Andy McKenzie for help with radio isotope assays, Anna Grabowska for her support and guidance, Jane Moffat for formatting, as well as fellow technicians and students Jane, Marian, Jamie, Jenny, Jacqueline and Mo.

Also those outside of my department, Raj Singh and Mark Aloysius who obtained samples for me and Abed Zaitoun for invaluable pathology advice and assistance.

Finally, thanks go to my family and husband, Barney, for their continuing support and encouragement.

Abstract

Introduction

The polypeptide hormone gastrin promotes the growth of cancer cells through the gastrin/CCK-2 receptor (CCK-2R) and is implicated in the development, progression and invasion of a number of cancer types, including all GI malignancies. The aim of this thesis was to establish correlations between gastrin, the CCK-2R and pro-carcinogenic factors in the oesophageal and pancreatic cancer setting with a view to assessing their importance as prognostic biomarkers. Further to this, transgenic animal models of gastric and intestinal cancer were used to demonstrate the therapeutic effects of gastrin neutralisation in comparison to a standard of care, COX-2 inhibition. The interactions of gastrin, the CCK-2R and *Helicobacter pylori,* the pro-carcinogenic bacterium were also investigated.

Methods

This thesis focused on correlations between gastrin and the CCK-2R and key tumourigenic pathways including, pro-angiogenic factors, e.g. blood vessel density and VEGF-R2 and anti-apoptotic factors e.g. Bcl-2 and XIAP. This involved use of (i) tumour microarrays of human primary pancreatic cancer and lymph node metastases and pancreatitis and (ii) biopsies of Barrett's oesophageal metaplasia and dysplasia together with normal oesophageal epithelium. Following immunohistochemical characterisation of the array of factors, correlations with gastrin parameters were performed using Spearman 2-tailed correlation with Bonferroni correction.

For therapeutic studies, a gastrin immunogen G17DT, which induces the production of neutralising anti-gastrin antibodies was administered to INS-GAS and *Apc*$^{Min/+}$ mouse models of gastric and colorectal carcinogenesis respectively in comparison with clinically used agents such as, COX inhibitors (aspirin and celecoxib). The INS-GAS model was also utilized to study the effect of *Helicobacter* infection and gastrin interactions on gastric cancer progression.

Blood was collected from Barrett's patients and used to determine serum gastrin levels using radio isotope assay.

Results

All Barrett's patients studied had elevated serum gastrin levels as a result of being on proton pump inhibitors (PPIs), as well as high levels of CCK-2 receptor protein. There were positive correlations between anti-apoptotic markers XIAP and p-Akt with serum gastrin, and between p-Akt and CCK-2 receptor protein expression. There were also positive correlations between the pro-angiogenic marker VEGF-R2 and CCK-2 receptor protein expression as well as blood vessel density, another indicator of angiogenesis, and serum gastrin. These results indicate a role for the CCK-2 receptor as a prognostic marker alongside other pro-carcinogenic factors and identify those patients in which hypergastrinaemia may have the potential to impact on the rate of progression.

In pancreatic cancer CCK-2 receptors were shown to be increased 4-fold in pancreatic cancer compared to the normal pancreas, with a key finding being that endothelial cells expressed greater levels of the CCK-2 receptor protein in pancreatic cancer tissue compared to normal pancreas. Also key was the positive correlation between CCK-2 receptor expression on endothelial cells and tumour recurrence along with the negative correlation between CCK-2 receptor expression on endothelial cells and survival. There was also a positive correlation between CCK-2 receptor protein expression and XIAP although no correlation was seen with the other anti-apoptotic marker Bcl-2, but Bcl-2 protein expression was significantly increased in pancreatic cancer compared to normal pancreas.

In vivo G17DT immunisation reduced angiogenesis, proliferation and COX-2 protein expression in INS-GAS mice, but only angiogenesis in *Apc*$^{Min/+}$ mice. COX inhibition significantly reduced proliferation and angiogenesis in both the *Apc*$^{Min/+}$ mice, and INS-GAS mice, suggesting that inhibition of gastrin maybe a partial substitute for the potential shown by COX-2 inhibitors, withdrawn due to cardiac toxicity. Results in the INS-GAS mouse also demonstrated that *Helicobacter* infection promotes carcinogenesis in this model to a greater extent than hypergastrinemia alone.

Conclusion

The expression of gastrin and its receptor the CCK-2R may provide an additional prognostic biomarker in GI cancer to demonstrate potential issues in patients with hypergastrinaemia through PPI treatment or *Helicobacter*

infection and its neutralisation or that of its receptor may provide a mechanism to limit any potential deleterious effects of hypergastrinaemia as well as provide some of the therapeutic benefits seen with COX-2 inhibitors.

Chapter 1

INTRODUCTION

Cancer, as defined by the American Cancer Society, is a

"group of diseases characterised by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death."

1.1 Carcinogenesis

Carcinogenesis is the process by which normal cells become cancerous. The division of cells occurs in all most all tissue and to insure the integrity of both organs and tissue the balance between cell proliferation and cell death , normal by apoptosis ,a form of programmed cell death, is tightly regulated under normal circumstances. However if this sensitive balance is disrupted then uncontrolled cell division occurs, this is due to mutations in the cells DNA, this mutated DNA is then replicated in resulting daughter cells further propagating uncontrolled cell division (Elmore 2007).

Original research indicated that one mutation was enough to lead to carcinogenesis, but more recent work has shown that multiple mutations are required to transform a normal cell to a cancer cell, and that these mutation have to occur in genes vital to cell division, apoptosis and DNA repair (Sieber, Heinimann *et al* 2005). There are two types of genes which hold the key to carcinogenesis, they are proto-oncogenes and tumour suppressor genes. Proto-oncogenes and tumour suppressor genes are discussed in more detail in section 1.1.1. Details a number of pathways that have been shown to be linked to carcinogenesis are shown in fig.1.1.

Substances which are directly linked to cancer or it's propagation are termed carcinogens. Carcinogens have the ability to either damage genomic DNA or disrupt metabolic processes within a cell. Common examples of carcinogens include asbestos, tobacco and gamma radiation. Cancers have

also been demonstrated to originate from viral infection, but this is more of a phanominom in other animals than humans, with 12% of human cancers being linked to viral infection (Carrillo-Infante, Abbadessa 2007). Genetic abnormalities which promote carcinogenesis can also occur randomly due to errors in DNA replication or can be inherited.

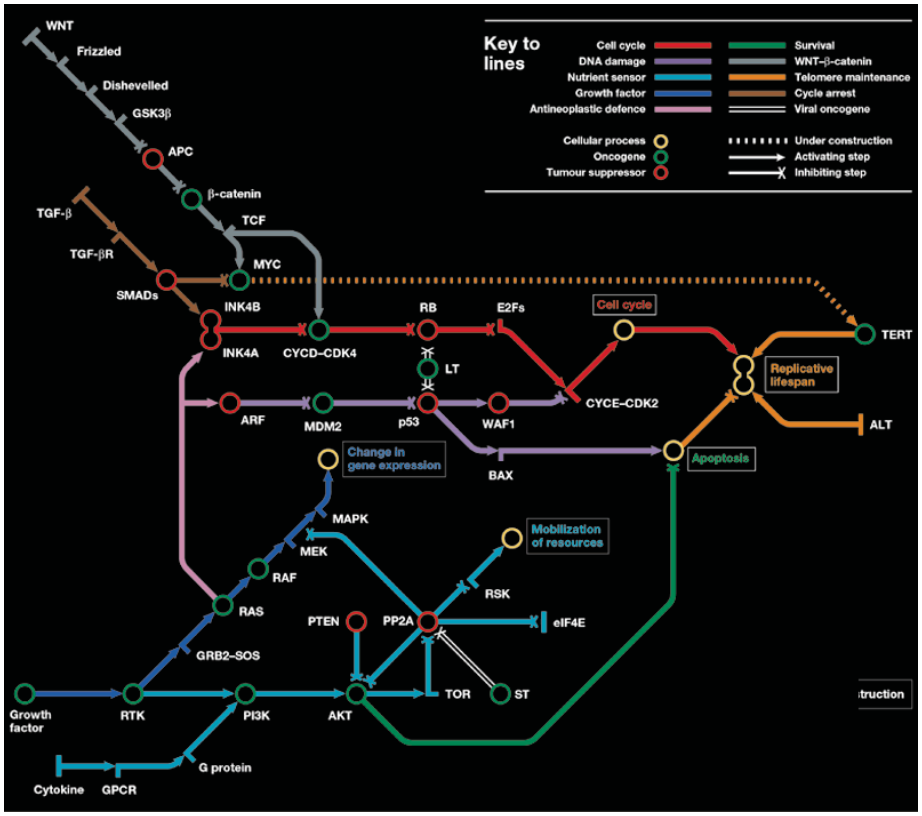


Fig. 1.1 A diagrammatic representation of cancer pathways (Hahn 2002).

1.1.1 Oncogenes and tumour suppressors

Proto-oncogenes are normal genes whose function is activated or enhanced by mutation. Mutated proto-oncogenes are referred to as oncogenes and their resulting protein is termed an oncoprotein. Oncogenes either cause an

over-expression of an oncoprotein which is structurally identical to the wild-type protein or structurally alter the protein and therefore its biochemical properties (Todd and Wong 1999). Examples of proto-oncogenes include *cMyc*, which was first identified in Burkitt's lymphoma and has since been associated with a number of human malignancies (Boxer and Dang 2001; Pelengaris, Khan *et al*. 2002) and *Ras* which has been identified in pancreatic, colon and lung cancers (Bos, Fearon *et al*. 1987).

Genes whose cellular function is inactivated following mutation are termed tumour suppressor genes. Mutations in tumour suppressor genes remove the natural cellular constraints on cell proliferation, cell adhesion and other cellular behavioural functions. The majority of cancers have been shown to have mutations in the tumour suppressor gene *p53* which encodes for p53 and is often referred to as the "guardian of the genome". Normal p53 monitors the integrity of the genome and in the event of DNA damage p53 accumulates to arrest cell development at the G1 stage to allow extra time for repair, if the cell is unable to repair then p53 prompts cell death by apoptosis (Yonish-Rouach, Resnitzky *et al*. 1991; Lane 1992). When p53 is inactivated by mutation or binding to host or viral proteins it is unable to promote programmed cell death, which leads to an accumulation of mutations and chromosomal rearrangements (Lane 1992). An example of the effects of defective p53 can be seen in p53 knockout mice which develop numerous tumours by the age of 6 months (Donehower, Harvey *et al*. 1992).

From early studies it appeared that cancer may arise from as few as one genetic change in the genome. However, later work has now indicated that cancer due to a single gene mutation is rare if not impossible, with work in rodents showing that oncogenes cooperate in pairs e.g. *Myc* and *Ras*, and that cells prepared *in vitro* with pairs of oncogenes drive tumour formation more rapidly than those observed in mice with only one oncogene (Hahn and Weinberg 2002).

1.1.2 Apoptosis

Apoptosis is a form of programmed cell death, which follows a series of biochemical events including blebbing, changes to the cell membrane, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation (Lowe and Lin 2000). An important feature of apoptosis is that there is no leakage of cell contents and therefore no inflammatory response.

The central component of apoptosis is a proteolytic family of proteases termed caspases. In response to pro-apoptotic signals enzymes participate in a cascade which culminates in the cleavage of a set of proteins and the disassembly of the cell (Thornberry and Lazebnik 1998).

There are a number of signals that appear important in initiating apoptosis these include, depletion of growth/survival factors, hypoxia, loss of cell

matrix interactions, DNA damage, telomere malfunction and inappropriate survival signals as a result of oncogenic mutations (Lowe and Lin 2000). In a high proportion of these examples it is the activation of p53 which triggers apoptosis e.g. when cells encounter hypoxic conditions it is the activation of p53 which promotes apoptosis, and if *p53* is mutated then cells can survive hypoxic conditions aiding clonal expansion within a tumour (McCurrach, Connor *et al*. 1997) and apoptosis due to telomere malfunction is triggered by activation of p53, therefore cells with mutant *p53* survive even though they are genetically unstable (Karlseder, Broccoli *et al*. 1999).

Activation of apoptosis can occur either through extrinsic (receptor) or intrinsic (mitochondrial) pathways (see fig 1.2). The extrinsic pathways are initiated by death receptors, which are members of the tumour necrosis factor (TNF) receptor gene superfamily and include CD95 (APO-1/Fas), TNF receptor 1 (TNFR1), TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1) and TRAIL-R2 (Fulda and Debatin 2006). Upon binding to their cognate ligand CD95 and the TRAIL receptors cause trimerization of the receptor and subsequent recruitment of adaptor molecules such as Fas-associated death domain (FADD) (Walczak and Krammer 2000). FADD then induces apoptosis through the activation of the cell death protease, caspase-8, which in turn initiates a protease cascade that cleaves cellular targets and causes apoptotic cell death (Lowe and Lin 2000). The intrinsic pathway is initiated by growth factors, cytokines and DNA damage. Its activation of caspase is tightly linked to permeabilisation of the outer mitochondrial membrane by members of the Bcl-2 family through the

permeability transition pore (PTP), the PTP is opened following apoptotic signalling and allows the release of cytochrome c from the mitochondria (Green and Reed 1998). Cytochrome c then interacts with Apaf-1 and pro-caspase-9 and initiates a protease cascade similar to that observed for the extrinsic pathway (Li, Nijhawan *et al*. 1997).

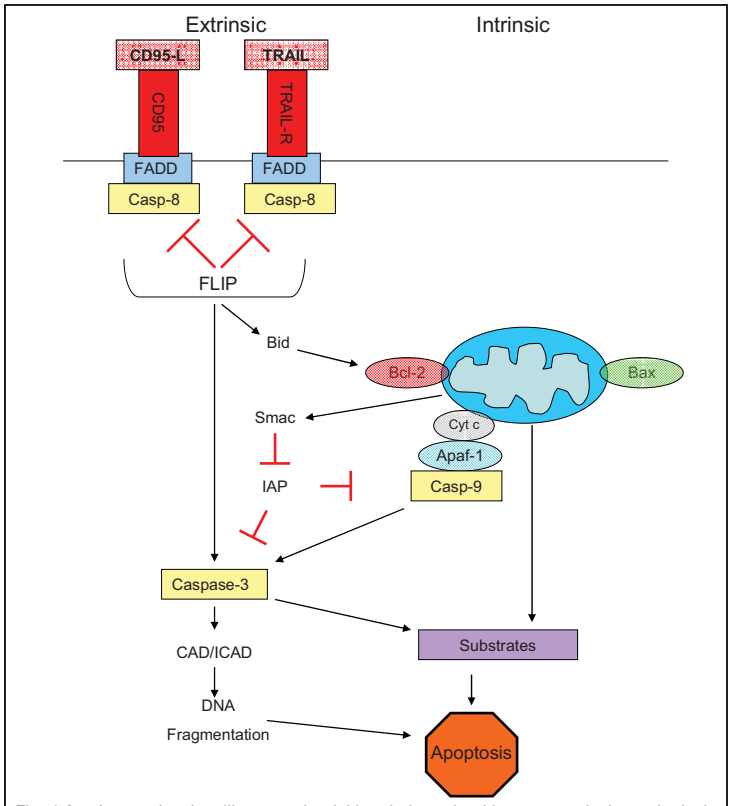


Fig 1.2 Apoptosis signalling can be initiated through either an extrinsic or intrinsic pathway. The extrinsic pathway involves the stimulation of death receptors e.g. CD95, and the recruitment of adaptor molecules and caspase-8. Caspase -8 is then activated and initiates apoptosis by direct cleavage of downstream effector caspases. The intrinsic pathway is initiated through stress signals resulting in the release of apoptogenic factors e.g. cytochrome c from the mitochondria into the cytosol and triggering caspase-3 activation. There is negative regulation of caspase activation in the extrinsic pathway by FLIP and the intrinsic pathway by Bcl-2 and IAP.

Studies in mice have shown a direct link between disruption of apoptosis and promotion of tumour development. For example Bcl-2 has been shown to promote lymphoproliferation in transgenic mice (McDonnell, Deane *et al*. 1989) and *bcl-2* transgenes have been shown to accelerate mammary carcinogenesis (Strasser, Harris *et al*. 1990). Bcl-2 is an oncogene, first

identified in the human leukaemia cell line 380, derived from a 16 year old male with acute pre-B-cell lymphoma (Tsujimoto, Finger *et al*. 1984), which promotes cell survival by blocking apoptosis (Hockenbery, Nunez *et al*. 1990).

1.1.3 Inflammation

It has long been thought that there is a link between inflammation and cancer with the observations by Virchow in the 1800's that tumours often arose at sites of chronic inflammation (Balkwill and Mantovani 2001). However, this view has not been favoured until recently with evidence immerging in support from both epidemiological studies in humans and molecular studies in animal models. Epidemiological studies have demonstrated that chronic inflammation can predispose individuals to some cancers. Worldwide 15% of all cancer deaths are due to infectious agents with chronic inflammation being a major contributing factor (Balkwill and Mantovani 2001) (see table 1.1). Animal models of induced gastric cancer via gastritis caused by infection with *Helicobacter pylori* (see section 1.2.5) (Houghton, Stoicov *et al*. 2004) and work in Mdr2-Knockout mice which develop hepatocellular carcinoma through the sequence of inflammation, dysplasia, dysplastic nodules, carcinoma and metastasis (Pikarsky, Porat *et al*. 2004) have also strengthened the argument for the role of inflammation in cancer development.

Malignancy	Inflammatory stimulus
Bladder cancer	Scistosomiasis, urinary catheters
Cervical cancer	Chlamydia
Colorectal cancer	Inflammatory bowel disease
Gastric cancer	*H. pylori* induced gastritis
Hepatocellular carcinoma	Hepatitis B and C virus
Kaposi's sarcoma	Human herpes virus type 8
Oesophageal cancer	Barrett's metaplasia
Ovarian cancer	Endometriosis

Table 1.1 Chronic inflammatory conditions linked to predispositions to cancer. Adapted from Balkwill *et al* 2005 and Coussens and Werb 2002.

The original theory was that it was enhanced cell proliferation which led to cancer (Balkwill and Mantovani 2001), but it has now been established that it is the sustained proliferation of cells within an environment which is rich in inflammatory cells, growth factors, activated stroma and DNA-damage promoting agents which can promote neoplastic risk (Coussens and Werb 2002). Under normal physiological conditions injury to a tissue activates the innate immune system leading to enhanced proliferation while the tissue regenerates. Once the injury is repaired then proliferation and inflammation reseed. However, if the proliferating cells have sustained DNA damage, they may not cease to proliferate after injury repair, but instead continue to proliferate in a microenvironment which is rich in inflammatory cells and growth/survival factors (Coussens and Werb 2002). As described by Dvorak (Dvorak 1986) tumours can be considered wounds that do not heal.

Macrophages are key components of the innate immune system, they are differentiated monocytes bought to the injury site by chemo-attractant cytokines termed chemokines and once activated are the main source of growth factors and cytokines in the local microenvironment which influence endothelial, epithelial and mesenchymal cells (Coussens and Werb 2002). Macrophages can change their functional profile repeatedly in response to their environment (Watkins, Egilmez *et al*. 2007) Depending on the response macrophages are either termed M1 or M2, M1 macrophages have anti-tumour activity and are triggered by exposure to lipopolysaccharides or IFN-γ, whereas exposure to IL-4 and IL-3 cause macrophages to become M2 and support tumour growth (Mantovani, Sozzani *et al*. 2002).

In nearly all tumours the major constituent of the infiltrate is tumour-associated macrophages (TAM), macrophages which reside in the tumour microenvironment. TAMs are M2 type macrophages, whose survival can be prolonged by cytokines produced by the tumour (Sica, Schioppa *et al*. 2006). Under normal activation conditions TAMs can kill tumour cells or destroy tissue based on vascular endothelium reactions following activation of IL-2, interferon and IL-12 (Balkwill and Mantovani 2001; Brigati, Noonan *et al*. 2002; Tsung, Dolan *et al*. 2002). However, TAMs have also been shown to promote tumour cell proliferation, angiogenesis, metastasis and activate oncogenes in cancer cells (Mantovani, Bussolino *et al*. 1992; Chen, Yao *et al*. 2003). The role of TAMs has been highlighted in the transgenic Polyoma virus middle T (PyMT) mouse model, which develop mammary cancer. When PyMT mice have a mutated CSF-1 gene, a gene

involved in macrophage recruitment, the development of late stage invasive carcinoma and pulmonary metastases is significantly attenuated (Wahl and Kleinman 1998).

Macrophages are not the only inflammatory cells to be linked to carcinogenesis, studies have also indicated the involvement of neutrophils, mast cells, eosinophils and activated T-lymphocytes, which are thought to contribute to malignancy by releasing extracellular proteases, pro-angiogenic factors and chemokines (Coussens and Werb 2002).

1.1.4 Angiogenesis

Angiogenesis itself is a normal process for the formation of new blood vessels in developing organs and as part of wound healing and in these circumstances it is a highly regulated process. However, unregulated angiogenesis has been linked to a number of diseases, including arthritis, diabetes related blindness and tumour development (Folkman 1990). In a normal situation blood vessels are established from endothelial precursors, this network is then expanded on by sprouting or intussusceptions, where interstitial tissue columns are inserted in to the lumen of pre-existing vessels and the lumen partitioned (Patan, Munn, *et al* 1996). In a tumour situation vessels develop by sprouting or intussusceptions of pre-existing vessels and tumour cells have also been shown to grow around existing vessels to form a perivascular cuff (Yancopoules, Davis *et al* 2000).

Angiogenesis allows tumours to grow beyond the limit otherwise imposed by the pre-existing vasculature and is considered a vital step in the evolution of solid tumours (Hanahan and Folkman 1996). The vascularisation of tumours has been a known phenomenon since the turn of the 1900's, but the mechanism of their eliciting new capillary growth from the host was not realised until the 1950's (Merwin and Algire 1956). Current understanding is that when levels of pro- and anti-angiogenic molecules are in a balanced state then the angiogenic switch is "off" and is only switched "on" when this balance is swayed in the favour of pro-angiogenic molecules (Bouck, Stellmach *et al* 1996). This switch can be triggered be a number of situations including hypoxia, inflammation, the activation of oncogenes and inactivation of tumour suppressor genes (Kerbel 2000).

Vessels created as a result of tumour angiogenesis are not as well constructed or organised as those created during normal angiogenesis due to a poorer coordination of regulatory factors. Tumour vessels lack some of the normal protective mechanisms usually acquired during growth, their walls tend to have numerous holes with a discontinuous or absent basement membrane and inter endothelial junctions are inferior (Hashizume, *et al* 2000, Hobbs, Monsky *et al* 1998).

Members of the pro-angiogenic vascular endothelial growth factor family (VEGF) have been shown to play a prominent role in tumour angiogenesis, causing the initial vessel dilation seen during angiogenic sprouting, as well

as stimulating endothelial cell proliferation, migration and assembly (Yancopoules, Davis *et al* 2000).

1.2 Gastrointestinal (GI) cancer

GI cancers account for 20% of all cancer incidences worldwide (MacFarlane and Stover 2007). GI cancer is a term that encompasses malignant conditions of the GI tract including the oesophagus, stomach, liver, intestines and anus as well as the biliary glands and pancreas. In basic terms the GI tract is a long hallow tube which extends from the oral cavity to the anus. This tube is comprised of 4 layers; mucosa, submucosa, muscularis externa and serosa (fig 1.3).

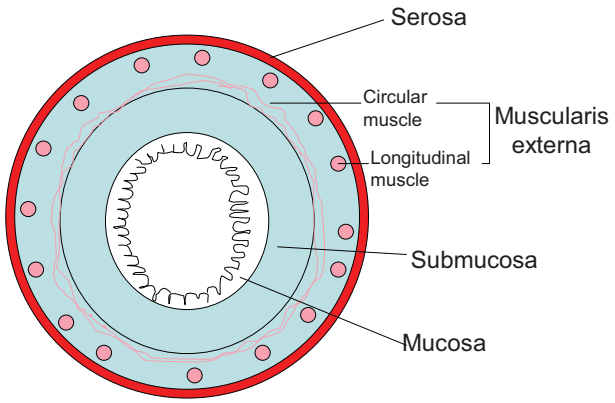


Fig. 1.3 Simplified diagrammatic representation of the layers which comprise the GI tract.

1.2.1 Incidence

Colorectal cancer is the 3rd commonest cause of cancer in the UK (www.cancerresearchuk.org) and the commonest form of GI cancer, followed by stomach (gastric), oesophageal and pancreatic (Fig 1.4).

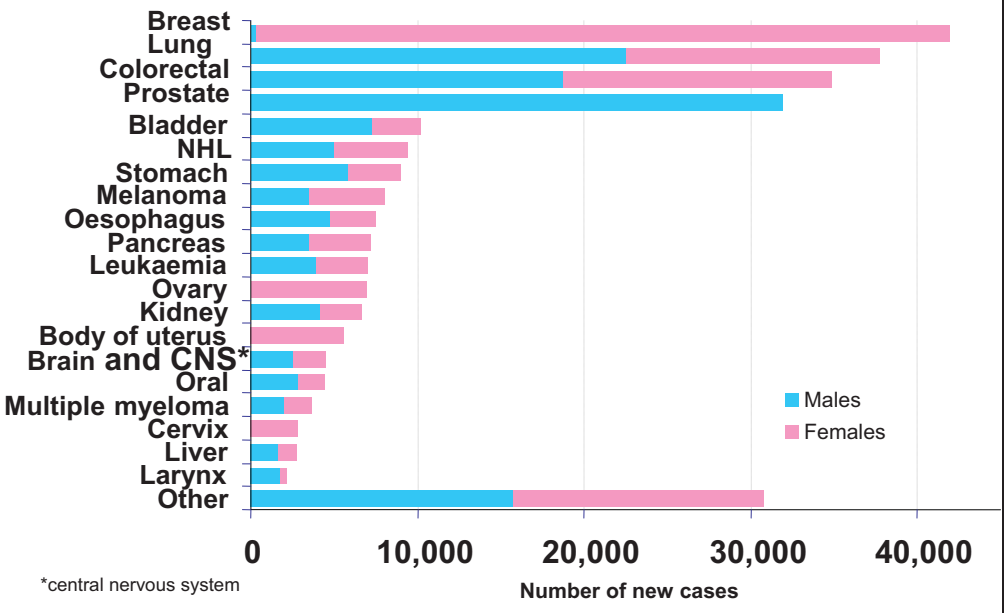


Fig. 1.4 The 20 most common cancers in the UK, 2002.

http://info.cancerresearchuk.org/cancerstats/incidence/commoncancers/

1.2.2 Prognosis and treatment

Overall the prognoses for GI cancers are poor; this is normally as a consequence of late presentation of the disease due to lack of symptoms in the early stages. Currently out of all the GI cancers the prognosis is best for

colorectal this is due to a number of screening programmes that have been established (Mitka 2008). The 5 year survival rate for GI cancers is poor and treatment follows the pattern of surgery, where appropriate, followed by either chemotherapy, radiotherapy or both. For these reasons therapeutic modalities are urgently needed in this area.

1.2.3 Molecular pathways involved in GI cancers

A number of molecular pathways have been highlighted as being important in the development, progression and metastasis of pancreatic cancer; these include Ras-Raf-MEK-ERK, PI3K/Akt and NFκB.

1.2.3.1 Ras-Raf-MEK-ERK pathway

The Ras-Raf-MEK-ERK pathway is one of the Mitogen-Activated Protein Kinase (MAPK) pathways that transfer and amplify messages from the cell surface to the nucleus, triggering a range of responses including cell proliferation, differentiation and survival (Kolch 2000). A systematic representation of the pathway can be seen in fig.1.5.

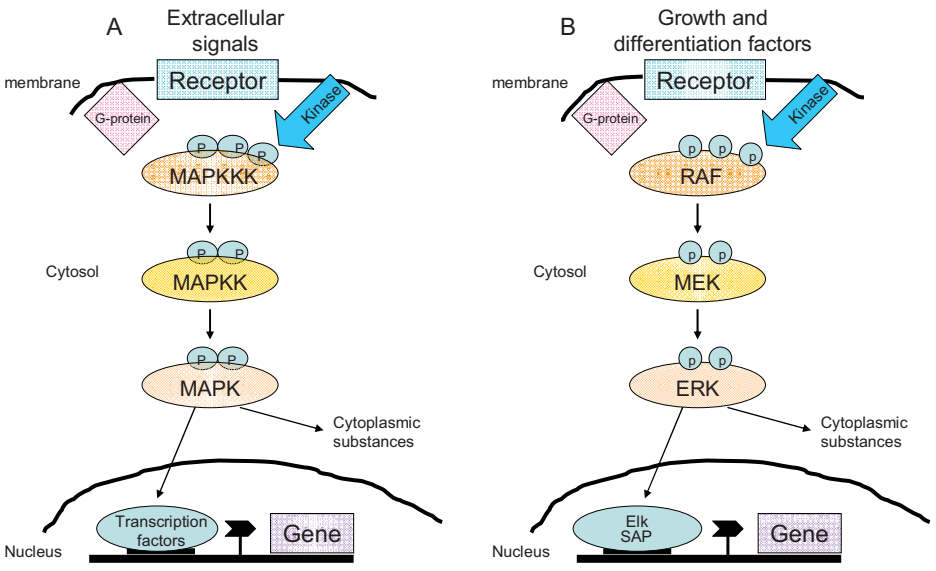


Fig. 1.5 A systematic representation of MAPK pathways adapted from Kolch 2000.

1.2.3.2 The PI3K/Akt pathway

The PI3K/Akt pathway, like the Ras-Raf-MEK-ERK, regulates many of the normal cellular processes that are critical for tumorigenesis. PI3K catalyses the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) at the membrane of the cell. PIP3 then helps in the recruitment and activation of a number of downstream targets including Akt (Luo, Manning *et al*. 2003) as represented in fig.1.6. Akt promotes cell survival by inhibiting proapoptotic factors e.g. Bad, forkhead and p53 and activating pro-survival protein such as NFκB. Akt regulates the cell cycle progression by indirectly stabilising cyclin D1 and Myc. Akt stimulates protein synthesis and cell growth by activating the MTOR through inhibition of tuberin/hamartin complex.

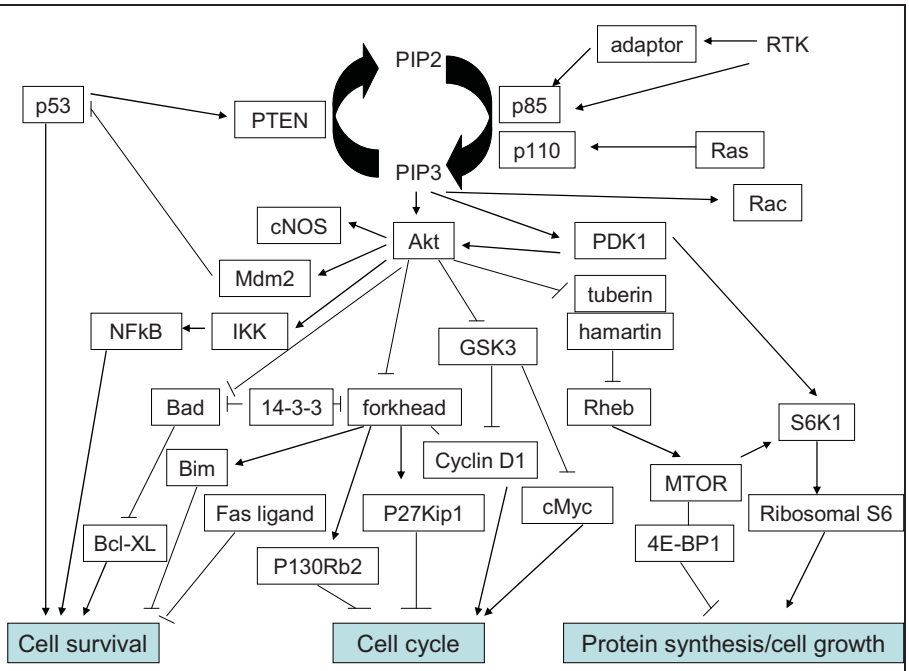


Fig.1.6 Systematic representation of PI3/Akt pathway adapted from Luo, Manning *et al* 2003 for more detailed description see text.

1.2.3.3 The NFκB pathway

NFκB is a family of transcription factors which are involved in the regulation of a number of biological processes. In normal cells activation of NFκB only occurs after appropriate stimuli and results in the up-regulation of transcription of the target genes, afterwards NFκB is returned to its inactive state. Therefore the activation of NFκB is both an inducible and transient process. In tumour cells molecular alterations cause impaired regulation of NFκB activation, resulting in a loss of inducability and constitutive activation of NFκB (Dolcet, Llobet *et al*. 2005).

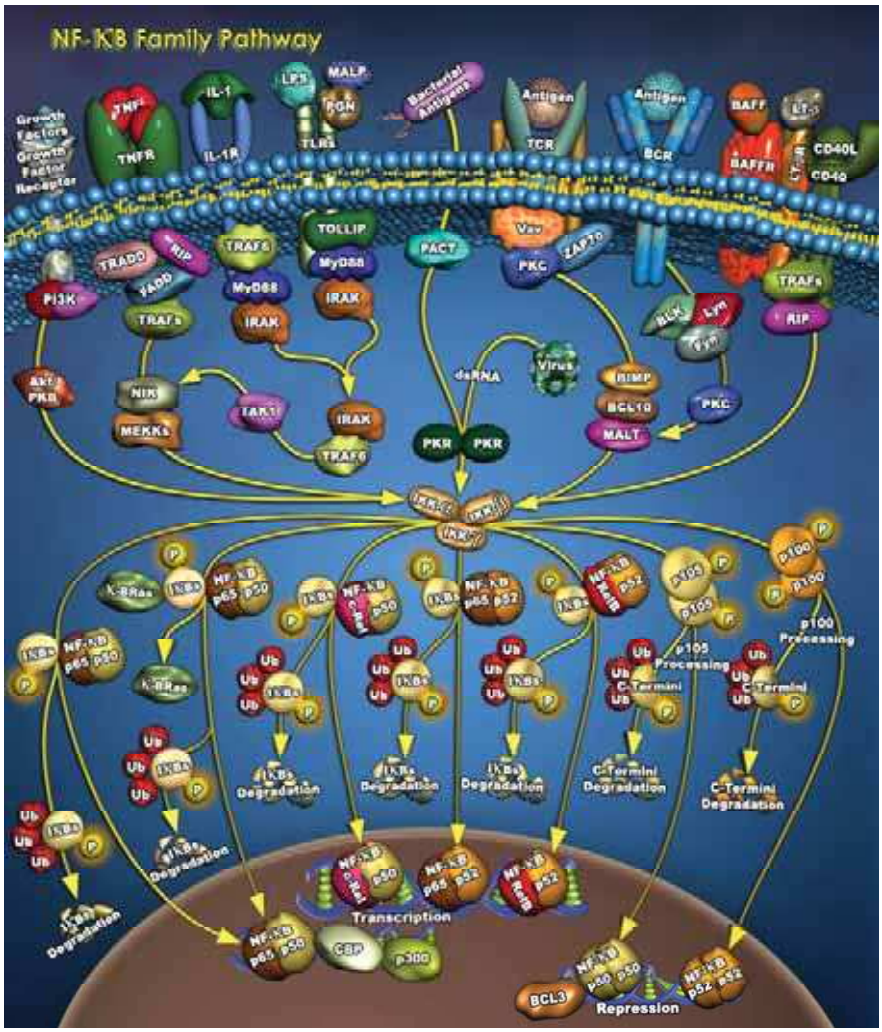


Fig. 1.7 Overview of the NF*k*B pathway taken from www.ambion.com/tools/pathway/pathway.php?pathway=NF-KappaB%20Family%20Pathway on 2.1.08

Fig 1.7 shows an overview of the NFκB pathway. A key step in this pathway is the phosphorylation of the IκB, which is regulated by IκB kinases (IKK). The IKK complex is comprised of two kinase subunits, IKKα and IKKβ, and an associated modulatory protein IKKγ. Phosphorylation of specific residues in the activation loop of each IKK subunit results in phosphorylation of the IκB proteins, causing ubiquitation and subsequent degradation by 26S proteasome (Brockman, Scherer *et al*.

1995; Lin, Brown *et al*. 1995). This process leads to NFκB translocation to the nucleus, where it binds to the promoter regions of the target genes resulting in gene expression (Yamamoto and Gaynor 2001).

The transcriptional activation of NFκB can also be regulated by the phosphorylation of p65, a member of the Rel family. IKK's have been shown to directly phosphorylate the serine residue in the p65 transactivation domain, as well as either directly or indirectly with the ability of PI3 inducible Akt to stimulate p65 transactivation (Yamamoto and Gaynor 2001). Work by Heissmeyer (Heissmeyer, Krappmann *et al*. 1999) showed that IKK can also be activated by phosphorylation of the NFκB precursor p105 which leads to enhanced processing and nuclear translocation of p50.

NFκB is involved in a number of cellular processes including cell cycle control, stress adaptation, inflammation and control of apoptosis. Dysregulation of NFκB in tumour cells is thought to lead to tumorigenesis and promote metastasis, as well as being linked to chemoresistance (Wang, Abbruzzese *et al*. 1999; Arlt, Gehrz *et al*. 2003).

1.3 Gastric cancer

1.3.1 Classification

Early classification of gastric cancer was based on histological appearance, which divided gastric cancer into 2 subtypes: (1) intestinal or well-differentiated adenocarcinoma and (2) diffuse gastric cancer. Intestinal-type gastric cancer consists of gland-like structures that mimic intestinal glands, whereas the diffuse type is more poorly differentiated and lacks any gland structures. Intestinal type is more strongly linked to environmental and dietary factors, compared to diffuse type which is often familial in distribution (Lauren 1965).

Since this early classification, gastric cancer has also been classified based on mucin expression and this distinguishes 4 types of gastric cancer: The gastric or foveolar type (G-type), the intestinal type (I-type), the gastric and intestinal mixed type (GI-type) and the neither gastric or intestinal phenotypes (*N*-type) (Fiocca, Villani *et al*. 1987; Tatematsu, Ichinose *et al*. 1990; Tatematsu, Tsukamoto *et al*. 2003).

1.3.2 Incidence

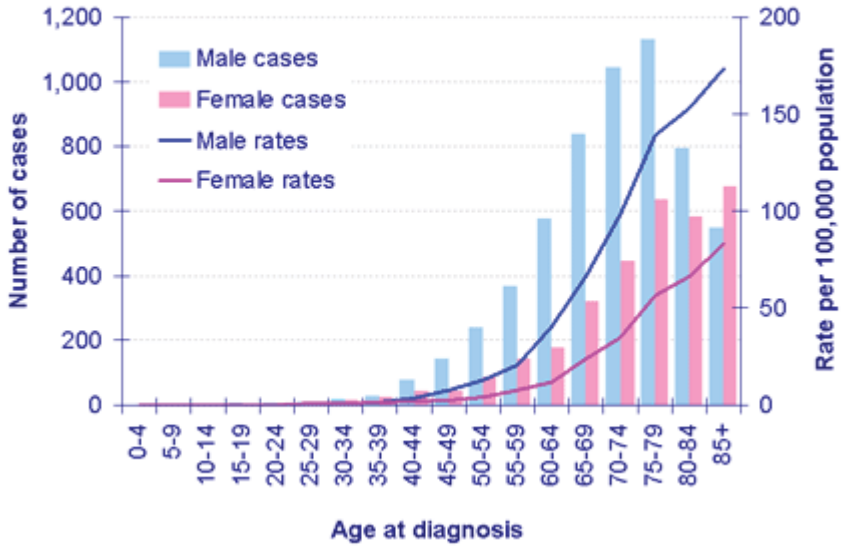


Fig. 1.8 The number of new cases of stomach cancer in the UK, 2002.

http://info.cancerresearchuk.org/cancerstats/incidence/commoncancers/

As shown in fig. 1.4, gastric (stomach) cancer is the 7th most common cancer in the UK, causing nearly 6000 deaths a year with risk increasing with age (fig. 1.8).

In recent years the incidence of gastric cancer has appeared to be falling in both the US and Western Europe. This is thought to be as a result of a decrease in the frequency of distal lesions and an increase in the frequency of adenocarcinoma of the proximal stomach. The reason for this fall in the US and Western Europe and not in the far East, South America, central Europe and developing countries, is thought to be in part due to dietary

change, for example increased consumption of fresh fruit and vegetables and a decrease in the consumption of salt (Levi, Lucchini *et al*. 2004).

Gastric cancer, specifically the intestinal type adenocarcinoma, develops through a series of discrete steps described by Correa (Correa 1992) now known as the atrophy-metaplasia-dysplasia-carcinoma sequence. This sequence involves the slow transformation of the gastric mucosa via chronic gastritis, multifocal and glandular atrophy, various degrees of intestinal metaplasia and dysplasia (fig. 1.9).

Diffuse type adenocarcinoma are not typically associated with these changes and are often present with diffuse thickening of the stomach wall, rather than a discernible mass (Solcia, Fiocca *et al*. 1996).

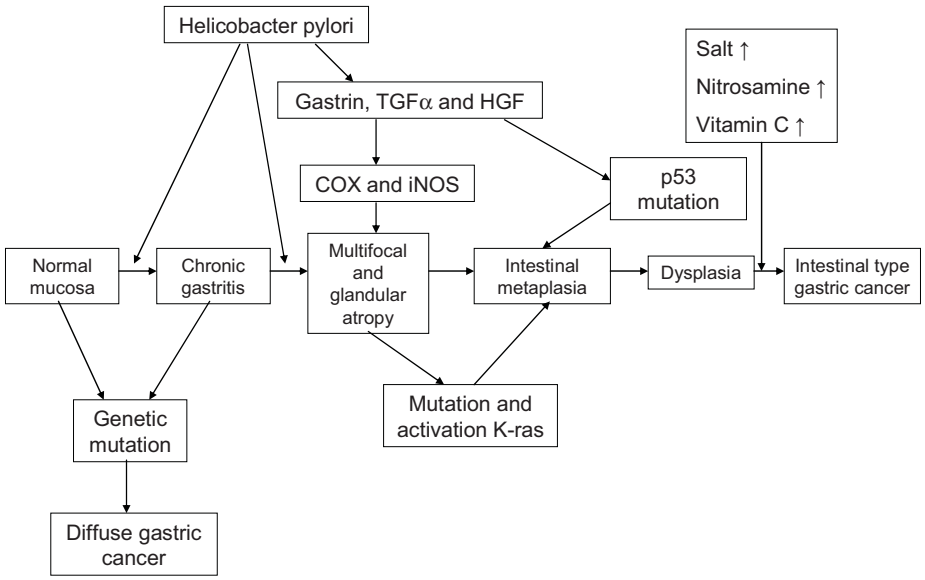


Fig. 1.9 Correas cascade showing pathological changes from gastritis to gastric dysplasia to gastric cancer. Adapted from Konturek 2003.

1.3.3 Prognosis

The prognosis for gastric cancer is poor, as 80% of patients are asymptomatic during the early stages, with most cases only being discovered after local invasion has advanced. It is due to these reasons that the 5 year survival rate for gastric cancer is low, being approximately 20%. However early tumour resection can achieve 5 year survival rates of around 90% (Karpeh M. 2001; Layke and Lopez 2004).

1.3.4 Genetics

Distinct genetic changes appear to be associated with distinct phenotypes, particularly in the case of the I-type and the G-type. In the I-type gastric cancer, *p53* mutations and allelic deletions of the *Adenomatous Polyposis Coli* (*APC*) gene are seen more frequently than in the G-type gastric carcinoma (Muller, Dammann *et al*. 1989; Uchino, Noguchi *et al*. 1993; Kushima, Muller *et al*. 1996; Wu, Kushima *et al*. 1998; Endoh, Sakata *et al*. 2000). In contrast, microsatellite instability is seen more often in the G-type than the I-type gastric cancer (Endoh, Sakata *et al*. 2000). As well as this, alterations of *p73* appear more predominately associated with the G-type gastric cancer (Yokozaki, Shitara *et al*. 1999).

The majority of gastric cancers are sporadic, but approximately 1-3% of all gastric cancer cases are linked to hereditary gastric cancer predisposition

(Inberg, Lauren *et al*. 1965). Germline mutations in the *CDH1* gene, which produces E-cadherin, account for a third of all hereditary diffuse gastric cancers. Other genetic mutations that are linked to an increased risk of gastric cancer are mutations of the *p53*, *APC* and *BRCA2* genes, which are all involved in tumour suppression (Watanabe, Enjoji *et al*. 1978; Jakubowska, Nej *et al*. 2002; Oliveira, Ferreira *et al*. 2004). The molecular nature of a major proportion of familial gastric cancer is presently unknown (Oliveira, Seruca *et al*. 2003).

1.3.5 Risk factors

As well as genetics there are also other risk factors associated with gastric cancer; they include age, gender, race and diet. Gastric cancer is observed more often in males over 60yrs of African American origin who have a high salt, low fruit and vegetable diet (Layke and Lopez 2004). In addition to this individuals with a history of gastritis have also been shown to be at increased risk (Sipponen, Kekki *et al*. 1985).

Since 1994, when the World Health Organisation (WHO) classified *Helicobacter pylori* (*H. pylori*) as a class 1 pathogen, it has become clear that this bacterium is one of the most prominent risk factors for spontaneous gastric cancer and is also linked to peptic ulcer disease (1994).

First isolated in 1982 by Australian scientists Barry Marshall and Robert Warren (Marshall and Warren 1984) and originally classified as *Campylobacter pylori* due to its similarities to the Campylobacter genus and being found in the pyloric region of the stomach (Pajares and Gisbert 2006). Subsequently a number of experiments were carried out to confirm this classification, these included electron microscopy (Jones, Curry *et al*. 1985), measuring the percentage of guanine and cytosine in bacterial DNA and fatty acid content, as well as sequencing the 16s ribosomal RNA and carrying out DNA-DNA hibridation. This led to its reclassification as *Helicobacter pylori* in 1989 (Pajares and Gisbert 2006).

H. pylori is a gram negative spiral shaped bacterium with a distinctive morphology (fig. 1.10). It is unipolar with a length of between 2.5-4μm and a width of 0.5-1μm (Goodwin and Armstrong 1990), with 1-4 flagella at one pole. Each flagella is 2.5μm long and 30nm wide with a membranous terminal bulb, which is essentially an extension of the outer membrane and is used for motility (Josenhans, Labigne *et al*. 1995).

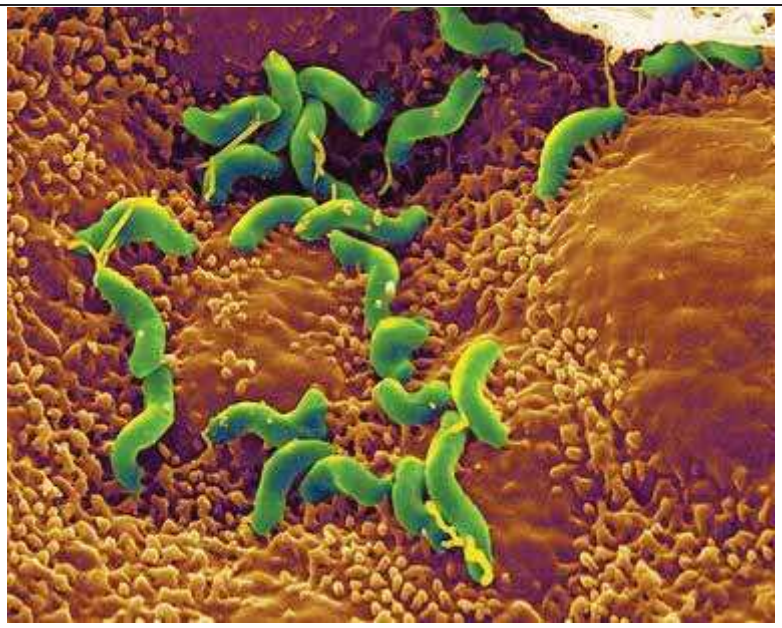


Fig 1.10 Electronmicrograph of *Helicobacter pylori* taken from www.migg.files.wordpress.com

Colonisation of the gastric mucosa by *H. pylori* alters the events of the gastric epithelial cell cycle, which may affect the threshold of carcinogenesis (Martin and Green 1995; Suzuki, Mori *et al*. 1997; Suzuki, Seto *et al*. 1998). *H. pylori* is thought to colonise around half the world's population, however not all those infected go on to develop gastric cancer, which may in part be due to strain specific virulence factors (Danesh 1999).

1.3.5.1 Helicobacter Pathology

In colonising the acidic environment of the stomach *H. pylori* has found a niche, in which most bacteria would not survive. One of the ways it overcomes the acidity of the environment gastric is to embed itself in the

mucus layer of the gastric mucosa, creating a physical barrier. However, before it can do this it first has to overcome the initial exposure to acidity, resist peristalsis and gain entry to the protective mucosa. When the circular genome sequence for *H. pylori* was completed it showed the presence of approximately 1500 proteins, many of which are responsible for overcoming the problems previously stated (Tomb, White *et al*. 1997).

1.3.5.2. Urease

H. pylori possess an urease enzyme, which since it is not an acidophile, is key to its survival in the median pH1.4 of the stomach. Urease is a cytosolic metalloenzyme with a dodecameric quaternary structure and a molecular weight of 550kDa. *H. pylori* expresses a urea transport protein with unique acid-dependent properties that activates the rate of urea entry to the cytoplasm The urea is taken up into the cytoplasm by a proton-gated channel and here the urease enzyme catalysis its hydrolysis to ammonia and carbon dioxide (Weeks, Eskandari *et al*. 2000). The resulting ammonia serves to buffer the cytosol and periplasm as well as creating a neutral layer around the bacterial surface (Scott, Marcus *et al*. 2002).

The significance of urease was demonstrated by Eaton *et al* (Eaton, Brooks *et al*. 1991) when they showed that bacteria deficient in the enzyme were unable to colonise the gastric mucosa. Further relevance of this enzyme was confirmed when its percentage of approximately 5% (already considered

high) of the bacteria's total protein content increased to 10% under certain culture conditions (Bauerfeind, Garner *et al*. 1997).

1.3.5.3*Cag* pathogenicity island (PAI)

H. pylori produces a number of virulence factors, one of which is the *cag* PAI, which is present in around 60% of US strains (Tomb, White *et al*. 1997), with *cag*+ strains linked to an increased risk of atrophic gastritis and distal gastric adenocarcinoma compared to *cag*- strains (Crabtree, Wyatt *et al*. 1993; Kuipers, Perez-Perez *et al*. 1995).

The *cag* PAI contains around 30 genes, which are inserted into the glutamate racemase gene. It is divided into two regions, termed cag I and cag II located either side of the intervening sequence (Censini, Lange *et al*. 1996; Busler, Torres *et al*. 2006).

Of the 30 plus genes on the cag PAI several of them encode products with similar homology to components of type IV secretion systems. The type IV secretion system acts as a syringe and following *H. pylori* adherence to epithelial cells *in vitro* the product of the terminal gene in the island, cytotoxin associated gene A (*cagA*), is translocated into the host cell where it under goes Src-dependent phosphorylation and activates a phosphatase (SHP-2), leading to cellular morphological changes of the host cell (Kusters, van Vliet *et al*. 2006).

The CagA protein is 145 kDa and causes a number of the cytoskeletal rearrangements which leads to the formation of the attachment pedestal, which is integral to the bacterium's adhesion ability (Segal, Cha *et al*. 1999; Figueiredo, Machado *et al*. 2005).

Orsini *et al* (Orsini, Ottanelli *et al.* 2003) showed higher gastric inflammation in patients infected with *cag* PAI positive *H. pylori* strains compared to *cag* PAI negative strains. Similar finding have also been reported in animal models (Thompson, Danon *et al*. 2004).

1.3.5.4 Vacuolating cytotoxin A (VacA)

Another important virulence factor is VacA, which causes cytoplasmic vacuolation of gastric epithelial cells (Smoot, Resau *et al*. 1996). VacA is approximately 95kDa and is produced by about 60% of the world's wild type *H. pylori*. Strains that produce VacA are classed as toxigenic and those that do not non-toxigenic.

The *vac*A gene is naturally polymorphic and differences are present mainly in two areas: the signal region, encoding the signal peptide and the N terminus of the mature protein (which maybe either s1 or s2 type) and the mid region, encoding the part of the p58 domain (m1 or m2 type) (Atherton, Cao *et al*. 1995). In HeLa cells s1/m1 strains have been shown to cause

more extensive vacuolation than s1/m2 strains, with s2/m2 being non-vacuolating (Atherton, Cao *et al*. 1995). This maybe of clinical importance as s2/m2 strains are less frequently associated with peptic ulceration and gastric carcinoma compared to s1/m1 or s1/m2 strains (Atherton, Peek *et al*. 1997; Rudi, Kolb *et al*. 1998; Strobel, Bereswill *et al*. 1998; van Doorn, Figueiredo *et al*. 1998)

Satin *et al* (Satin, Norais *et al*. 1997) showed that VacA increases extracellular secretion of acidic hydrolases inducing large acidic vacuoles, it also impairs the degradative power of late endosomes and lysosomes. They concluded that a deficiency in intracellular digestion may create a condition similar to starvation in gastric cells, worsened by the associated deficiency in the lysosomal-targeting hydrolases, which in turn would contribute to cell damage induced by other bacterial products or by factors released during chronic inflammation.

1.3.6 Insulin-Gastrin (INS-GAS) mouse model of gastric carcinogenesis

INS-GAS mice are a transgenic model of gastric cancer that over-express the amidated form of gastrin (G17), and therefore have elevated levels of circulating G17. The increase in G17 is under the control of a rat insulin I promoter. These mice spontaneously develop atrophic gastritis, intestinal metaplasia and dysplasia, with adenocarcinoma observed in 75% of mice at an approximate age of 20 months. The development of adenocarcinoma can

be induced in 85-100% of mice in 8 months when infected with *H. felis* and in 100% of mice in 8 months when infected with *H. pylori* (Wang, Dangler *et al*. 2000).

1.4 Colorectal cancer

1.4.1 Development

Colorectal cancer is the result of malignant transformation of the colorectal epithelium resulting from a multistep, multipathway, multifocal process that requires sequential or concomitant damage to several genes within and across cellular generations. Colorectal cancers arise from single crypt lesions and progress to adenomatous polyps and later to invasive carcinomas (Fearon, Hamilton *et al*. 1987; Groden, Thliveris *et al*. 1991). The *adenomatous polyposis coli* (*APC*) gene mutation, either inherited or acquired, is considered an early and central event in the majority of cancers, which is then followed later by an accumulation of somatic mutations (Fearnhead, Wilding *et al*. 2002).

1.4.2 Incidence

Colorectal cancer is a common disease with an incidence rate of 5%, making it the third leading cause of cancer death worldwide and therefore an important public health issue. There have been significant improvements in five year survival from colon cancer over the last 30 years (fig. 1.11).

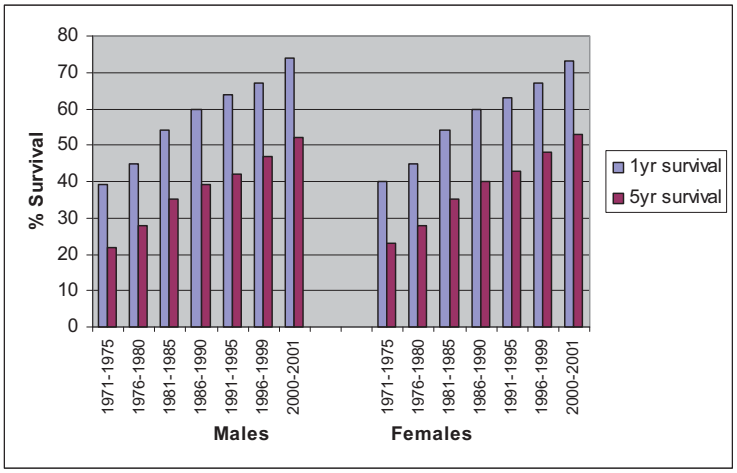


Fig 1.11 Relative age standardised 5 year survival for patients diagnosed with colorectal cancer in England and Wales. Adapted from http://info.cancerresearchuk.org/cancerstats/incidence/commoncancers/

Incidence rates of colorectal cancer vary widely in different geographical areas, with relatively low rates in Asia, Africa and parts of Latin America, but with high rates in "Western" countries including Northern Europe, Australia, New Zealand and the US (Parkin and Muir 1992). Epidemiological studies have suggested that specific components of the Western diet are risk factors in colorectal cancer pathogenesis; these include dietary fat and red meat, but fruit, vegetables and dietary fibre are thought to be protective (Potter 1997).

Colorectal cancer can be observed in one of three specific patterns; sporadic, inherited and familial. Sporadic disease with no familial or inherited predisposition accounts for 70% of colorectal cancer in the population. Probably as a result of the normal ageing process, as well as environmental and dietary factors, sporadic cancer is most common in those over 50 years

of age. Fewer than 10% of colon cancer patients have an inherited predisposition for the disease. Up to 25% of colon cancer cases fall into the least understood category of familial colon cancer. Familial colon cancer is described as colon cancer, which occurs in families at an incidence that is too frequent to be classed as sporadic but not in a pattern consistent with inherited cases (Calvert and Frucht 2002).

1.4.3 Prognosis

With early detection and/or improved treatments the general prognosis for those diagnosed with colon cancer is improving. Figures released by Cancer Research UK in 2003 suggest that survival rates for colorectal cancer are increasing (fig. 1.6).

1.4.4 Genetics

The molecular basis for sporadic colon cancer was described by Fearon and Vogelstein (Fearon and Vogelstein 1990) as a multistep model for carcinogenesis. This model describes an accumulation of genetic events, each conferring a selective growth advantage to an affected colon cell as follows:

- Colorectal cancer is a result of mutational activation of oncogenes and the inactivation of tumour suppressor genes.
- Somatic mutation in at least 4 or 5 genes of a cell are required for malignant transformation.
- The biological behaviour of the tumour is determined by the accumulation of multiple genetic mutations rather than the sequence of the mutations.
- Features of the tumorigenic processes of colon cancer are applicable to other solid tumours.

A common form of inherited colon cancer is familial adenomatous polyposis (FAP) and is a result of a germline mutation in the tumour suppressor gene *APC*. FAP is a dominantly inherited syndrome where the affected person develops hundreds to thousands of colonic polyps. Although the transition of polyps to cancer is low the vast number virtually assures development of colon cancer at an early age (Fearnhead, Wilding *et al*. 2002).

An example of familial colon cancer can be seen in the Ashkenazi Jewish population, which is probably due to an I1307K *APC* germline mutation. In this case the relative risk of tumour is much lower than in FAP because, unlike this germline mutation, which causes protein structure abnormalities, the I1307K *APC* germline mutation causes a predisposition to sporadic mutations at distant sites of the gene (which then cause protein structure abnormalities) at a later stage of development (de la Chapelle 2004).

1.4.5 Hyperplastic polyps and colorectal cancer

Hyperplastic polyps are the most common type of polyp in the human colon and rectum, but relatively little is known about the aetiology, natural history or growth rates of these polyps. The main reason for this is due to an accepted belief that they are benign and have little or no malignant potential (Winawer, Fletcher *et al*. 1997). The recommendation of the American College of Gastroenterology is that hyperplastic polyps found during endoscopy are not an indication for subsequent colonoscopy (Bond 1993).

1.4.6 *Apc*$^{Min/+}$ mouse model of intestinal carcinogenesis

As previously described (Section 1.3.4) the *APC* gene is a tumour suppressor gene, the loss of which has been shown to play a key role in colorectal carcinogenesis (Powell, Zilz *et al*. 1992). The *Apc*$^{Min/+}$ mouse lineage was originally established by treating a C57BL/6 male mouse with the mutagen ethylnitrosourea, which resulted in a mutation that predisposed the mouse to spontaneous intestinal cancer. The mutant gene was found to be dominantly expressed and fully penetrant with affected mice developing multiple intestinal neoplasms (Min) throughout the length of the intestinal tract within several weeks of birth (Moser, Pitot *et al*. 1990). The dominant mutation is located in *Apc,* the mouse homolog of the human *APC* gene, and causes a truncation of the gene product at amino acid 850, homozygous *Apc*$^{Min/Min}$ mice die as embryos (Su, Kinzler *et al*. 1992).

1.5 Pancreatic cancer

In industrialised western countries pancreatic cancer is the fourth leading cause of cancer death, and although in recent years the five year survival rate has significantly increased, it is still at a very low 5% (Jemal, Siegel *et al*. 2006). There are a number of factors which account for this high death rate; early diagnosis is rare, the tumours grow rapidly and aggressively and are unresponsive to chemotherapy, radiotherapy and immunotherapy (Buchler, Friess *et al*. 1991).

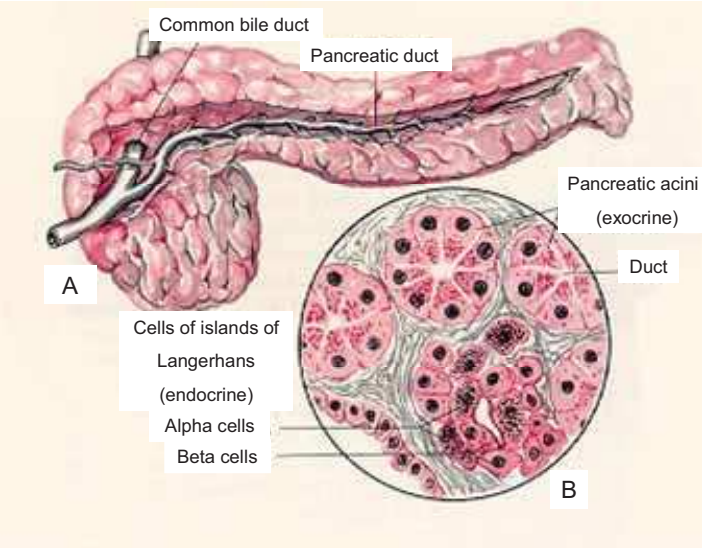


Fig 1.12 Diagrammatic representation of the human pancreas (A) showing a cross section of islands of Langerhans (B).

Taken from http://www.ucpancreas.org/pancreas.htm 1.12.07

As shown in fig. 1.12 the pancreas is composed of numerous acini (exocrine) cells grouped around central ducts and endocrine cells, which make up the islets of Langerhans.

1.5.1 Classification

The commonest form of cancer affecting the exocrine pancreas, and also the most difficult to treat, is pancreatic ductal adenocarcinoma (Parkin, Bray *et al*. 2001). This form of pancreatic cancer gives rise to three distinct cancer precursor lesions: Mucinous cystic neoplasms, intraductal papillary mucinous neoplasms (IPMNs) and pancreatic intraepithelial neoplasia (PanINs). PanINs can be further classified into early and late lesions, beginning with PanIN-1A, 1B (hyperplasia) and progressing to PanIN-2 and then to PanIN-3 or carcinoma in situ (Ghaneh, Costello *et al*. 2007). A comprehensive list of different histological variants of malignant tumours of the exocrine pancreas is shown in table 1.2.

Table 1.2 The frequency and prognosis for different histological types of malignant exocrine pancreatic tumours

Histological type	Frequency (%)	Prognosis
Ductal adenocarcinoma	80	Long term survival rare
Ductal adenonocarcinoma variant		
Undifferentiated carcinoma	5	Worse than ductal
Mucinous non cystic	2	Poor
Adenosquamous	2	Poor
Mucinous non-cystic carcinoma	<1	Poor
Signet ring cell carcinoma	<1	Poor
Adenosquamous carcinoma	<1	More aggressive than ductal
Mixed ductal-endocrine carcinoma	<1	Poor
Osteoclast-like giant cell tumour	<1	Poor
Other malignancies		
Serous cystadenocarcinoma	<1	Similar to ductal
Mucinous cystadenacarcinoma	3	Similar to ductal
Intraductal pappillary-mucinous neoplasm-invasive carcinoma	1-3	Patients tend to present with preinvasive lesions
Acinar cell carcinoma	2	Variable
Pseudopapillary carcinoma	<1	Favourable
Pancreatablastoma	Rare	good

Adapted from Ghaneh, Costella *et al* 2007 using WHO statistics

1.5.2 Risk factors

There are a number of risk factors associated with the development of pancreatic cancer, the largest being increasing age, new onset diabetes mellitus, obesity, chronic and hereditary pancreatitis, and an inherited predisposition for pancreatic cancer (Ghaneh, Costello *et al*. 2007).

A single centre, medical-surgical, prospective cohort study in 2002 showed that the risk of pancreatic cancer is significantly ($p<0.001$) increased in patients with chronic pancreatitis compared to age and sex matched controls (Malka, Hammel *et al*. 2002). Observations have shown that patients may have chronic pancreatitis up to 20yrs before developing pancreatic cancer and these patients are more likely to have a severe form of the disease with higher calcification of the glands and an increased risk of complications (Ghaneh, Costello *et al*. 2007). There is also a 70-fold greater risk in patients with hereditary chronic pancreatitis, an autosomal dominant disease caused by a mutation in the *PRSS1* gene (Howes, Lerch *et al*. 2004).

1.6 Oesophageal cancer

The incidence of oesophageal cancer has dramatically increased in the last three decades and is the 9th leading cause of cancer death in the UK. It is twice as common in men as women, with a 2% increase year on year in men, while the incidence remains static in women (http://info.cancerresearchuk.org/cancerandresearch/ cancers/oesophageal/ last updated 25.6.07). Recent advances in surgical technique and new therapeutic approaches have slightly increased the 5 year survival rate from 4% in the 1960's to 14% now, although the rate of relapse remains high (Syrigos, Zalonis *et al*. 2008).

1.6.1 Classification

Oesophageal cancer progression follows the metaplasia-dysplasia-adenocarcinoma sequence (MCS) and the progression and severity are classified using the tumour, node, metastasis (TNM) system, which characterises primary tumour size, lymph node involvement and cancer spread. Tumour size is scored 1-4; 1 being small tumour and 4 being large; lymph nodes are scored 0-3; 0 being no cancer positive lymph nodes and 3 being many positive lymph nodes and metastasis is scored 0-1; 0 being no spread and 1 being spread (www.cancerhelp.org.uk/help/default.asp?page=102#the_TNM_ last updated 25.6.07). The TNM system is used to break oesophageal cancer progression into four major pathological stages:

Stage I invasion of cancer cells up to the submucosa; stage 2 invasion into but not through the musculari propria; stage 3 invasion beyond the muscularia propria into the adventitia; stage 4 invasion into adjacent structures (ie, aorta, pleura, lung) (Shimpi, George *et al*. 2007).

1.6.2 Risk factors

Oesophageal cancer is more frequent in people over 50, with, as previously mentioned, higher occurrence in males (see section 1.5). Obesity is associated with oesophageal cancer with increased risk linked to a body mass index (BMI) over 25. This risk is then increased at a BMI of 30-35 with an even greater risk with a BMI over 35 (Abnet, Freedman *et al*. 2008). Diets high in red and/or processed meat and low in fruit and vegetables are also linked to increased risk (Gonzalez, Jakszyn *et al*. 2006; Cross, Leitzmann *et al*. 2007). Finally, prolonged gastroesophageal reflux (GERD), a condition where there is abnormal reflex of gastric acid and bile into the oesophagus causing mucosal damage, is associated with progression to oesophageal cancer (DeVault and Castell 2005). The initial injury from acid reflux causes oesophagitis characterised by inflammation and irritation, this can then develop into metaplastic, premalignant lesions referred to as Barrett's oesophagus (BE). GERD-derived BE is considered a major risk factor of oesophageal cancer (Isomoto, Nishi *et al*. 2007).

1.6.2.1 Barrett's oesophagus

First described in 1950, Barrett's oesophagus is the metaplastic change in the epithelium of the distal oesophagus from normal squamous epithelium to specialised columnar-intestinal type epithelium that contains goblet cells (Barrett 1950). This change in epithelial cell type is a result of chronic gastric reflux (Spechler and Goyal 1986; Winters, Spurling *et al*. 1987), which causes injury to the epithelium initiating the release of inflammatory cytokines, resulting in an altered chemical environment of the oesophagus. The oesophageal epithelium is maintained by stem cells, the only permanent resident of the oesophagus, through their production of daughter cells. However, the changes in the chemical environment cause the stem cells to produce intestinal columnar type cells, which are more resistant to acid than the normal squamous epithelium (Jankowski, Wright *et al*. 1999). Increased proliferation rates lead to more efficient colonisation of the distal oesophagus, and therefore there is a selection pressure for cells which have acquired *p53* mutations, an anti-oncogene that normally restricts cell growth (Wijnhoven, Tilanus *et al*. 2001).

1.6.2.1.1 Progression of Barrett's oesophagus to adenocarcinoma

The prevalence of Barrett's oesophagus in the general population aged over 50 years is between 5-9% (Geboes and Van Eyken 2000) with an estimated 4% of these going on to develop oesophageal adenocarcinoma (Louis 2007).

The length of the Barrett's segment is roughly correlated to the risk of progression to dysplasia and adenocarcinoma, with longer Barrett's segments corresponding with increased risk (Weston, Krmpotich *et al*. 1997).

The progression of Barrett's metaplasia to adenocarcinoma is associated with a number of gene structure and expression, and protein expression changes. One of the earliest molecular events is thought to be the selection and propagation of metaplastic clones with specialised intestinal metaplasia via pathways involving mitogen activated protein kinases (MAPKs) (Souza, Shewmake *et al*. 2002) and protein kinase C (PKC) (Triadafilopoulos 2001). These effects are further amplified by the inhibition of apoptosis and promotion of cellular proliferation by cyclooxygenase-2 (COX-2) (Shirvani, Ouatu-Lascar *et al*. 2000). The inhibition of apoptosis occurs late in Barrett's and then only in a select few cells with high grade dysplasia, but the previously mentioned alterations, along with the acquired mutations in p53, push metaplastic cells along the MCS (Jankowski, Wright *et al*. 1999).

Approximately 75% of patients presenting with low grade dysplasia regress and show no signs of dysplasia at subsequent biopsies, however around 20% go onto develop high grade dysplasia, from which development of oesophageal adenocarcinoma is inevitable (Chang and Katzka 2004). The progression from metaplasia to dysplasia is in part, due to alterations in the expression of cytokines and growth factors (Jankowski 1993), as well as alterations in cell cycle-associated genes, such as an increase in cyclin D1,

hypermethylation and mutation of p16, and the push of cells from G_0 to G_1 (Jankowski, Wright *et al*. 1999). Mutations are also seen in the following genes; *APC*, mutated in colorectal cancer (*MCC*), deleted in pancreatic cancer (*DPC4*), and retinoblastoma (*Rb*). Random errors in replication become more frequent and multiple aneuploidy is seen. As well as these alteration there is also an increase in; microsatellite instability, expression of metalloproteinases (MMPs), epidermal growth factor receptor (EGFR) and transforming growth factor α (TGFα), all of which continue to push the oesophageal epithelium along the MCS towards malignance (Wijnhoven, Tilanus *et al*. 2001). The progression from normal oesophagus to adenocarcinoma is summarised in fig. 1.13.

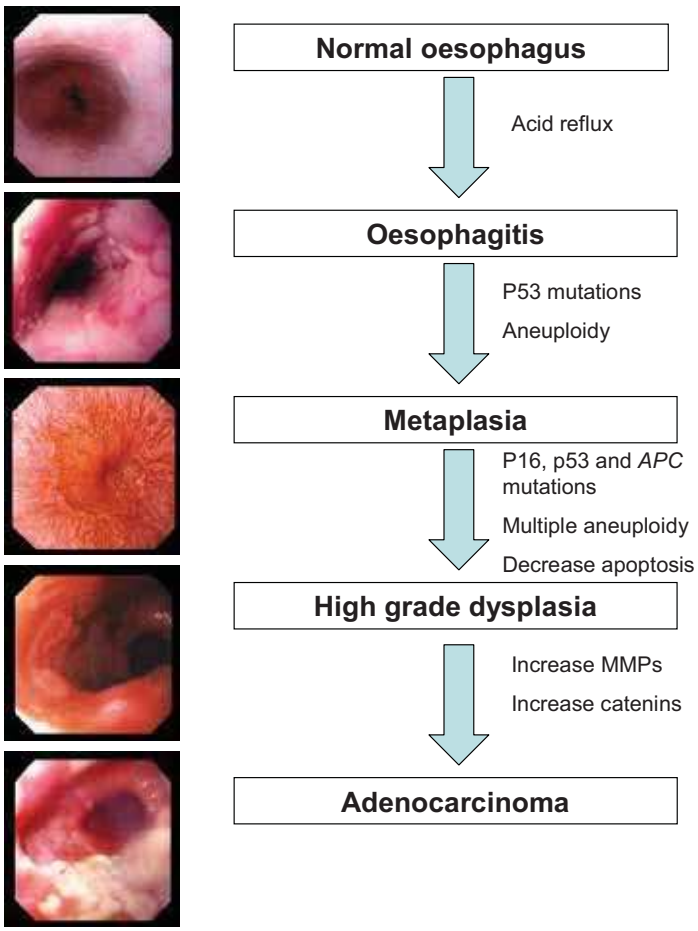


Fig. 1.13 Progression from normal oesophagus to adenocarcinoma. Endoscopic pictures from www.endoatlas.com/atlas_es downloaded 4.2.08

1.7 Gastrin

Gastrin, a polypeptide hormone, is the principle mediator of food stimulated gastric acid secretion, responsible for at least 50% of the postprandial phase acid release in the stomach (Kovacs, Walsh *et al*. 1989). Under normal conditions gastrin is synthesised primarily by G-cells located in antral mucosal region of the stomach and its synthesis and secretion is tightly regulated. In the antral mucosal region gastrin is found in storage granules at the base of the cell. The G-cells possess a typical flask-like endocrine cell shape and secrete gastrin onto the mucosal surface via the narrow neck of the cell (Watson and Steele 1993). Gastrin also plays a more general role in the control of the acid-secreting capacity of the stomach, through control of gastric cell proliferation, differentiation and maturation (Dockray, Varro *et al*. 2001). Another important action of gastrin is the stimulation of mucosal growth in the stomach that results in hyperplasia of the enterochromaffin-like (ECL) and parietal cells (Crean, Marshall *et al*. 1969).

Located on chromosome 17q the *gastrin* gene (Lund, Geurts van Kessel *et al*. 1986) and its transcription is regulated by both somatostatin and the somatostatin receptor (SSTR), as well as by various signalling molecules including ligands of epidermal growth factor (EGF) (Merchant, Demediuk *et al*. 1991; Howell, Ziober *et al*. 1995).

Following transcription of the *gastrin* gene the resulting messenger RNA (mRNA) is transcribed to pre-progastrin, a 101 amino acid peptide (Wiborg,

Berglund *et al*. 1984). This is an immature gastrin peptide, which contains a signal peptide at its N-terminal end, which targets it to the endoplasmic reticulum (ER). When the pre-progastrin arrives at the ER the signal peptide is cleaved and this results in an 80 amino acid progastrin which moves into the Golgi secretary pathway (Walter and Lingappa 1986). Depending on cell type the progastrin is either stored, as described earlier in secretary granules or, in a cancer situation, it can be secreted in an uncontrolled constitutive fashion as non-processed gastrin. The stored progastrin is converted via enzyme catalysed reactions into glycine-extended gastrin-34, a 34 amino acid protein, which can be processed further to either glycine-extended gastrin-17 (gly-17) or matured amidated gastrin-17 (G17) (Smith and Watson 2000). This conversion of pre-progastrin is represented diagrammatically in fig.1.14.

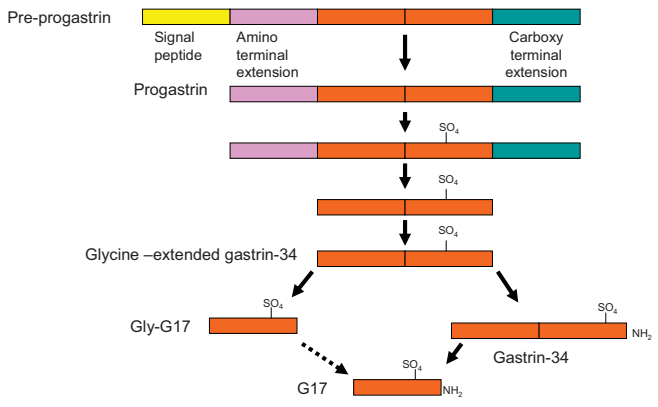


Fig 1.14 Conversion of pre-progastrin to mature amidated gastrin 17 (G17)

1.7.1 The gastrin receptor cholecystokinin-2(CCK-2)

On the basis of their pharmacological properties and specificities for binding, CCK receptors have been sub-divided into two types CCK-1 (CCK-A) and CCK-2 (CCK-B) receptors, both of which belong to the seven transmembrane G-protein-coupled receptor superfamily. The two sub-types of receptors share 50% amino acid homology. CCK-1 receptors are located mainly in the periphery, but are also found in some regions of the brain. The main population of central CCK receptors are of the CCK-2 subtype, which are also found in the stomach and vagus nerve (Hill, Campbell *et al*. 1987). In the stomach CCK-2 receptors are mainly expressed in the parietal and ECL cells. They are distributed throughout the human GI tract, pancreas, lung and some neuroendocrine tissues (Zhou, Chen *et al*. 2004).

It has been shown that the CCK 2 receptor also serves as a gastrin receptor, and binds both gastrin and CCK with almost the same affinity, compared to CCK-1 receptor which has a much greater affinity for CCK than gastrin. The receptor is highly conserved across a number of species including human, dog, guinea pig, rabbit and rat, with an overall amino acid identity of 72% (Noble and Roques 1999).

The CCK-2 receptor mediates the effects of gastrin, and is therefore principally involved in controlling gastric acid secretion and maintaining the normal cellular composition and function of the gastric mucosa.

1.7.1.1 Structure of CCK-2R

The human CCK-2 receptor is encoded for by a 5 exon gene over 10 kilobases (Nishida, Miyata *et al*. 1994) that has been identified on chromosome 11. Further to this *in situ* hybridisation has specifically shown the human *CCK2R* gene to be on the distal short arm of chromosome 11 (11p15.4) (Song, Brown *et al*. 1993). There are four different isoforms of the receptor.

1.7.1.1.1 Classical CCK2R

Under physiologically normal conditions the human *CCK-2R* gene encodes for the classical 74kDa glycoprotein, which is found mainly on GI derived cell-lineages; these include parietal and neuroendocrine cells (Schmitz, Goke *et al*. 2001), ECL cells (Bakke, Qvigstad *et al*. 2001) and islet cells in the pancreas (Reubi 2003). As highlighted by Reubi (Reubi 2003) there are discrepancies in the incidence of CCK2-receptor in stomach and colon carcinomas, with early studies reporting a presence compared to more recent work which has failed to show CCK2-receptor proteins in these tumour types. An explanation to this may lay in the existence of different isoforms, long, truncated and splice 4, of the receptor.

1.7.1.1.2 Long isoform

The long isoform, which differs from the classical receptor by the addition of a 5 amino acid cassette, GGAGP, insertion at the end of exon 4 (Song, Brown *et al*. 1993) was the first CCK2R isoform to be identified. As represented diagrammatically in fig.1.15 the addition of this pentapeptide cassette is considered to be due to the existence of two different splice donor sites at the end of exon 4. When the receptor is spliced at the G/gt consensus site then the 15 nucleotides after this junction are excluded from the mRNA, therefore encoding for the short classical form of the receptor. However, if the receptor is spliced at C/gt then the resulting mRNA contains the additional 15 nucleotides and results in the long isoform (Song, Brown *et al*. 1993).

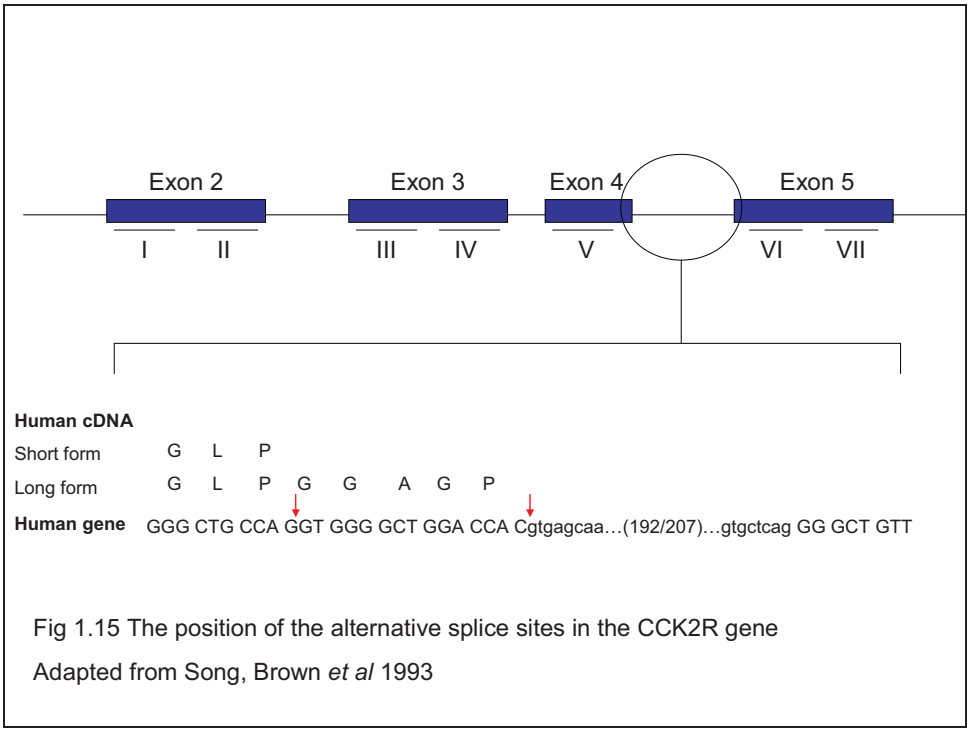


Fig 1.15 The position of the alternative splice sites in the CCK2R gene
Adapted from Song, Brown *et al* 1993

1.7.1.1.3 Truncated isoform

The truncated isoform of the CCK2 receptor is a result of alternative usage of the novel exon 1b, which gives this isoform different binding properties compared to the classical short isoform. Exon 1b is approximately 5.5kb upstream of exon 2 (Miyake 1995). This isoform is truncated due to the lack of an N-terminal extracellular domain, the actual amino acid sequence is the same as the classical isoform and therefore there is no change in the translated protein (McWilliams, Watson *et al*. 1998). The truncation causes the loss of three putative N-glycosalation sites and therefore a change in molecular size and structure. Work by Miyake (Miyake 1995) using competition assays points to the N-terminal extracellular domain not being involved in ligand binding but being important for ligand selectivity.

The truncated and classical isoforms have been shown to be co-expressed in the human brain and gastric fundus, which may suggest involvement in the same physiological pathways (McWilliams, Watson *et al*. 1998). The truncated isoform has been shown to be exclusively expressed in the gastric tumour cell line, AGS (Miyake 1995), compared to the classical isoform which is exclusively expressed in LIM 1215, RD 19 and MCF 7B (McWilliams, Watson *et al*. 1998). McWilliams (McWilliams, Watson *et al*. 1998) suggests that, "tumour cells may uncouple the co-expression of the receptor isoforms and that their malignant functions may differ".

1.7.1.1.4 Splice 4 variant isoform

First described in 2000 and designated CCK-BRi4sv (now CCK-2Ri4sv) by Hellmich *et al* (Hellmich, Rui *et al*. 2000), the CCK-2R intron 4 splice variant was identified in colorectal cancers and adenomatous polyps but not normal colonic mucosa, and it was hypothesised that it may stimulate colorectal cancer cell proliferation through a gastrin independent mechanism. CCK-2Ri4sv has since been identified in pancreatic cancer cells, but not normal pancreas (Ding, Kuntz *et al*. 2002).

It has been demonstrated that this isoform of CCK-2R is as a result of a decrease in U2AF35. U2AF35 is the small subunit of the heterodimeric splicing factor U2AF which is also composed of the larger subunit U2AF65. U2AF35 specifically recognises the 3'-end dinucleotide AG and is essential for pre-mRNA splicing and the removal of intron 4, therefore a decrease in U2AF35 generates receptors that retain the 69 amino acids encoded for by intron 4 of the gene, with the overall result being a receptor with an elongated intracellular third loop (Ding, Kuntz *et al*. 2002).

1.7.2 Gastrin and GI cancers

It is now widely accepted that gastrin promotes the growth of cancer cells both *in vitro* and *in vivo* through CCK-2 receptors, and that the expression of both the *gastrin* gene and the CCK-2 receptor are closely related to the

development, progression and invasion of cancer cells in GI cancers (Watson, Durrant *et al*. 1989; Clerc, Leung-Theung-Long *et al*. 2002; Watson, Morris *et al*. 2002).

1.7.2.1 Gastrin and gastric cancer

The exact role of gastrin in the development of gastric cancer has yet to be elucidated, but as discussed in section 1.2.6, INS-GAS mice that over-express G17 develop gastric adenocarcinoma a process which is accelerated by *H. felis* infection (Wang, Dangler *et al*. 2000) and hypergastrinemia has been shown to occur early in the course of human *H. pylori* infection (Levi, Beardshall *et al*. 1989). Added to this the *gastrin* gene has been shown to be activated in cells isolated from human gastric tumours (Watson, Durrant *et al*. 1991) and *in vitro* stimulates growth of gastric cancer cell lines TMK-1 and AGS, (Ochiai, Yasui *et al*. 1985; Iwase, Evers *et al*. 1997) as well in xenograft models (Watson, Durrant *et al*. 1991). Iwase *et al* (Iwase, Evers *et al*. 1997) also showed that gastric carcinoma growth could be stimulated by gly-G17.

Gastrin has not only been shown to directly promote the growth of the gastric epithelium, but also to increase the expression of other members of the epidermal growth factor family, including HB-EGF, TGF-α and amphiregulin, which are linked to gastric carcinogenesis (Henwood, Clarke *et al*. 2001).

1.7.2.2 Gastrin and colorectal cancer

Hypergastrinemia has been reported in colorectal cancer patients, suggesting that gastrin may act as an endocrine proliferative agent, although the source of gastrin is undefined (Aly, Shulkes *et al*. 2004). Elevated levels of gastrin precursors in the serum of colorectal patients have also been reported when *H. pylori* status was controlled for (Ciccotosto, McLeish *et al*. 1995; Siddheshwar, Gray *et al*. 2001). This fits with work by Konturek *et al* (Konturek, Bielanski *et al*. 2002), which showed a decrease in serum concentrations of gastrin precursors, but not G17 after colon cancer resection.

Two studies have shown that CCK-2 receptor mRNA is only expressed in a small sub-set of colorectal carcinomas (Reubi, Waser *et al*. 1999; Schmitz, Otte *et al*. 2001). The CCK-2Ri4sv was reported in 100% of colorectal carcinomas by Hellmich *et al* (Hellmich, Rui *et al*. 2000), but this was a small sample size (n=8); in a larger sample size (n=79) Schmitz *et al* (Schmitz, Otte *et al*. 2001) was unable to detect CCK-2Ri4sv.

Recent work now suggests that progastrin maybe synthesised by colorectal carcinoma tissue, but it is unable to process it to G17, and that the level of processing is dependent on the tumour. Of interest gastrin precursors are found in high concentrations in developing embryonic colorectal tissue, but not G17, suggesting that there maybe a developmental link between colon cancer and gastrin (Aly, Shulkes *et al*. 2004).

1.7.2.3 Gastrin and pancreatic cancer

Gastrin has been shown to stimulate the growth of pancreatic cancer through the CCK-2 receptor (Smith, Rickabaugh *et al*. 1993; Smith, Liu *et al*. 1994) and further work by Smith (Smith, Shih *et al*. 1996) showed not only that gastrin is produced by pancreatic tumour cells *in vitro,* but also in xenografts of pancreatic cancer, demonstrating that gastrin acts as an autocrine growth factor.

A number of studies have shown gastrin transiently in neonatal pancreatic islets, suggesting a role for gastrin in pancreatic development and that it may play a part in mediating some developmental changes (Brand and Fuller 1988). There is conflicting data in the literature regarding the expression of CCK-2 receptor in normal pancreatic tissue and pancreatic adenocarcinoma. Tang *et al* (Tang, Biemond *et al*. 1997) showed that the CCK-2 receptor was expressed in normal pancreatic tissue, but not in pancreatic cancer, whereas work by de Weerth *et al* (de Weerth, von Schrenck *et al*. 1999) concluded that most human pancreatic cancer cell lines of ductal origin express CCK-2 receptor mRNA. Work by Caplin *et al* (Caplin, Savage *et al*. 2000) demonstrated that normal pancreatic tissue did not express the CCK-2 receptor, nor progastrin, glycine-extended or amidated gastrin, but that pancreatic cancer cells showed moderate to high CCK-2 receptor expression in 95% of cases, progastrin in 91%, glycine extended in 55% and amidated in 23%. This led them to conclude that

gastrin fits with the oncofoetal expression hypothesis; i.e. gastrin is expressed in pancreatic tissue during foetal development and oncogenesis.

1.7.2.4 Gastrin and oesophageal cancer

Based on the similarities between the genetic alterations seen in the development of oesophageal and colonic adenocarcinomas, principally the APC mutations, it is fair to hypothesise that, as in colonic carcinogenesis, gastrin may play a role in the progression of Barrett's oesophagus to oesophageal adenocarcinoma. However, data on this topic is conflicting.

CCK-2 receptor mRNA has been shown to be expressed in normal and inflamed squamous mucosa, Barrett's metaplasia and oesophageal adenocarcinoma. The levels of expression in Barrett's mucosa were 2-fold higher than those of the normal squamous mucosa (Haigh, Attwood *et al*. 2003). Moore *et al* (Moore, Jepeal *et al*. 2004) have identified CCK-2 receptor mRNA in three human oesophageal cell lines, SEG-1, BIC and SKGT-4 and our group has also shown CCK-2 receptor mRNA expression in OE19 (oesophageal adenocarcinoma stage III), OE21 (oesophageal squamous carcinoma stage III) and OE33 (Barrett's metaplasia-derived oesophageal adenocarcinoma stage II) cell lines, and that there is an increase in the expression of gastrin, CCK-2 receptor and CCK-2 receptor i4sv gene mRNA in Barrett's samples compared to normal oesophagus (Harris, Clarke *et al*. 2004). A dose-dependent increase in proliferation in SEG-1 cells

when incubated with G17 has been shown (Haigh, Attwood *et al*. 2003). This fits with work in male Sprague-Dawley rats that showed gastrin increased proliferation in the oesophageal mucosa (Van Nieuwenhove, De Backer *et al*. 1998; Van Nieuwenhove, Chen *et al*. 2001).

However, there is other data which states that CCK-2 receptor could only be detected in normal oesophagus and tissue surrounding oesophageal tumour, and not in oesophageal cancer samples (Clerc, Dufresne *et al*. 1997). Also one group were unable to observe gastrin protein expression in Barrett's metaplasia using immunohistochemistry (Griffin and Sweeney 1987). Some of these differences could be attributed to the type of tumours examined e.g. the work by Clerc *et al* (Clerc, Dufresne *et al*. 1997) looked mainly at squamous cell carcinoma and, unlike oesophageal adenocarcinoma, it is not derived from Barrett's metaplasia.

The role of gastrin in Barrett's progression is important, because individuals with GERD are often prescribed proton pump inhibitors (PPI's), which lower gastric acid secretion and as a result have been shown to increase gastrin levels resulting in hypergastrinemia. PPI's have been associated with increased risk of oesophageal adenocarcinoma which may be mediated through the CCK-2 receptor (Lagergren, Bergstrom *et al*. 1999).

1.7.3 Gastrin and cyclooxygenase

Cyclooxygenase (COX), also known as prostaglandin H synthetase, is a rate limiting enzyme involved in prostaglandin synthesis through the conversion of arachadonic acid into prostaglandins. There are two isoforms of the COX enzyme which share over 60% identity at the amino acid level. COX-1, which is constitutively expressed in many tissues and regulates and maintains normal cellular function, and COX-2, which is the inducible form of the enzyme, and is only expressed after stimulation by a variety of growth factors (Smith, Hawcroft *et al*. 2000). More recently a third COX isoform has been described, COX-3. COX-3 shares all the of the catalytic and important structural features of COX-1 and -2, but has an insertion of intron 1 downstream of the initiating methionine (Chandrasekharan, Dai *et al*. 2002).

COX-2 has been shown to be over-expressed in a number of pre-malignant tissues and malignant tumours, including gastric, colorectal, pancreatic and oesophageal (Dannenberg, Altorki *et al*. 2001). In the $APC^{\Delta 716}$ mouse model when the *COX-2* gene is knocked down there is an 86% reduction in tumour burden this is reduced to 66% when there is only single gene knockdown (Oshima, Dinchuk *et al*. 1996). Inhibition of COX-2 by specific COX-2 inhibitors reduces proliferation and angiogenesis and induces apoptosis in human gastric cell lines (Sawaoka, Tsuji *et al*. 1999).

Gastrin has been shown to increase expression of COX-2 in a dose dependent manner in KATO III cells, a gastric epithelial cell line which have a genomic deletion of *p53* and mutations of *k-sam* and *c-met* (Konturek, Kania *et al*. 2003). Yao *et al* (Yao, Song *et al*. 2002) showed that specific inhibition of COX-2 with NS-398 inhibited the trophic effects of gastrin in colon cancer MC-26 cells that possess both COX-2 and functional gastrin receptors.

1.8 Hypothesis

Down-stream effects of amidated gastrin on angiogenesis and apoptosis are mediated via the CCK-2 receptor by multiple factors within different signalling pathways. Therefore it is hypothesised that:

- Correlations exist between gastrin and CCK-2 receptor expression and pro-carcinogenic factors and their expression could act as biomarkers of progression. This will be investigated in the oesophageal setting with Barrett's metaplasia and dysplasia, since the department has access to screening samples.
- Gastrin and CCK-2 receptor expression correlations with pro-angiogenic and anti-apoptotic factors are established in full blown cancer. The department has access to a large pancreatic tissue microarray (TMA) which will be used to assess this in the pancreatic cancer setting.
- Inhibition of gastrin will reduce multiple pro-carcinogenic factors concurrently. Mouse models will be used to evaluate this; the INS-GAS mouse model of gastro-oesophageal cancer and the *Apc*$^{Min/+}$ mouse model of intestinal carcinogenesis. The latter model will also be used to compare gastrin neutralisation to a standard of care modality; COX-2 inhibition.

Chapter 2

MATERIALS AND METHODS

2.1 Sample collection and preparation

2.1.1 Sample processing

All samples were collected in formal calcium and fixed (approx 24hrs depending on sample thickness).

Fixed samples were placed into histology cassettes and processed overnight on the TP1020 tissue processor (Leica microsigation, UK).

Samples were processed on the following programme under vacuum and agitation;

- 60 minutes in 70% ethanol
- 60 minutes in 90% ethanol
- 60minutes in 100% ethanol
- 60minutes in 100% ethanol
- 60minutes in 100% ethanol
- 60minutes in 100% ethanol
- 60minutes in 100% xylene
- 60minutes in 100% xylene
- 60minutes in 100% xylene
- 120 minutes in 60^{o}C embedding wax
- 120 minutes in 60^{o}C embedding wax

The samples, permeated with molten wax were transferred to the embedding station.

2.1.2 Sample embedding

The samples were placed in pre-heated (60^{o}C) moulds and then molten wax poured over the top, a labelled cassette was then placed on top and the assembly left to cool evenly to 4 ^{o}C. Once set the wax forms a solid matrix to aid sectioning.

2.1.3 Cutting of sections

Sample blocks were trimmed and then chilled, 4µm sections cut using a rotary microtome (Leica microsigation, UK). Sections were then placed in a 37^{o}C flotation bath to stretch and then transferred onto polysine slides and air dried overnight.

2.2 Immunohistochemistry

2.2.1 Deparaffinising samples

In order to stain the samples for analysis with immunohistochemistry, the samples were first deparaffinised. Samples were placed in a slide rack and immersed in two changes of 100% xylene for 3 minutes each, followed by two immersions in 100% ethanol for 2 minutes each. If samples were to be blocked immediately for endogenous peroxidase activity they went straight into the hydrogen peroxide block if they were under going antigen retrieval first they were rinsed in running tap water.

2.2.2 Endogenous peroxidase activity

Endogenous peroxidase activity was blocked with hydrogen peroxide solution diluted in methanol and rinsed in two washes of 100% ethanol followed by running tap water if the blocking step was carried out before antigen retrieval or distilled water followed by a rinse in distilled water and then two rinses of phosphate buffered saline (PBS) pH 7.6 if the blocking step was carried out after antigen retrieval.

2.2.3Antigen Retrieval

Antigen retrieval was undertaken because the configuration of the tissue proteins, and significantly, for immunohistochemistry, the antigenic sites are changed during tissue processing (Thomas Boenisch 2001). Samples were placed in a plastic slide rack and dish and immersed in the appropriate solution and the appropriate antigen retrieval protocol followed (table 2.1). The samples were then quenched in running tap water for 2-3 minutes and rinsed in 2 washes of PBS pH 7.6 for a further minute each.

2.2.4 Serum block

To prevent any cross reactivity with the species that the secondary antibody was raised in samples were incubated with the appropriate serum (table 2.1) and then rinsed in two washes of PBS pH7.6. In addition to the serum block

a streptavidin/biotin block (Vector) was also used prior to staining for COX-2, CD34 and XIAP.

2.2.5 Positive and negative antibodies

Samples were incubated with the required primary antibody and a corresponding sample was incubated with the appropriate negative IgG at matched concentration to the primary (table 2.1). Where incubation was over-night at 4^{o}C, samples were covered with parafilm coverslips and placed in a humid chamber to prevent drying out. After incubation samples were rinsed in two washes of PBS pH 7.6.

2.2.6 Secondary antibodies

Samples were incubated with the appropriate secondary (table 2.1) diluted 1 in 300 in distilled water for 30 minutes at room temperature and then rinsed twice in PBS pH 7.6.

2.2.7 (strept)Avidin-biotin complex (ABC) protocol

To amplify the signal from the bound antibody, an ABC kit (Dako Cytomation, UK) was used. The (strept)avidin and biotinylated enzymes were mixed at an optimal ratio and incubated at 4^{o}C for 30 minutes before use. The complex was then dropped onto the samples and incubated at room temperature for 30 minutes and then rinsed twice in PBS pH7.6.

2.2.8 Visualisation, counterstaining and cover-slipping

For all antibodies, except CCK-2R, 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was used to visualise the staining. DAB reacts with ABC and produces a brown colour, incubation time ranged from 3-10 minutes depending on intensity. Following incubation with DAB samples were rinsed in running tap water and counter-stained with filtered Meyers haematoxylin (RA Lamb, UK) before being rinsed in running tap water and dehydrated through 3 times 100% ethanol rinses and 2 xylene rinses and cover-slipped with DPX mounting media. CCK-2R staining was visualised with 3-amino-9-ethylcarbazole (AEC), which reacts with ABC to produce a red colour Samples were incubated for 10 minutes and then rinsed in running tap water and counterstained with filtered Meyers haematoxylin (RA Lamb, UK) before being rinsed in running tap water and cover-slipped with aqueous Vectormount (Vector, UK).

2.2.9 Mouse on mouse antibodies

Where antibodies raised in mice were used on mouse tissue (BrdU and Bcl-2) the animal research kit (ARK^{TM}) (Dako Cytomation, UK) was used. Samples were deparaffinised as described in section 2.2.1 and antigen retrieval preformed if required (table 2.1). Samples were then incubated with a pre-diluted hydrogen peroxidase solution for 5 minutes and rinsed twice in PBS pH 7.6. Then the primary antibody, which has been pre-incubated with modified biotinylated anti-mouse immunoglobulin and a blocking reagent containing normal mouse serum, that binds any residual

biotinylation reagent and prevents interactions between immunoglobulins endogenous to the sample was added to the sample for 15 minutes. Samples were then rinsed twice in PBS pH 7.6 and streptavidin-peroxidase added for 15 minutes before rinsing twice in PBS pH 7.6 and visualising with DAB, counterstaining and cover-slipping as described in section 2.2.8.

2.3 Staining analysis

2.3.1 Staining intensity

Intensity of staining was assessed using the Leica QWin image analysis software (Leica Microsystems, UK) and the area of interest defined. The programme gave units of absorption; these results were then subtracted from the maximum absorbance value possible (255) to give the final result in reflectance units, the lower the reflectance unit the lower the intensity of staining.

2.3.2 Area of staining

Area of staining was assessed using the Leica QWin image analysis software (Leica Microsystems, UK). The stained area is expressed relative to the whole tissue.

2.3.3 H-scoring staining analysis

The semi-quantitative H-scoring system was used were the intensity and area of staining needed to be assessed. H-scoring was preformed using a pre-designed programme on the Leica QWin image analysis software (Leica Microsystems, UK). This programme measures intensity as well as area of staining. Areas of high staining are multiplied by 3, areas of medium staining by 2 and areas of low staining by 1, these results are then added together to give the H-score (Lessey, Yeh *et al*. 1996; Wang, Ko *et al*. 2008).

2.3.4 Proliferation analysis

BrdU/Mib1 labelled proliferating cells in the normal and pre-cancerous lesions were manually counted per gland to give a BrdU/Mib1 labelling index (percentage proliferating cells per gland).

2.3.5 Blood vessel staining analysis

BVD hotspots were assessed manually by counting the number of "events" (the number of times a blood vessel appears within the field of view) per field at 40x magnification, with a total of 10 fields of view analysed per section.

2.4 Radio Isotope Assay

A set of standards were prepared using human G17 (Ananspec) which were serially diluted from 1mg/ml stock solution to 10000, 5000, 1000, 750, 500,250, 100, 75, 50, 25, 10, 5 and 1pg/ml in gastrin depleted, heat inactivated human serum (Sigma, UK) containing 200KIA Trasylol/ml (Bayer, UK). These standards were then stored on ice until further use. Rabbit anti-human G17 (Dako cytomation, UK) was diluted 1 in 5000 in PBS and stored on ice until required. ^{125}I-G17 (PerkinElmer, UK) was diluted from 2 x 10^{-8}M stock solution to 4 x 10^{-11}M (decay adjusted) and stored on use until required.

The following PEG (MW 10000)-coated glass tubes were prepared in triplicate:

- Standards/samples: 50µl standard/sample, 50µl antibody, 50µl ^{125}I-G17, 50µl PBS
- Total binding: 50µl mouse serum, 50µl antibody, 50µl ^{125}I-G17, 50µl PBS
- Reagent blank: 50µl mouse serum, 50µl l ^{125}I-G17, 100µl PBS
- Background: 50µl mouse serum, 50µl antibody, 100µl PBS
- Total counts: 50µl l ^{125}I-G17, 150µl PBS

All samples were vortexed, covered with parafilm and incubated at 4oC for 72hrs.

50µl newborn calf serum (Sigma, UK) containing 250µl of 25% PEG (8000) was added to the tubes and vortexed, followed by centrifugation at 1500rpm at 4^{o}C for 30 minutes to harvest the samples. The supernatant was then aspirated off and the pellet counted on the gamma counter.

2.5 In vivo mouse studies

All *in vivo* experiments were performed in the Division of Pre-Clinical Oncology, University of Nottingham and were carried out under the Home Office licence number 40/2323 granted after local ethical approval.

Mice were housed in sterile insolators illuminated by fluorescent lights set to give 12 hours light/dark cycle. The room was air-conditioned by a system designed to maintain an air temperature range of 23 ± 2ºC. Mice were group housed in plastic cages with irradiated bedding and provided with nesting materials and environmental enrichment. They were fed irradiated 2019 rodent diet (Harlan) and autoclaved water was offered *ad libitum.*

2.5.1 INS-GAS model

The INS-GAS mouse model of gastric adenocarcinoma as described by Wang *et al* in 1993 (Wang, Bonner-Weir *et al*. 1993; Wang, Koh *et al*. 1996) was re-derived in the Division of Pre-clinical Oncology to remove specific pathogens. End-stage disease was characterised and found to occur

at week 40 in the colony, of note a side effect of the INS-GAS transgene is obesity.

At termination the stomach was removed and opened up along the longest curvature and any contents scraped out. The stomach was then rinsed twice in sterile PBS, before being sectioned and placed flat in biopsy bags. The tissue was then left to fix overnight in formal calcium before being processed, embedded and sectioned as previously described.

2.5.2 *Apc*$^{Min/+}$ model

An in-house *APC*^{Min} mouse breeding colony used in this study (Watson and Smith 2001). The model has been used experimentally, since 1984 onwards when CP Holroyde used it to represent terminal colorectal cancer and associated cachexia (Holroyde, Skutches *et al*. 1984). The mice exhibit multiple intestinal neoplasia (Min) and concurrent hypergastrinaemia. Genetic screening by PCR ensured that all the mice entering the breeding colony had the mutant allele.

Genotyping was carried out using DNeasy tissue kit (Qiagen) to extract DNA from tail snips. 2µl of the resulting DNA was then added to 44µl megamix blue (Helena Biosciences), along with 2µl of the forward primer, 5' TCT CGT TCT GAG AAA GAC AGA AGC T 3'(MWG Biotech) and 2µl of the reverse primer, 5' TGA TAC TTC TTC CAA AGC TTT GGC TAT 3'(MWG Biotech). PCR reaction was then carried out on ABI

thermocycler under the following conditions initialisation at 95°C for 5mins then 30 cycles of denaturing at 95°C for 30secs, annealing at 54°C for 60sec and extension at 72°C for 2mins samples were then held at 4°C until being stored The PCR product was then digested using restriction enzyme HindIII (Roche) overnight at 37°C before being run out on a 2% agarose gel and visualised with ethidium bromide.

Depending on the study, at termination, the stomach was removed as described in section 2.5.1 or the intestine was dissected out. For the intestine tumour burden in each mouse was counted using a dissecting microscope in the small and large intestine respectively. The small and large intestines were collected, stored and fixed separately in formal calcium for approximately 24 hrs. They were then rolled up into a "swiss roll" and placed in histology cassettes and processed and embedded as previously described.

2.6 Therapeutic intervention

2.6.1 G17DT immunisation

Mice were given a course of either 250mg/kg G17DT or DT vehicle at 0, 1 and 3 weeks and then at 3-weekly intervals thereafter, this was based on data achieved in previous studies with the Division of Pre-Clinical Oncology (Smith, Justin *et al*. 2000; Gilliam, Watson *et al*. 2004). The DT part was used as a control to determine that any effects seen were as a result of a response to the G17 and not the DT. At the study end point mice were

fasted overnight and then administered with bromodeoxyuridine (BrdU) 1hr before termination. Unfortunately due to the volume of serum required for measuring serum gastrin levels it was not possible to do so in all studies.

2.6.2 COX inhibitors

Mice were given either 37.5mg/ml aspirin in drinking water (1.5mg/kg), 0.75mg/ml celecoxib in drinking water (3mg/kg) or placebo for duration of 9 months. At the study end point mice were fasted overnight and then administered with bromodeoxyuridine (BrdU) 1hr before termination.

2.7 Helicobacter infection

The following *H. pylori* strains were used Sydney Strain 1 (SS1), NCTC 11637 and B128 7.13 rederived from B128 by Rick Peek and cultured on 5% horse blood agar. *H. felis* (strain: ATCC49179), was cultured on 5% horse blood agar slopes with Brucella broth overlays. All bacteria were incubated kept at 37ºC with 5% CO_2 and 86% N_2. Swabs of these were taken and put in Brucella broth (Oxoid UK) and the optical density adjusted. Mice were given 0.1ml containing $1x10^8$ colony forming units. All bacteria were provided by the Institute of Infections, Immunity and Inflammation, University of Nottingham. The mice were orally dosed on days 1, 3 and 5 of the study with 1ml bacterial suspension by oral gavage. Control mice were given an equal volume of uninfected Brucella broth.

Antibody	Hydrogen peroide block	Antigen retrival solution	Antihgen retrival protocol	Serum block	Primary antibody concentration	Matched IgG concentration negative	Secondary antibody
Rabbit anti-human COX-2 (Cayman, USA)	0.6% in methanol 30mins	None	None	20% swine serum (Dako Cytomation, UK) 30mins	10mg/l 1hr at RT	Rabbit serum (Dako Cytomation, UK)	Swine anti-rabbit biotinylated (Dako Cytomation, UK)
Rabbit anti-mouse COX-2 (Cayman, USA)	0.6% in methanol 30mins	Trypsin	37°C for 20mins	5% swine serum (Dako Cytomation, UK) 10mins	5mg/l 2hrs at RT	Inactivated primary antobody (Dako Cytomation, UK)	Swine anti-rabbit biotinylated (Dako Cytomation, UK)
Mouse anti-BrdU (Dako Cytomation, UK)		10mM Citric acid pH 6.0	microwave at 98°C for 15mins followed by1M HCl acid at 55°C for 6-8mins		5.5mg/l	Mouse serum (Dako Cytomation)	
Mouse anti-Mib-1 (Dako Cytomation, UK)	0.6% in methanol 30mins	10mM Citric acid pH 6.0	microwave at 98°C for 20mins	20% rabbit serum (Dako Cytomation, UK) 15mins	2.7mg/l 1hr at RT	Univeral mouse negative	Rabbit anti-mouse biotinylated (Dako Cytomation, UK)
Rat anti-mouse CD34 (Becton Dickinson, UK)	3% in methanol 15mins	10mM Citric acid pH 6.0	Pre-heat solution to 98°C then microwave for 10mins at 98°C	none	1.25mg/l O/N at 4°C	Rat IgG (Becton Dickinson, UK)	anti-rat IgG biotinylated (Becton Dickinson, UK)
Mouse anti-CD31 (Dako Cytomation, UK)	6% in methanol 15mins	10mM Tris plus 1mM EDTA pH 9.0	microwave at 98°C for 15mins	20% rabbit serum (Dako Cytomation, UK) 30mins	4.5mg/l 1hr at RT	Universal mouse negative (Dako Cytomation, UK)	Rabbit anti-mouse biotinylated (Dako Cytomation, UK)
Mouse anti-Bcl-2 (Dako Cytomation, UK)	0.6% in distilled water 15mins	10mM Citric acid pH 6.0	microwave at 98°C for 20mins	20% rabbit serum (Dako Cytomation, UK) 30mins	5.6mg/l 1hr at RT	Universal mouse negative (Dako Cytomation, UK)	Rabbit anti-mouse biotinylated (Dako Cytomation, UK)
Rabbit anti-VEGF-R2 (Abcam, UK)	1% in distilled water 15mins	Protinase K	10mins at RT	20% swine serum (Dako Cytomation, UK) 15mins	2.5mg/l 1hr at RT	Rabbit serum (Dako Cytomation, UK)	Swine anti-rabbit biotinylated (Dako Cytomation, UK)
Mouse anti-XIAP (Cayman, USA)	1% in methanol 15mins	10mM Citric acid pH 6.0	Pre-heat solution to 98°C then microwave for 10mins at 98°C	10% rabbit serum (Dako Cytomation, UK) 15mins	1mg/l O/N at 4°C	Universal mouse negative (Dako Cytomation, UK)	Rabbit anti-mouse biotinylated (Dako Cytomation, UK)
Mouse anti-Akt (Dako Cytomation, UK)	1% in distilled water 15mins	10mM Citric acid pH 6.0	microwave at 98°C for 15mins	10% rabbit serum (Dako Cytomation, UK) 30mins	20mg/l O/N at 4°C	Universal mouse negative (Dako Cytomation, UK)	Rabbit anti-mouse biotinylated (Dako Cytomation, UK)
Mouse anti-phospho-Akt (Dako Cytomation, UK)	1% in distilled water 15mins	10mM Citric acid pH 6.0	microwave at 98°C for 15mins	10% rabbit serum (Dako Cytomation, UK) 30mins	20mg/l O/N at 4°C	Universal mouse negative (Dako Cytomation, UK)	Rabbit anti-mouse biotinylated (Dako Cytomation, UK)
Goat anti-CCK2R (Everset biotech, UK)	0.6% in methanol 15mins	10mM Citric acid pH 6.0	microwave at 98°C for 15mins	20% rabbit serum (Dako Cytomation, UK) 30mins	4mg/l O/N at 4°C	Goat serum (Dako Cytomation, UK)	Rabbit anti-goat biotinylated (Dako Cytomation, UK)
Goat anti-HB-EGF (Santa Cruz, USA)	3% in methanol 15mins	10mM Citric acid pH 6.0	Pre-heat solution to 90°C then microwave for 20mins at 90°C	20% rabbit serum (Dako Cytomation, UK) 30mins	4mg/l 1hr at RT	Goat serum (Dako Cytomation, UK)	Rabbit anti-goat HRP (Dako Cytomation, UK)

Table 2.1 immunohistochemistry antibodies, concentrations, blocks, antigen retrieval and secondary antibodies.

(RT = Room temperature, O/N = Over night)

Chapter 3

PROGRESSION OF BARRETT'S OESOPHAGUS A ROLE FOR GASTRIN

3.1 Barrett's oesophagus

Barrett's oesophagus is a recognised pre-malignant condition (Hirota, Loughney *et al*. 1999), which can progress down the multistep pathway of metaplasia to dysplasia and finally to oesophageal adenocarcinoma (Jankowski, Wright *et al*. 1999). To reduce the number of individuals that progress from Barrett's to adenocarcinoma, surveillance programmes are in place; however, unlike other cancer surveillance programmes, they do not appear to be having a great effect on reducing the number of individuals that progress to adenocarcinoma (Gerson and Triadafilopoulos 2002). Therefore, an alternative approach is required, such as identifying possible biomarkers that can predict an individual's chance of progressing from Barrett's to adenocarcinoma.

With gastrin and its receptor emerging as players in the development of pre-malignant and malignant lesions of the GI tract (Aly, Shulkes *et al*. 2004), this study aimed to evaluate the role of gastrin and its receptor on the progression of Barrett's oesophagus to oesophageal adenocarcinoma. This was achieved by analysis of anti-apoptotic and angiogenic factors in relation to gastrin and CCK-2R.

Pinch biopsies and whole blood were obtained with informed ethical constant from Barrett's patients attending Dr. Ragunath's endoscopy clinic. The biopsies were independently classified by a pathologist according to histological type; columnar lined oesophagus (CLO), intestinal metaplasia (IM) and high grade dysplasia (HGD). Staining intensity was assessed using reflectance units as described in section 2.3.1. Whole blood was centrifuged to obtain the serum and biopsies were formalin fixed and paraffin embedded. A gastrin radioimmune assay was used to quantify serum

levels of G17 and immunohistochemistry was used to determining the protein levels of various markers.

3.1.1 Proliferation

Proliferation was assessed using the MIB1 antibody (Dako Cytomation, UK) that stain for the Ki-67 antigen. Ki-67 is preferentially expressed by cells during all active phases of the cell cycle (G_1, S, G_2 and M phases), but not during the resting phase (G_0) or DNA repair (Gerdes, Lemke *et al*. 1984). Proliferation was assessed in the columnar cells of CLO and the lesions of IM and HGD.

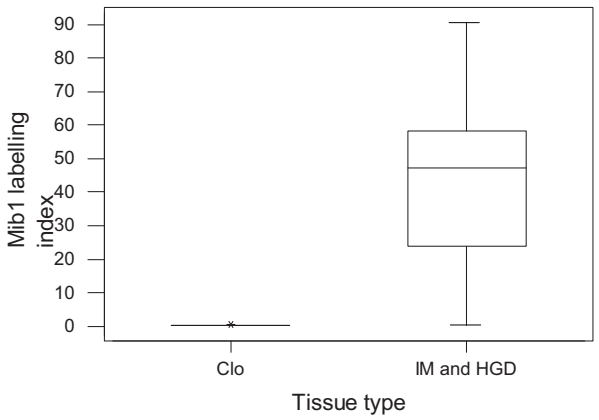


Fig. 3.1 comparison of proliferation in different grades of Barrett's oesophagus (CLO n=5, IM and HGD n=19). $p<0.001$ using Wilcoxon signed rank. Boxes represent spread of data from third to first quartile, horizontal line represents the median; error bars extend to highest and lowest data values within upper and lower limits, * represent outliers.

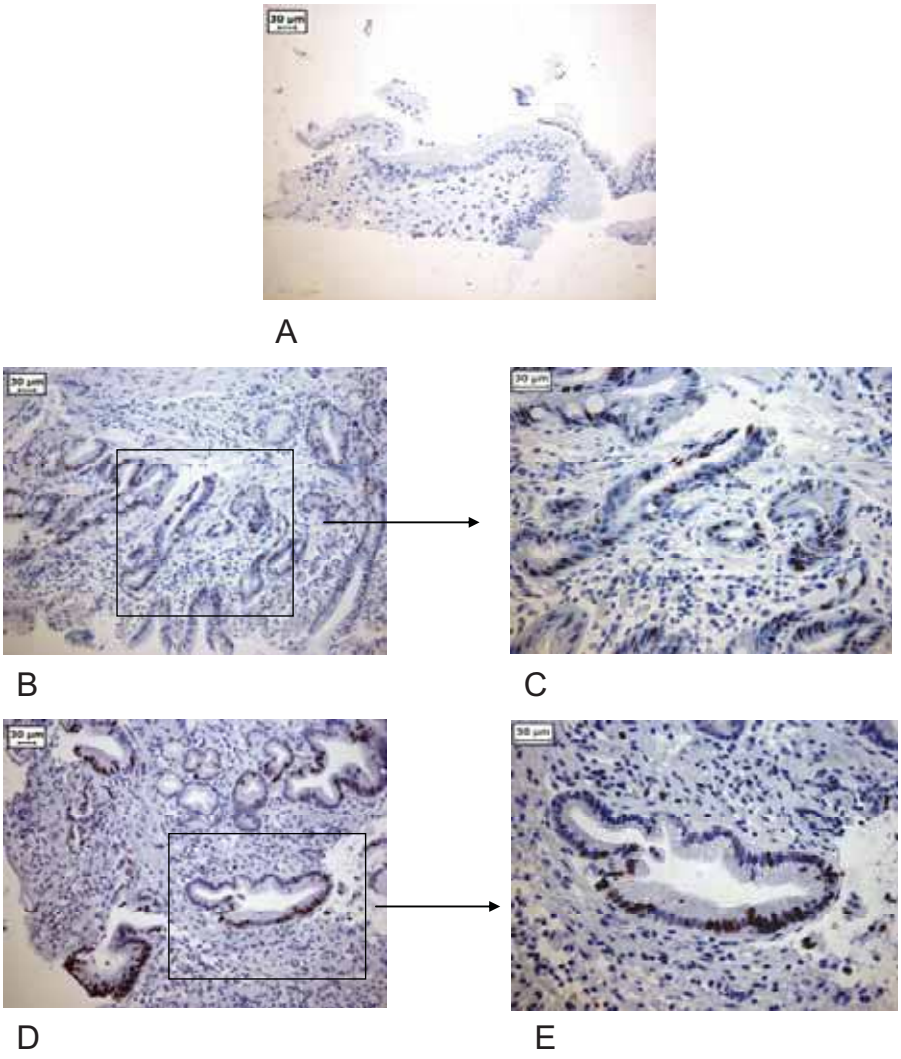


Fig. 3.2 Examples of MIB-1 staining of proliferating cells in CLO (A), IM (B-C) and HGD (D-E).

There was a significant 1300-fold ($p<0.001$) increase in proliferation in IM and HGD combined, compared to CLO (fig 3.1). In the CLO tissue there was very little staining of the columnar cells. In IM and HGD there was specific nuclear staining of the goblet and dysplastic cells respectively; there was also occasional staining of stromal cells (fig 3.2).

3.1.2 Serum gastrin and CCK-2 receptor levels

A RIA was used to quantify serum levels of G17 and a specific CCK-2 receptor antibody was used to stain for the gastrin receptor.

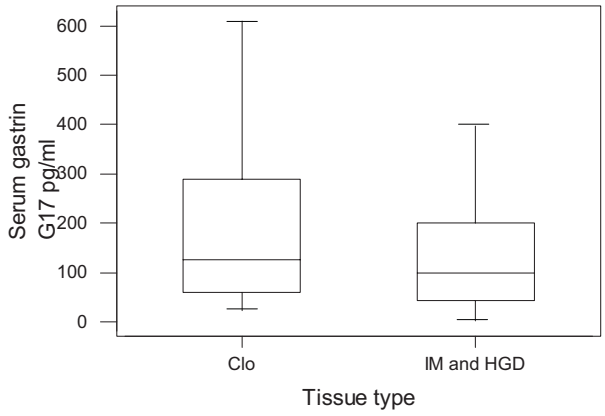


Fig. 3.3 Comparison of serum gastrin levels in patients with different grades of Barrett's oesophagus (CLO n=5, IM and HGD n=19). Boxes represent spread of data from third to first quartile, horizontal line represents the median, error bars extend to highest and lowest data values within upper and lower limits.

There was no significant difference between serum gastrin levels in individuals with CLO compared to those with IM and HGD (see fig 3.3). All the individuals in this study had elevated serum gastrin levels compared to normal fasting levels of <40pg/ml (Corleto, Goebel *et al*. 2003) with 90% of individuals being hypergastrinaemic.

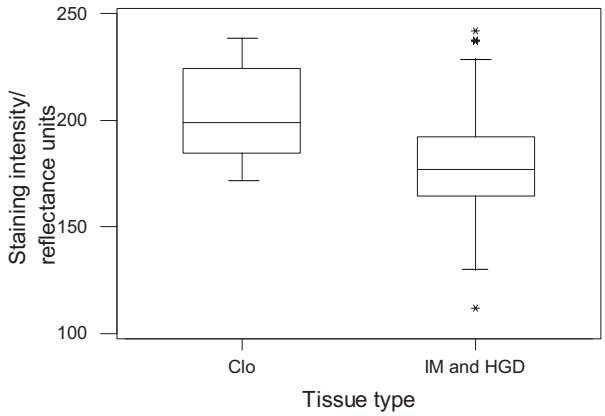


Fig. 3.4 Comparison of CCK-2R protein expression in different grades of Barrett's oesophagus (CLO n=5, IM and HGD n=19). $p<0.005$ using Wilcoxon signed rank. Boxes represent spread of data from third to first quartile, horizontal line represents the median, error bars extend to highest and lowest data values within upper and lower limits, * represent outliers.

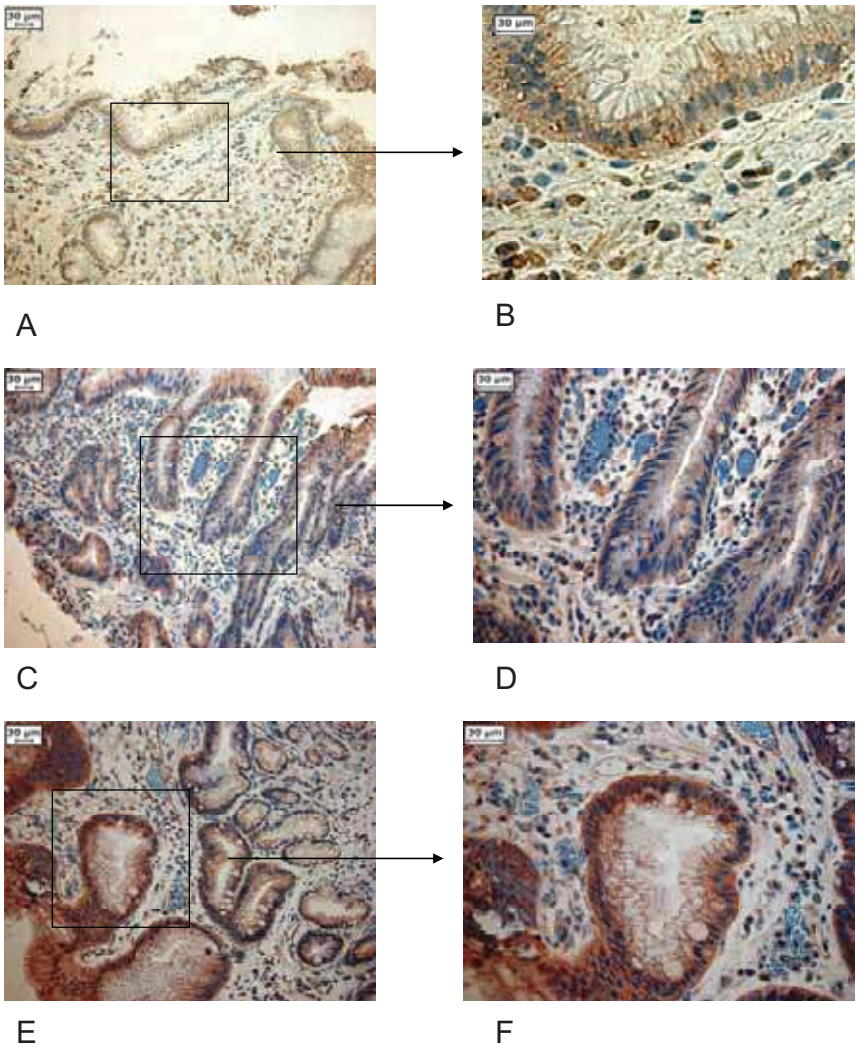


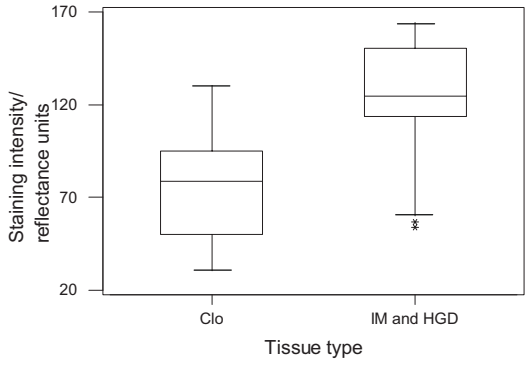
Fig. 3.5 Examples of CCK-2R staining in CLO (A-B), IM (C-D) and HGD (E-F).

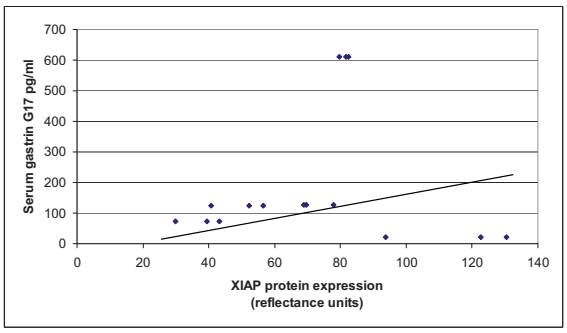
There was a significant 1.1-fold decrease ($p<0.005$) in CCK-2 receptor protein expression in IM and HGD compared to CLO (see fig 3.4). Examples of CCK-2 receptor staining patterns are shown in fig. 3.5; in the CLO tissue staining of the columnar cells was cytoplasmic, there was also diffuse staining of the stromal tissue. In the IM samples there was a similar pattern of staining in the goblet cells as seen in the columnar, but the stromal staining was weaker. The most intense staining was

seen in HGD, although only in a few samples, in both the cytoplasm and nucleus of dysplastic cells.

3.1.3 X-linked Inhibitor of Apoptosis (XIAP) protein expression

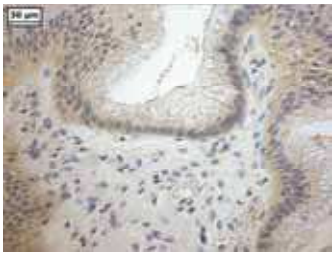
XIAP is a member of the inhibitor of apoptosis (IAP) protein family and inhibits cellular apoptosis via inhibition of caspase -3, -7 and cytochrome c-mediated activation of caspase-9 (Kluger, McCarthy *et al*. 2007). A specific XIAP antibody (AbCam, UK) was used to stain for XIAP protein.



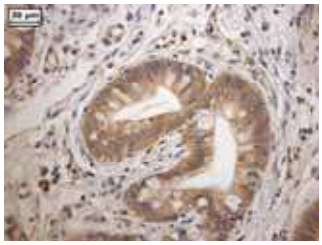


A B

Fig. 3.6 comparison of levels of XIAP protein expression in different grades of Barrett's oesophagus (CLO n=5, IM and HGD n=19). $p<0.005$ using Wilcoxon signed rank. Boxes represent spread of data from third to first quartile, horizontal line represents the median, error bars extend to highest and lowest data values within upper and lower limits, * represent outliers (A). Positive correlation between XIAP protein expression and serum gastrin levels in CLO tissue $p<0.05$, $r_s=0.582$ using spearman 2-tailed correlation (B).



A



B

C

Fig 3.7 Examples of XIAP protein staining in CLO (A), IM (B) and HGD (C) tissue.

There was a significant 1.6-fold increase in XIAP protein expression in IM and HGD compared to CLO (see fig. 3.6A) with a positive correlation, although not significant when Bonferroni correction was applied, between XIAP protein expression and serum gastrin levels in CLO ($p<0.05$, $r_s=0.582$) (see fig. 3.6B). The pattern of staining in CLO was weak cytoplasmic staining at the base of the columnar cells and there was no stromal staining. In IM and HGD there was moderate to strong staining of the goblet cells and dysplastic cells respectively, with weak staining of cytoplasm in the stromal cells; also in the IM there was some nuclear staining of the stromal cells (See fig 3.7).

3.1.4 Bcl-2 protein expression

Bcl-2 is an anti-apoptotic member of the Bcl-2 family and protein levels were assessed using a specific Bcl-2 antibody.

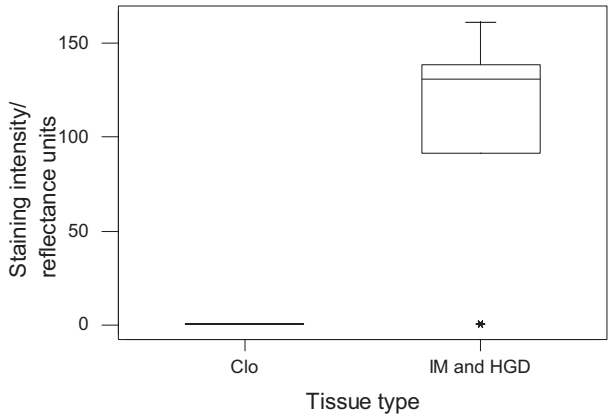
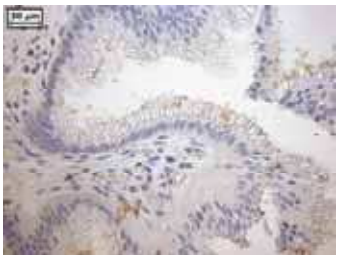
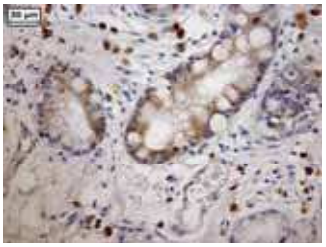


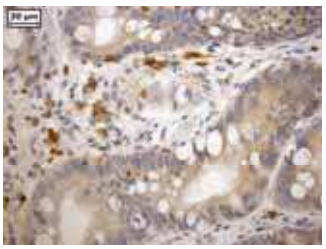
Fig. 3.8 comparison of Bcl-2 protein expression in different grades of Barrett's oesophagus (Clo n=5, IM and HGD n=19). $p<0.001$ using Wilcoxon signed rank. Boxes represent spread of data from third to first quartile, horizontal line represents the median, error bars extend to highest and lowest data values within upper and lower limits, * represent outliers.



A



B



C

Fig. 3.9 Examples of Bcl-2 staining in CLO (A), IM (B) and HGD (C) tissue.

Fig 3.8 shows that there was a significant 85.5-fold increase ($p<0.001$) in Bcl-2 protein expression the IM and HGD compared to CLO. As shown in fig. 3.9 there was no staining in the columnar cells of the CLO samples, but there was strong cytoplasmic staining in the goblet cells of IM with moderate cytoplasmic and strong nuclear staining of certain stromal cells. In the HGD there was moderate cytoplasmic staining of the dysplastic and some stromal cells, but no nuclear staining.

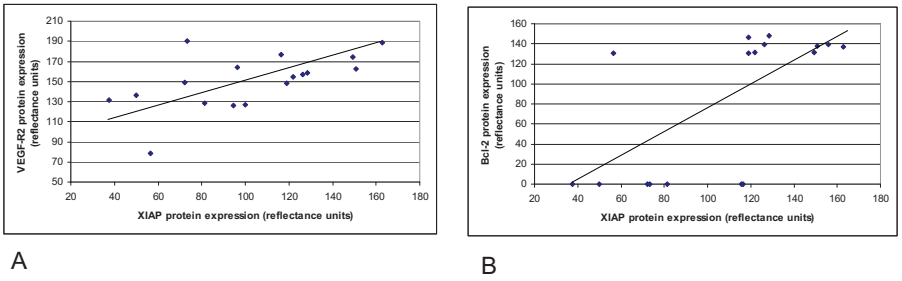


Fig 3.10 Correlation between XIAP and VEGF-R2 protein expression $p=0.024$, $rs=0.544$ (A) and XIAP and Bcl-2 protein expression $p<0.001$, $rs=0.757$ (B) using Spearman 2-tailed correlation.

There was a significant positive correlation (after Bonferroni correction) $p<0.001$ between Bcl-2 and XIAP protein expression and a positive correlation between VEGF-R2 and XIAP protein expression (Fig. 3.10).

3.1.5 Akt and Phosphorylated-Akt (P-Akt) protein expression

Akt is involved in the regulation of cell cycle, survival and metabolism by binding and regulating a number of downstream effectors including NFκB and members of the Bcl-2 protein family. P-Akt is the phosphorylated activated form of Akt (Song, Ouyang *et al*. 2005). Akt and P-Akt protein expression levels were assessed by staining with specific antibodies (Dako Cytomation, UK).

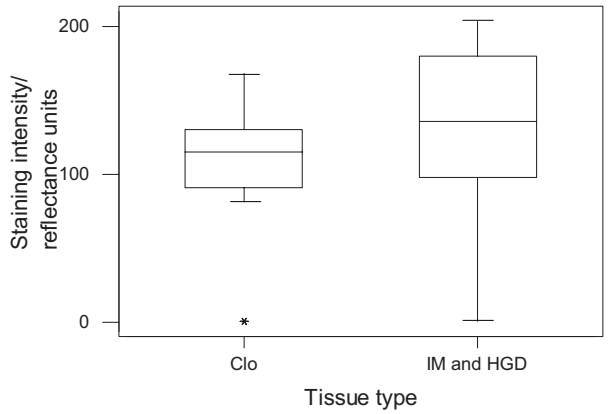
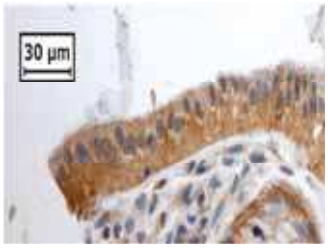
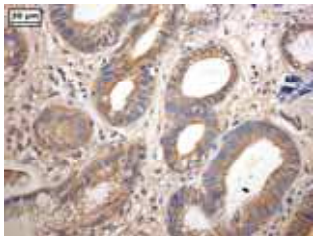


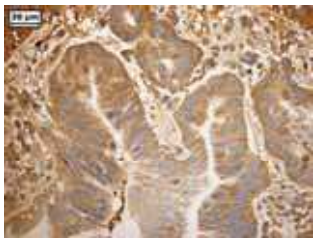
Fig. 3.11 comparison of Akt protein expression in different grades of Barrett's oesophagus (Clo n=5, IM and HGD n=19). Boxes represent spread of data from third to first quartile, horizontal line represents the median, error bars extend to highest and lowest data values within upper and lower limits, * represent outliers.



A



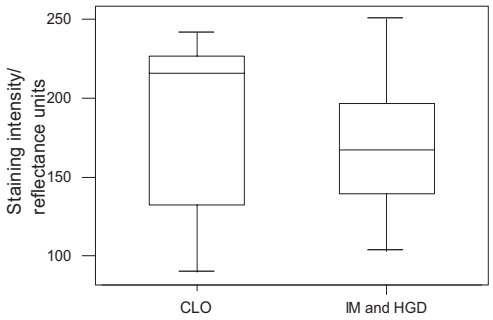
B



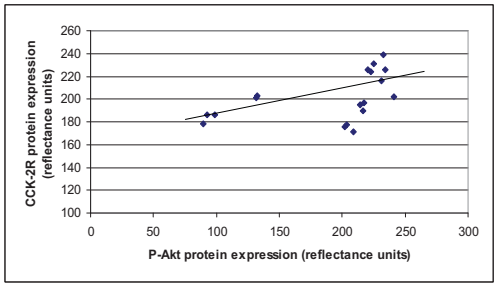
C

Fig. 3.12 Examples of Akt staining in CLO (A), IM (B) and HGD (C).

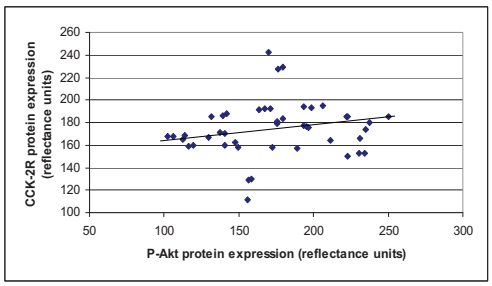
The intensity levels of Akt protein expression were similar in all three grades of Barrett's tissue assessed, with no significant difference (see fig. 3.11). There was intense staining in the cytoplasm of columnar, stromal and endothelial cells, with clear gradation from intense to weak from the base to the apex of the columnar cells. Staining was also intense in the dysplastic cells of HGD (fig 3.12).



A

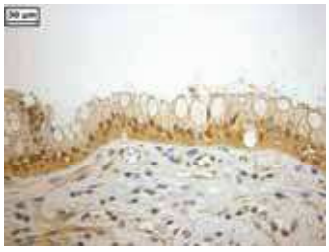


B

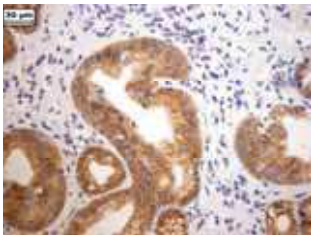


C

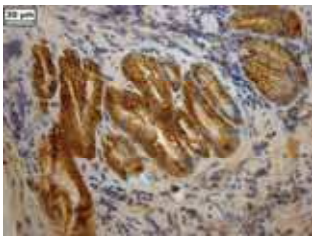
Fig. 3.13 comparison of P-Akt protein expression in different grades of Barrett's oesophagus (CLO n=5, IM and HGD n=19) Boxes represent spread of data from third to first quartile, horizontal line represents the median, error bars extend to highest and lowest data values within upper and lower limits, * represent outliers (A). There were positive correlations between CCK-2R and P-Akt protein expression in CLO, $p<0.05$, r_s 0.582(B) and IM and HGD $p<0.001$, r_s 0.5 (C) using Spearmans 2-tailed correlation.



A



B



C

Fig. 3.14 Examples of P-Akt staining in CLO (A), IM (B) and HGD (C).

As shown in Fig. 3.13A, as for Akt there is no significant difference between the intensity of staining of P-Akt between the different histological tissue types, but there was a significant positive correlation between P-Akt and CCK-2R protein expression in CLO ($p<0.005$, $r_s=0.582$) and IM and HGD ($p<0.001$, $r_s=0.5$) (see fig. 3.13B and C). In the CLO there was graduated cytoplasmic staining of the columnar cells with the strongest at the base. There was also nuclear staining of the columnar cells and weak staining of the stromal cells and some endothelial cells. In comparison, in both the IM and HGD there was no nuclear staining, only cytoplasmic staining of the goblet cells and dysplastic cells respectively. There was some weak stromal staining in the HGD, but not the IM (fig. 3.14).

3.1.6 Vascular endothelial growth factor-receptor 2 (VEGF-R2) protein expression

VEGF-R2 is a member of a receptor tyrosine kinase family and its activation plays an important role in a number of biological processes including; embryonic development, cell proliferation, migration and differentiation (Holmqvist, Cross *et al*. 2004). A specific VEGF-R2 antibody (AbCam, UK) was used to assess the level of VEGF-R2 protein expression.

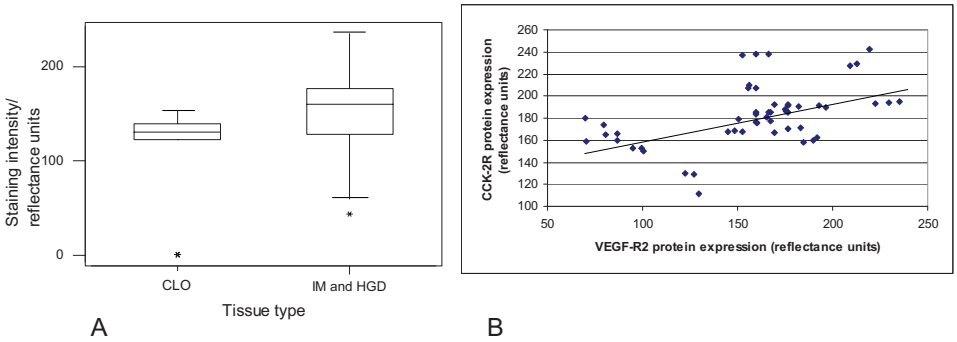


Fig. 3.15 comparison of VEGF-R2 protein expression in different grades of Barrett's oesophagus (CLO n=5, IM and HGD n=19), $p<0.001$ using Wilcoxon signed rank. Boxes represent spread of data from third to first quartile, horizontal line represents the median, error bars extend to highest and lowest data values within upper and lower limits, * represent outliers (A). Significant positive correlation between VEGF-R2 and CCK-2R protein expression in IM and HGD $p<0.001$, r_s 0.522 (B).

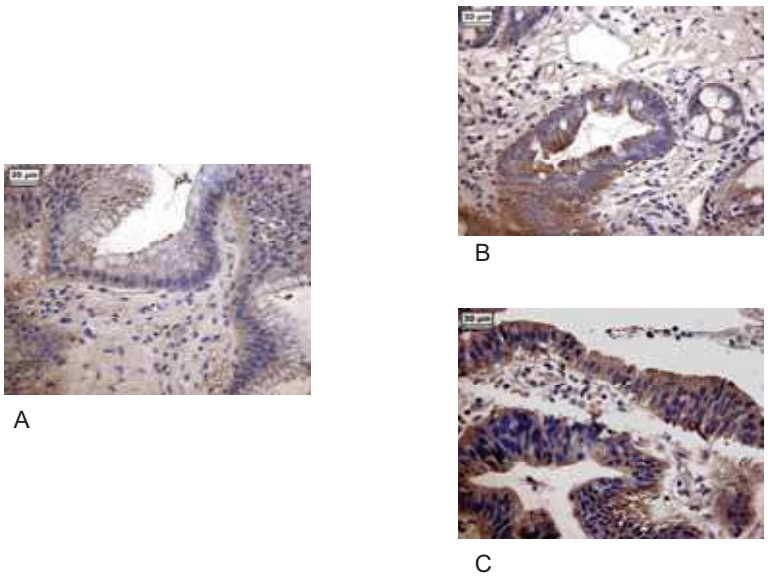


Fig. 3.16 Examples of VEGF-R2 staining in CLO (A), IM (B) and HGD (C).

There was a stepwise increase in VEGF-R2 protein expression from CLO to HGD with a significant 1.3-fold increase ($p<0.001$) in VEGF-R2 protein expression in IM

and HGD compared to CLO, with a significant positive correlation between VEGF-R2 and CCK-2R protein expression in IM and HGD ($p<0.001$, $r_s=0.522$) results are summarised in Fig. 3.15. In CLO there was very weak diffuse staining of the columnar and stromal cells. In IM, staining was localised to the membrane of the goblet cells with strongest staining at the apex of the cell; there was also very weak diffuse staining of the stromal cells. Like IM, HGD also showed strongest staining at the apex of the cells, this time the dysplastic cells, but staining was also cytoplasmic as well as membranous and again there was weak stromal staining (see fig 3.16).

3.1.7 Heparin-binding epidermal growth factor (HB-EGF) protein expression

HB-EGF is a member of the epidermal growth factor (EGF) superfamily ligands and is linked to angiogenesis (Mehta and Besner 2007). A specific HB-EGF antibody (Santa Cruz, USA) was used to assess HB-EGF protein expression.

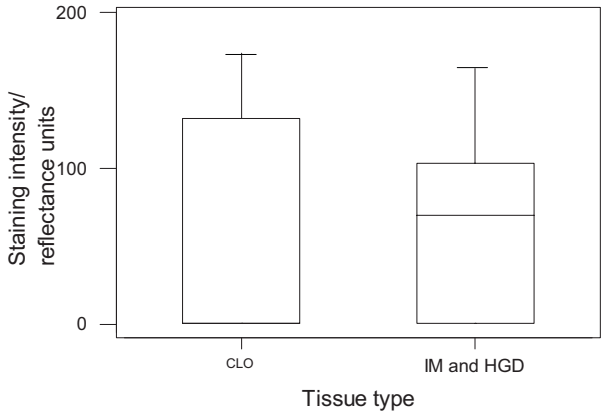
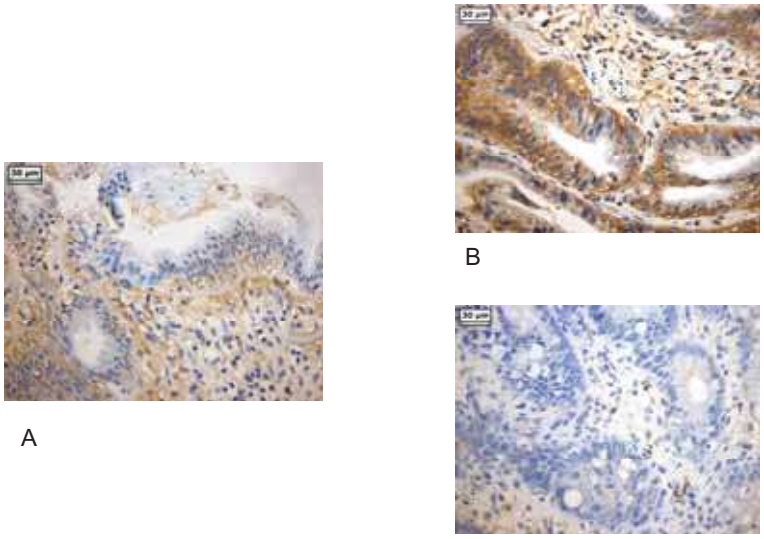


Fig. 3.17 comparison of HB-EGF protein expression in different grades of Barrett's oesophagus (CLO n=5, IM and HGD n=19). Boxes represent spread of data from third to first quartile, horizontal line represents the median, error bars extend to highest and lowest data values within upper and lower limits.



A

B

C

Fig. 3.18 Examples of HB-EGF staining in CLO (A), IM (B) and HGD (C).

There was no significant difference between levels of HB-EGF protein expression in CLO compared to IM and HGD (fig 3.17). In the CLO there was moderate staining in the stroma including endothelial cells. In the columnar cells, staining was graduated

with the strongest staining as the base of the cell. The IM showed strong cytoplasmic staining of the goblet cells and diffuse staining in the stroma. In the HGD there was no staining of the dysplastic cells, only cytoplasmic staining of occasional stromal cells (fig 3.18).

3.1.8 Blood Vessel Density (BVD)

CD31 was used to assess BVD as a measure of angiogenesis. Blood vessels are comprised of endothelial cells and it is these which express the epitope which binds CD31. As shown in fig. 3.20 cross-sectioned blood vessels appear as circles and longitudinal blood vessels appear as tubes.

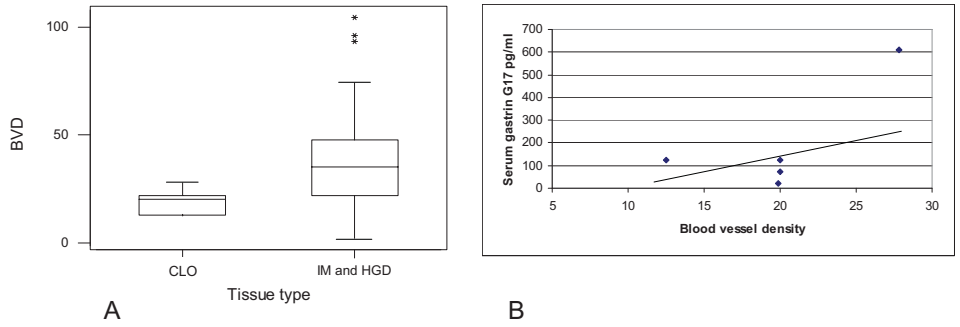


Fig. 3.19 comparison of blood vessel density in different grades of Barrett's oesophagus (CLO n=5, IM and HGD n=19), $p<0.005$ using one way ANOVA. Boxes represent spread of data from third to first quartile, horizontal line represents the median, error bars extend to highest and lowest data values within upper and lower limits, * represent outliers (A). Significant positive correlation between blood vessel density and serum gastrin levels in Clo tissue, $p<0.05$, $r_s=0.632$ using Spearman 2-tailed correlation (B).

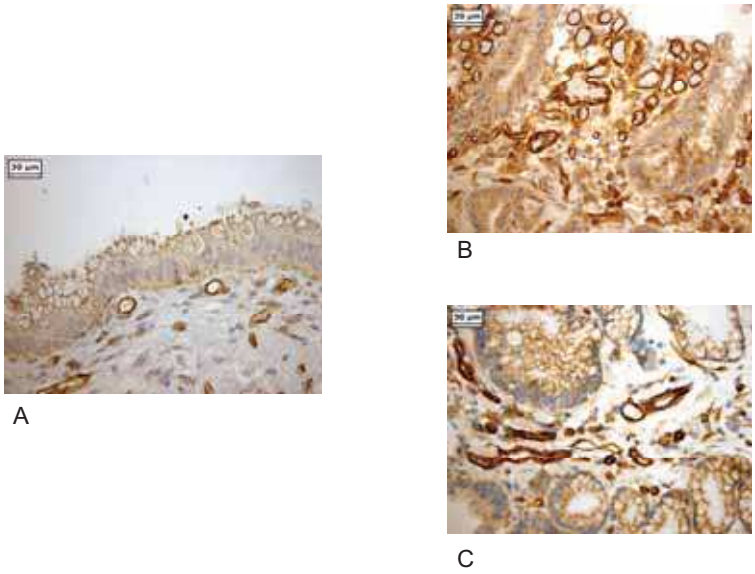
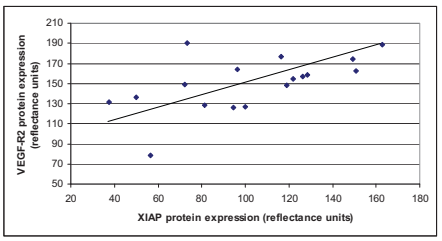
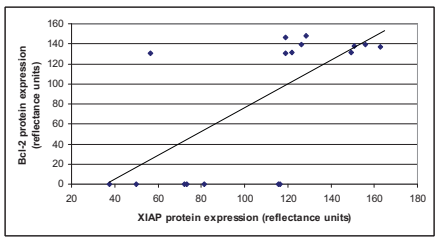


Fig. 3.20 Examples of blood vessel staining in CLO (A), IM (B) and HGD (C).

As fig. 3.19 shows there was a significant 1.9-fold increase ($p<0.005$) in BVD in IM and HGD compared to CLO with a positive correlation, although not significant when Bonferroni correction was applied, between BVD and serum gastrin levels in CLO ($p<0.05$, $r_s=0.632$). Fig. 3.20 clearly shows that there are less new blood vessels in the CLO than the IM and to a lesser extent the HGD.



A



B

Fig 3.21 Correlation between XIAP and VEGF-R2 protein expression p=0.024, rs=0.544 (A) and XIAP and Bcl-2 protein expression p<0.001, rs=0.757 (B) using Spearman 2-tailed correlation.

There were significant positive correlations between BVD and proliferation ($p<0.02$) and BVD and XIAP protein expression ($p<0.01$) after Bonferroni correction (fig. 3.21).

3.1.9 COX-2 protein expression

COX-2 the rate limiting enzyme in the synthesis of prostaglandin from arachadonic acid is over-expressed in a number of pre-malignant tissues and malignant tumours (Dannenberg, Altorki *et al*. 2001). A specific COX-2 antibody (Caymen, USA) was used to measure COX-2 protein expression.

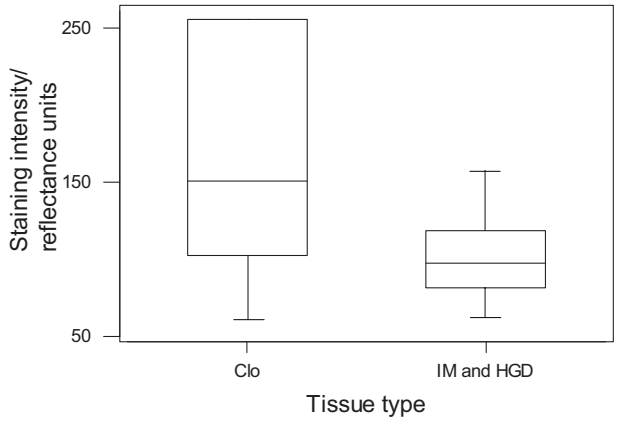


Fig. 3.22 comparison of COX-2 protein expression in different grades of Barrett's oesophagus (Clo n=5, IM and HGD n=19). $p<0.001$ using Wilcoxon signed rank. Boxes represent spread of data from third to first quartile, horizontal line represents the median, error bars extend to highest and lowest data values within upper and lower limits.

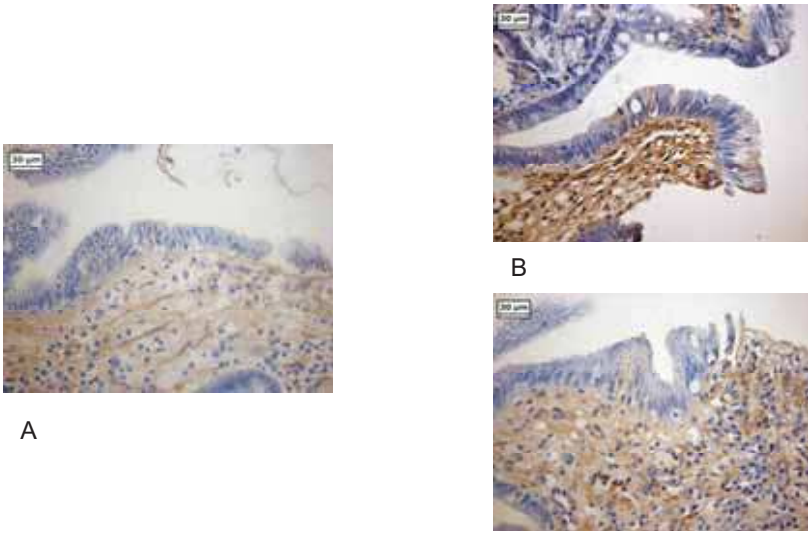


Fig. 3.23 Examples of COX-2 protein staining in CLO (A), IM (B) and HGD (C).

There was a significant 1.6-fold decrease ($p<0.001$) in COX-2 protein expression in IM and HGD compared to CLO (fig. 3.22). Staining was mainly stromal with some endothelial staining in all tissue types. In the CLO there was weak cytoplasmic

staining of occasional columnar cells. In the IM staining was more intense in the stroma and there was cytoplasmic staining of the goblet cells. The HGD showed mainly stromal staining with some staining of dysplastic cells (fig 3.23).

3.2 Discussion

Across all patients in this study, serum gastrin levels were elevated above those of normal fasting serum gastrin levels. An explanation for this is that all patients, as is standard with Barrett's patients, were on proton pump inhibitors (PPIs). PPIs work by blocking gastric acid secretion, but this then causes an increase in serum gastrin as shown in a number of studies on the long term effects of the PPI Lansoprazole (Muller, Dammann *et al*. 1989; Brunner, Hell *et al*. 1995).

High expression of the CCK-2 receptor protein was seen in all tissue types, which is in agreement with work by Haigh *et al* (Haigh, Attwood *et al*. 2003) which showed that CCK-2 receptor expression was increased in Barrett's metaplastic tissue compared to normal oesophageal tissue. However, there was slightly lower CCK-2 receptor protein expression in IM and HGD compared to CLO, possibly because the high levels of circulating gastrin reduce the need for high levels of the receptor or lead to down regulation of the receptor.

The anti-apoptosis markers XIAP and Bcl-2 were significantly increased in IM and HGD compared to CLO, with positive correlation between XIAP protein expression and serum gastrin in CLO. XIAP is the best-characterised and considered the most

potent member of the Inhibitor of Apoptosis Protein (IAP) family and differs from other members by its ability to directly bind to and inhibit activated caspase-3, -7 and -9 (Eckelman, Salvesen *et al*. 2006). There is currently nothing in the literature about the role of XIAP in the progression of Barrett's oesophagus, but overexpression of XIAP has been observed in a number of cell lines including those of colon, ovarian, renal and prostate origin (Tamm, Kornblau *et al*. 2000). However, the results presented here relating to Bcl-2 protein expression are in agreement with current research, which demonstrates an up-regulation of Bcl-2 expression from Barrett's metaplasia to neoplasia (Shimizu, Vallbohmer *et al*. 2006), and therefore supports the data observed for XIAP and the significant positive correlation seen between Bcl-2 and XIAP protein expression in individual patients.

Another anti-apoptotic marker Akt and its activated form P-Akt, a member of the serine/threonine specific protein kinase family, have been shown to be increased in the progression of Barrett's adenocarcinoma (Beales, Ogunwobi *et al*. 2007), but they were not up-regulated in progression from CLO to IM and HGD in this study. Previous work within our group has shown that Akt is activated by gastrin in Barrett's metaplastic tissue (Harris, Clarke *et al*. 2004) and therefore due to the elevated levels of serum gastrin in the individuals in this study any trend may have been masked. There was a positive correlation between p-Akt and CCK-2 receptor protein expression in both CLO and IM and HGD, and between p-Akt and serum gastrin in IM and HGD, suggesting that activation of Akt by gastrin occurs later in the progression of Barrett's.

VEGF-R2 is established as a receptor expressed on endothelial cells, which binds VEGF and stimulates angiogenesis (Ferrara, Gerber *et al*. 2003). However, as shown in this study, VEGF-R2 has also been shown to be expressed on non-endothelial cells (Liang and Hyder 2005), suggesting a possible role for VEGF in the progression of tumours through proliferation and cell survival (Masood, Cai *et al*. 2001). Blocking binding of VEGF to VEGF-R2 has been shown to reduce proliferation in a number of breast cancer cell lines and VEGF may protect cells from apoptosis by the up-regulation of Bcl-2 (Liang, Brekken *et al*. 2006). In this study there was a significant increase in VEGF-R2 protein expression in IM and HGD compared to CLO and a positive correlation between VEGF-R2 and CCK-2 receptor protein expression in IM and HGD, which may indicate that high levels of gastrin acting through the CCK-2 receptor results increase VEGF-R2 expression, which in turn up-regulates Bcl-2 and XIAP in Barrett's tissue. Although in this study no correlation was seen between VEGF-R2 and Bcl-2 protein expression, a positive correlation was seen between VEGF-R2 and XIAP protein expression.

Angiogenesis is an essential part of the carcinogenesis process and previous work in our group has shown that HB-EGF expression can be up-regulated by the binding of gastrin to the CCK-2 receptor leading to increased angiogenesis (Clarke, Dickson *et al*. 2006), but in this study no clear trend was observed between HB-EGF and progression to dysplasia, although there was a correlation between BVD and progression. There was also a positive correlation between BVD and serum gastrin in CLO, indicating that serum gastrin promotes angiogenesis early in Barrett's oesophagus, but not via an increase in HB-EGF.

This work showed a significant decrease in COX-2 protein expression in IM and HGD compared to CLO. Current data on COX-2 expression and progression in Barrett's suggests that there is no relationship (Abdalla, Lao-Sirieix *et al*. 2004). *In vivo* studies have shown that gastrin stimulation can increase COX-2 mediated prostaglandin expression and proliferation via the CCK-2 receptor in Barrett's oesophagus (Abdalla, Lao-Sirieix *et al*. 2004), but no correlation between the gastrin parameters and COX-2 were observed in this study.

In summary serum gastrin levels positively correlate with XIAP protein expression and BVD, and CCK-2R protein expression positively correlates with P-Akt and VEGF-R2 protein expression in Barrett's tissue.

Chapter 4

A role for CCK-2R in the pancreatic cancer environment

4.1 Pancreatic cancer

Pancreatic cancer is the 4th highest cancer killer in the USA with the highest fatality rate of all cancers, although the five year survival rate has increased significantly between 1974-2001 it still remains around 5% (Jemal, Siegel *et al*. 2006). This poor prognosis is in part due to the late presentation of the disease and one of the reasons why a better understanding and a more targeted therapeutic approach to the disease is required.

Studies have shown that gastrin via the CCK-2 receptor can stimulate pancreatic cancer growth (Smith, Rickabaugh *et al*. 1993; Smith, Liu *et al*. 1994). However, data on the expression of CCK-2 receptors in the normal pancreas and pancreatic adenocarcinoma is conflicting. Therefore, formalin-fixed paraffin-embedded tissue microarrays of human pancreatic cancer, pancreatitis, cancer positive and negative lymph nodes and resectioned normal tissue obtained from Mr. D. Lobo, Medical and Surgical Sciences, University of Nottingham were used in this study to determine the levels of CCK-2 receptor protein expression in the pancreatic cancer environment, as well as levels of anti-apoptotic factors and COX-2 using immunohistochemistry.

4.1.1 CCK-2 receptor expression

As in section 3.1.1 a specific CCK-2 receptor antibody was used to stain for the gastrin receptor.

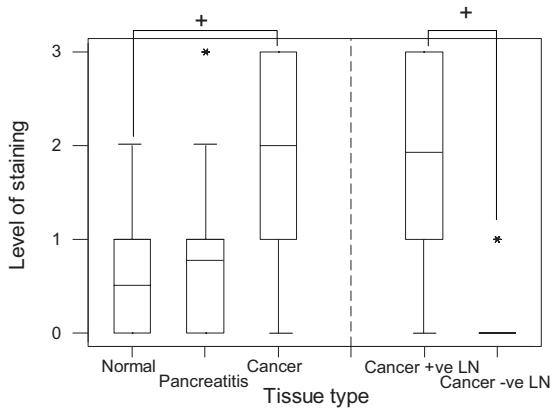


Fig. 4.1 CCK-2R protein expression. Boxes represent spread of data from third to first quartile, horizontal line represents the mean, error bars extend to highest and lowest data values within upper and lower limits, * represent outliers (normal n=31, pancreatitis n=106, cancer n=95, lymph node negative n=64, lymph node positive n=50). +p<0.001 using one way ANOVA.

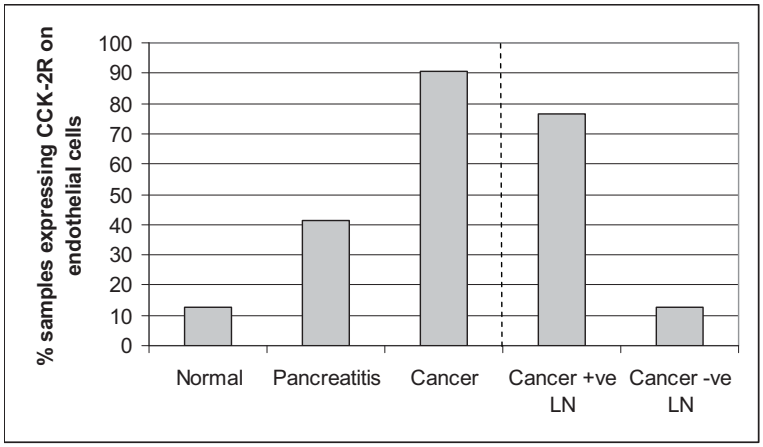


Fig. 4.2 Comparison in the expression of the CCK-2R on blood vessels across the different tissue samples. Bars represent percentage of sections showing positive CCK2R expression on blood vessels.

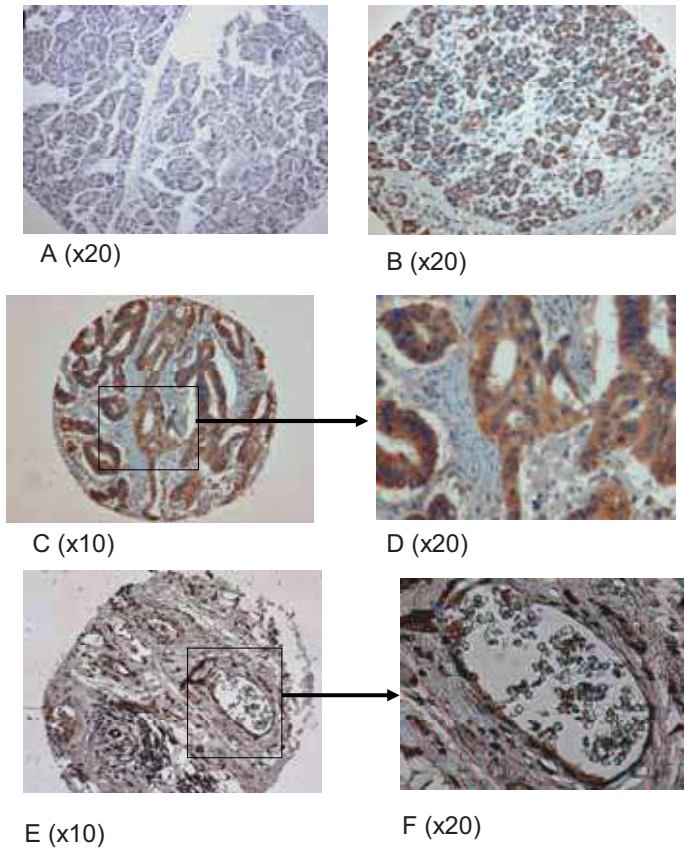


Fig 4.3 Examples of CCK-2R staining in normal pancreas (A), Pancreatitis (B), cancer tissue (C-E) and endothelial cells (D).

Fig. 4.1, shows that there was a significant increase ($p<0.001$) in CCK2R protein expression in pancreatic cancer tissue compared to normal pancreas and in lymph nodes that contain cancer cells compared to those that did not. In conjunction with this fig. 4.2 shows that there was an increase in the number of samples that expressed the CCK-2 receptor on endothelial cells in cancer tissue (7-fold), lymph nodes that contain cancer cells (5.9-fold) and pancreatitis (3.2-fold) compared to normal pancreatic tissue.

Examples of CCK-2 receptor staining in normal, pancreatitis and pancreatic cancer tissue are shown in fig 4.3. Staining can clearly be seen in the abnormal cancer cells and in the endothelial cells (fig 4.3F).

Along side this work analysis of patient data was carried out on the same samples by Dr. Mark Aloysius (personal communication), which demonstrated that in the pancreatic cancer samples there was a significant negative correlation between patient survival and CCK-2R protein expression (r_s=-0.188, p=0.033), and between patient survival and CCK-2R localisation on endothelial cells (r_s=-0.188, p=0.033). There was also a significant positive correlation (r_s=0.233, p=0.008) in cancer samples between recurrence and CCK-2R protein expression, and between recurrence and CCK-2R localisation on endothelial cells. All analysis was carried out using Spearman's 2-tailed correlation with Bonferroni correction.

4.1.2 XIAP protein expression

A specific XIAP antibody was used to stain for XIAP protein expression.

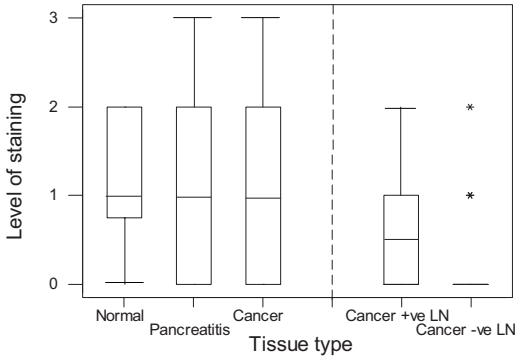


Fig. 4.4 XIAP protein expression. Boxes represent spread of data from third to first quartile, horizontal line represents the mean, error bars extend to highest and lowest data values within upper and lower limits, * represent outliers (normal n=26, lymph node negative n=49, lymph node positive n=40, pancreatitis n=85, cancer n=73).

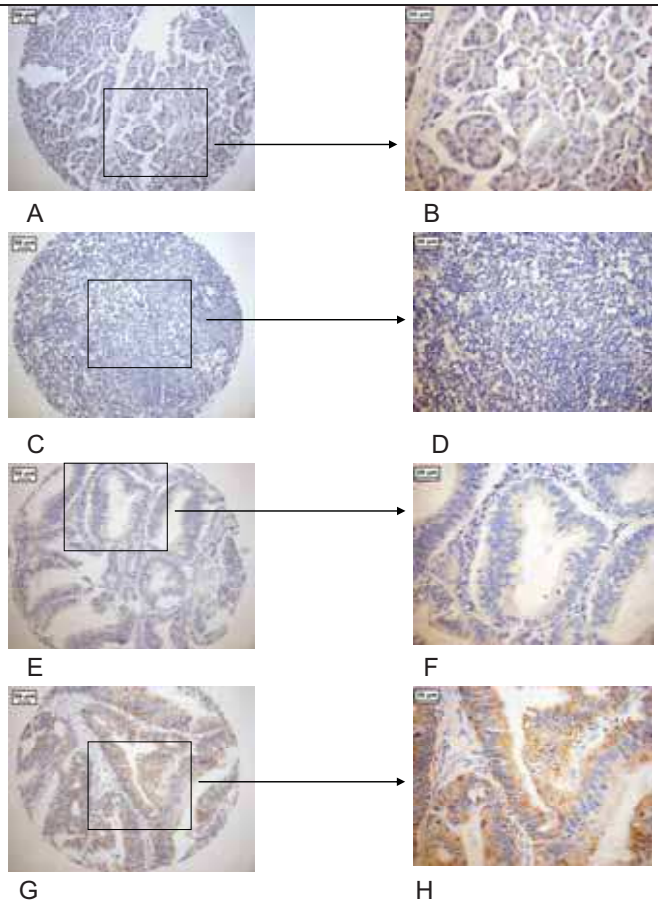


Fig. 4.5 Examples of XIAP staining in normal pancreatic tissue (A and B), negative lymph node (C and D) and well differentiated pancreatic cancer (E-H).

As shown in fig. 4.4 the level of XIAP protein expression in pancreatitis and cancer is similar to that seen in the normal tissue. There was a 21.3 fold decrease in XIAP protein expression in lymph node tissue which does not contain cancer cells compared to lymph node tissue which does. High levels of staining were only seen in pancreatitis and cancer and not normal pancreas. There was weak cytoplasmic staining of occasional acinar cells in normal pancreatic tissue (fig 4.5A and B) and the negative lymph nodes showed very weak or negative staining for XIAP (fig 4.5C and D). In pancreatic cancer there was cytoplasmic staining of the cancer cells, which tended to be weak (fig 4.5E and F), but occasional samples showed strong cytoplasmic staining (fig 4.5G and H).

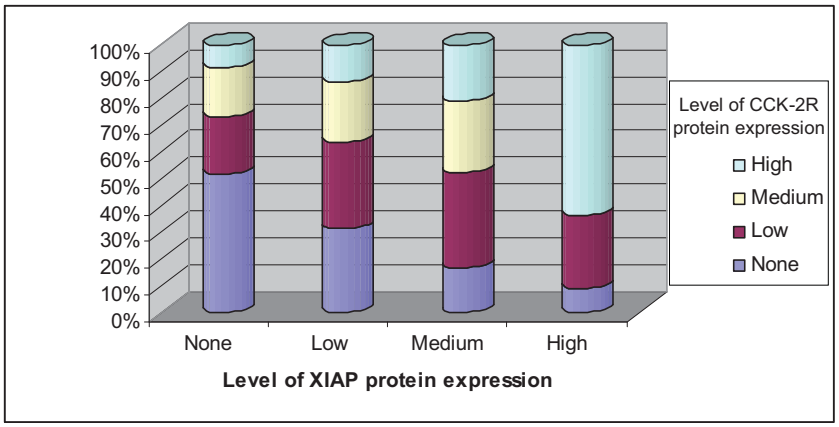


Fig. 4.6 positive correlation between XIAP protein expression and CCK-2R protein expression r_s=0.354, p=0.003 using spearman 2-tailed correlation.

There was a significant (p=0.003) positive correlation, after Bonferroni correction was applied, between XIAP protein expression and CCK-2R protein expression (fig. 4.6). There was also a significant (p=0.003, r_s=0.354) positive correlation, after Bonferroni correction was applied, between XIAP protein expression and CCK-2R localisation on endothelial cells.

4.1.3 Bcl-2 protein expression

A specific Bcl-2 antibody was used to stain for Bcl-2 protein expression.

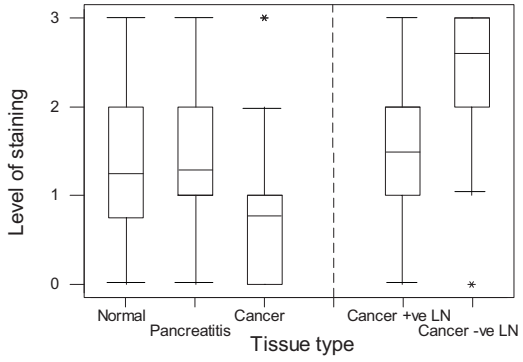


Fig. 4.7 Bcl-2 protein expression. Boxes represent spread of data from third to first quartile, horizontal line represents the mean, error bars extend to highest and lowest data values within upper and lower limits, * represent outliers (normal n=26, lymph node negative n=49, lymph node positive n=40, pancreatitis n=88, cancer n=73). +$p<0.05$ using one way ANOVA.

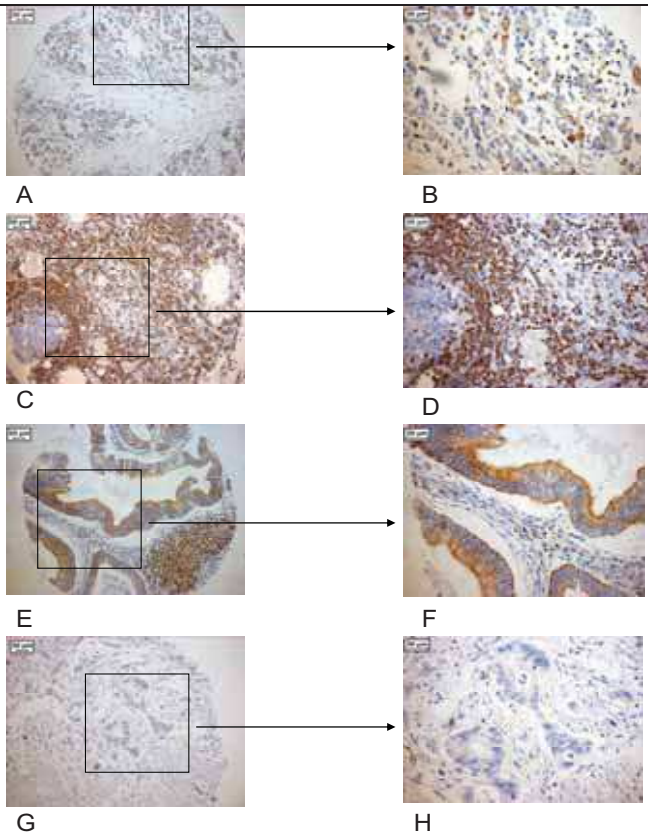


Fig. 4.8 Examples of Bcl-2 staining in normal pancreatic tissue (A and B), negative lymph node (C and D), well differentiated pancreatic caner (E and F) and poorly differentiated pancreatic cancer (G and H).

Fig 4.7 shows that there was a significant 1.59-fold ($p<0.05$) reduction in Bcl-2 protein expression in pancreatic cancer tissue compared to normal pancreatic tissue. Although when the level of staining was averaged across samples of each tissue type there was a decrease in Bcl-2 staining in pancreatic cancer, high levels of staining were observed in occasional pancreatitis and cancer samples but no examples of high staining were seen in samples of normal pancreas. Normal pancreas showed weak to medium cell membrane staining for Bcl-2 (fig 4.8A and B). In cancer negative lymph nodes there was strong staining of the lymphoid cells (fig 4.8C and D). The level of staining seen in the pancreatic cancer samples was dependent on the type of cancer, with well differentiated cancer showing high levels of staining, and poorly differentiated cancers showing low levels of staining as illustrated in fig 4.8E-F.

4.1.4 COX-2 protein expression

A number of studies have shown increased COX-2 expression in pancreatic cancer tissue. Molina *et al* 1999 (Molina, Sitja-Arnau *et al*. 1999) showed elevated levels of both COX-2 mRNA and protein in human pancreatic adenocarcinomas and cell lines, derived from pancreatic tumours, this work is supported by similar findings by Okami *et al* 1999 (Okami, Yamamoto *et al*. 1999) that showed that 54% of pancreatic carcinomas over-expressed COX-2 mRNA and 44% showed strong COX-2 protein expression compared to normal pancreas. Therefore it was decided use a specific COX-2 antibody to ascertain if these pancreatic samples also showed this correlation in relation to COX-2 protein expression.

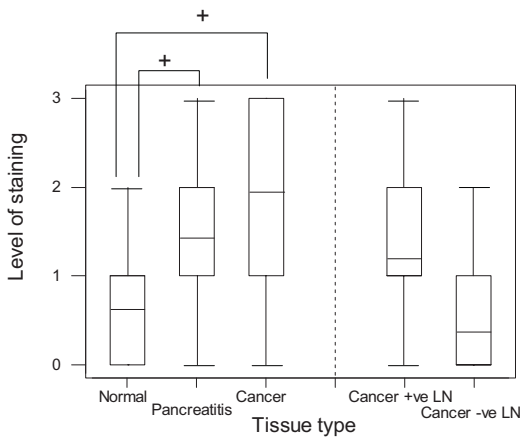


Fig. 4.9 COX-2 protein expression. Boxes represent spread of data from third to first quartile, horizontal line represents the mean, error bars extend to highest and lowest data values within upper and lower limits, * represent outliers (normal n=22, lymph node negative n=45, lymph node positive n=31, pancreatitis n=73, cancer n=55). *p<0.001 using one way ANOVA.

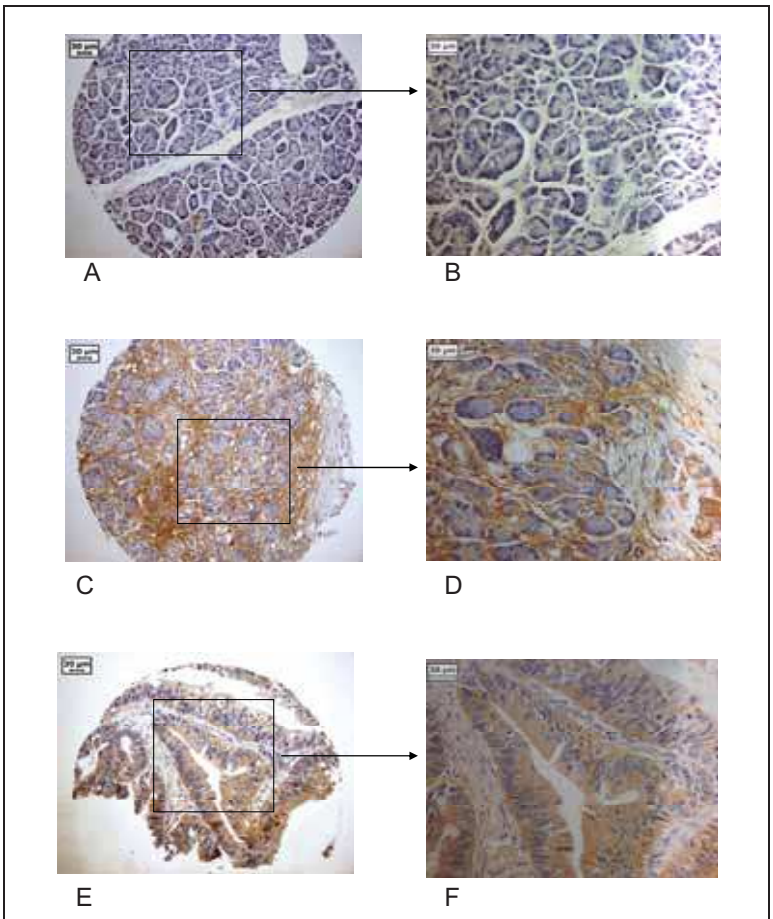


Fig. 4.10 Examples of COX-2 staining in normal pancreatic tissue (A and B), pancreatitis (C and D) and well differentiated pancreatic cancer (E and F).

COX-2 protein expression showed a significant ($p<0.001$) 2.2 fold increase in pancreatitis and a 2.9-fold increase in pancreatic cancer tissue, compared to normal pancreatic tissue (see fig. 4.9). High levels of staining were only detected in pancreatitis and cancer and not normal pancreas. There appeared to be no COX-2 protein expression in the acinar cells of the normal pancreas (fig 4.10A and B) as is the case in pancreatitis, but there was staining of the connective tissue between the acinar cells (fig 4.10C and D). In pancreatic cancer there was diffuse cytoplasmic staining of the cancer cells, but no stromal staining (fig 4.10 E and F).

4.2 Discussion

The current standard of care for patients presenting with advanced pancreatic cancer is gemcitabine, which increases 12 month survival from 2% with the previous standard of care agent 5-Fluorouracil to 18% (Burris, Moore *et al*. 1997). For these reasons a better understanding of this disease is required in order to develop more targeted therapeutic approach.

Caplin *et al* (Caplin, Savage *et al*. 2000) showed that CCK-2 receptors were only expressed in pancreatic cancer cells and not normal pancreatic cells. This has been confirmed in this work, which showed there was a 4-fold increase in CCK-2 receptor protein expression in pancreatic cancer as well as an increase in positive lymph nodes compared to resectioned normal tissue. There was some expression of CCK-2 receptor protein in the normal pancreatic tissue. This has also been described by another group and an explanation for this is likely to be due to the normals being resection normals, which also suggests that locally mediated growth by gastrin may also be of relevance to surrounding tissue and regulation mediated by factors from nearby neoplastic cells (Goetze, Nielsen *et al*. 2000).

Results presented in this work showed that CCK-2 receptors were present on endothelial cells and that the presence of endothelial cells expressing CCK-2 receptors was higher in cancer than the normal pancreas. Work in rats has shown that CCK-2 receptors are present on vascular endothelial cells and macrophages in the lungs (Cong, Li *et al*. 2003; Alvarez, Ibiza *et al*. 2006). The presence of CCK-2 receptors on endothelial cells in pancreatic cancer supports other work within our group, which

has shown that gastrin acting through the CCK-2 receptor enhances endothelial cell activity in models of angiogenesis (Clarke, Dickson *et al*. 2006) further supporting the role of gastrin and its receptor in angiogenesis.

COX-2 a downstream target of gastrin, has been shown to be over-expressed in pancreatic cell lines and pancreatic cancers compared to the normal pancreas (Ding, Tong *et al*. 2001). The data presented in this work supports this, showing a significant increase in COX-2 protein expression in pancreatic cancer compared to normal pancreas. The pattern of COX-2 staining presented in this work is in accordance with that observed by Yip-Schneider *et al.,* (Yip-Schneider, Barnard *et al*. 2000) who showed that COX-2 protein was not present in normal acinar cells, and in pancreatic cancer expression is localised to the cancer cells with no expression in the surrounding stroma. No significant correlation was seen between COX-2 and CCK-2 receptor protein expression in pancreatic cancer, indicating that up-regulation of COX-2 in pancreatic cancer is not mediated through the CCK-2 receptor.

Another down stream target of gastrin, XIAP, considered the most potent member of the Inhibitor of Apoptosis Protein (IAP) family, blocks apoptosis through interactions with initiator caspase-9 and effector caspases 3 and 7 (Mori, Doi *et al*. 2007) and is expressed at low levels in most normal adult tissue (Yang, Cao *et al*. 2003). Moderately high levels of XIAP protein expression have been described in pancreatic cancer cell lines MIA PaCa, BXPC-3 and Panc-1 (Yang, Cao *et al*. 2003) and XIAP has been shown to be over-expressed in 77% of pancreatic cancers (Karikari, Roy *et al*. 2007).

In this study high levels of XIAP protein expression were only seen in pancreatitis and cancer tissue and not in normal pancreatic tissue, but overall there was no significant increase in XIAP expression in pancreatitis or cancer tissue compared to normal pancreas. This lack of significance maybe due to the normal pancreas biopsies being taken from individuals that had pancreatic cancer and are therefore not true normals. There was a significant positive correlation with CCK-2 receptor protein expression and XIAP expression in pancreatic cancer. In gastro-oesophageal cell lines gastrin has been linked to increased expression of XIAP (Harris 2005). Therefore in a subset of patients gastrin may drive over-expression of XIAP via the CCK-2 receptor in pancreatic tissue. There was also a significant positive correlation in pancreatic cancer with CCK-2 receptor protein localisation in endothelial cells and XIAP protein expression highlighting a possible role in angiogenesis. Endothelial cell expression of CCK-2 receptors and XIAP has not previously been linked, however, the proangiogenic factor VEGF has been linked to up-regulation of XIAP (Tran, Rak *et al*. 1999), and as shown in my work in Barrett's oesophagus, VEGF-R2 positively correlates with serum gastrin levels. It is therefore plausible, that in pancreatic cancer serum gastrin acting via the CCK-2 receptor on endothelial cells up-regulates VEGF-R2 expression, which leads to an increase in XIAP and an overall increase in angiogenesis.

Another mediator of apoptosis, which may also be a downstream target of gastrin, is Bcl-2, an anti-apoptotic member of the Bcl-2 family. In the large intestine high gastrin expression has been linked to increased Bcl-2 expression (Mao, Wu *et al*. 2005). In this work no correlations were seen between CCK-2 receptor protein expression or endothelial cell localisation and Bcl-2, but a significant decrease in Bcl-

2 protein expression was seen in pancreatic cancer compared to normal pancreas. There was also higher Bcl-2 protein expression in well differentiated cancer samples compared to poorly differentiated.

The results relating to Bcl-2 expression in this work are in agreement with work published by Campani *et al* (Campani, Esposito *et al*. 2001) who showed lower levels of Bcl-2 protein expression in pancreatic cancer compared to normal pancreas, with 25% of cancers showing high expression. They also showed that expression was higher in well differentiated tumours compared to poorly differentiated. Other work has also shown that Bcl-2 protein expression is absent from pancreatic cancer tissue with higher expression in the normal pancreas (Evans, Cornford *et al*. 2001).

Pancreatic carcinomas, which express high levels of Bcl-2 have been linked to better overall survival (Sinicrope, Evans *et al*. 1996) and cells with a high Bcl-2 protein content are more susceptible to gemcitabine cytotoxicity. However, it is unclear whether gemcitabine exposure increases levels of Bcl-2 protein expression (Bold, Chandra *et al*. 1999). Furthermore, there is evidence to suggest that it's the relationship between Bcl-2 and pro-apoptotic member of the BCL-2 family, Bax, which is important and not Bcl-2 on its own. Combined high levels of Bcl-2 and Bax have been linked to improved survival and proposed as strong prognostic markers in pancreatic cancer patients that have undergone marginal resection (Magistrelli, Coppola *et al*. 2006). For this reason in future work it maybe prudent to measure Bax expression along side Bcl-2.

In pancreatic cancer tissue CCK-2 receptor protein expression significantly positively correlated with tumour recurrence following surgical resection, as did CCK-2 receptor protein localisation in endothelial cells. Added to this there was a significant negative correlation in pancreatic cancer samples between survival and both CCK-2R protein expression and CCK-2R localisation in endothelial cells. There is currently no other data available on the link between CCK-2 receptor over-expression and cancer recurrence and survival in pancreatic cancer. These results therefore indicate that CCK-2 receptor expression could be a marker for recurrence and reduced survival in pancreatic cancer. This is supported by work in gastric cancer, which has shown that gastrin producing tumour cells in intestinal type gastric cancer have been linked to cancer recurrence (Stephens, Hopper *et al*. 2007).

In summary CCK-2R protein expression and CCK-2R localisation on endothelial cells was significantly elevated in pancreatic cancer compared to normal pancreas. CCK-2R protein expression significantly positively correlated with XIAP protein expression, as well as cancer recurrence.

Chapter 5

Helicobacter infection and therapeutic intervention studies in the INS-GAS mouse model

5.1 The INS-GAS mouse as a gastric cancer model of infection and therapeutic intervention

Fig.5.1 Example of an INS-GAS mouse

The transgenic INS-GAS mouse (fig. 5.1), which over-expresses amidated gastrin, was developed to investigate the importance of gastrin secretion in gastric carcinogenesis. The INS-GAS mouse contains a gastrin transgene, made up of an insulin promoter upstream of the human gastrin coding sequence, resulting in an over expression of gastrin in the pancreatic β-cells and elevated (around 150pM) serum levels of human amidated gastrin. INS-GAS mice show hyperplasia of the gastric mucosa associated with significant increases in the levels of both transforming growth factor α (TGF-α) and heparin binding epidermal growth factor (HB-EGF). From 5 months onwards initial hyperacidity is replaced with hypoacidity accompanied by a steady decline in parietal cells. By 20 months there is a marked gastric hypertrophy due to foveolar hyperplasia, with invasive carcinomas seen in the gastric fundus.

Although INS-GAS mice develop atrophy followed by the onset of gastric cancer the process is relatively slow requiring nearly the entire lifetime of the mouse, suggesting that other cofactors and/or genetic events are needed. Neoplastic progression appears to progress through the intermediate stages of intestinal metaplasia, dysplasia, carcinoma *in situ* and invasive cancer, similar to that seen in human gastric carcinogenesis(Correa 1992).

Wang *et al* (Wang, Dangler *et al*. 2000) showed that infection with *Helicobacter* resulted in the rapid (~ 6 month) onset of atrophy and gastric cancer, and in the study more than half of the infected INS-GAS mice developed invasive carcinoma. Some degree of atrophy was observed in *Helicobacter* infected FVB/N wild-type mice in the absence of any increase in plasma gastrin levels, but this was less than the atrophy seen with hypergastrinemia in the absence of infection.

Comparison of the INS-GAS mouse with the MTI/G-gly mouse, a transgenic model, which over-expresses the glycine extended form of gastrin (G-gly), and with a double transgenic mouse obtained by crossing the two lines of mutant mice to give an INS-GAS/G-gly mouse, which over-expresses both the amidated and the glycine extended forms of gastrin, has shown that G-gly synergises with amidated gastrin to stimulate acid secretion and inhibit parietal cell loss (Cui, Koh *et al*. 2004).

Infection of INS-GAS mice with *H. pylori* and *H. felis* has been shown to be gender specific with gastric cancer developing more readily in males than females, which appears consistent with the greater incidence of gastric carcinoma in men (Fox, Rogers *et al*. 2003; Cui, Koh *et al*. 2004).

5.2 G17-diptheria toxoid (G17DT)

G17DT is an anti-gastrin vaccine that neutralises G17 and gly-G17 hormones, which as discussed in section 1.6.2 are implicated in a number of GI cancers. G17DT is comprised of nine *N*-terminal amino acids of G17; that are attached by spacer peptides to a diphtheria toxiod (DT), a large carrier protein (fig. 5.2). G17DT is administered in a liquid suspension and elicits an immune response resulting in the production of antibodies, which cross react with and neutralise the target hormones G17 and gly-G17 preventing them from stimulating cells (2003).

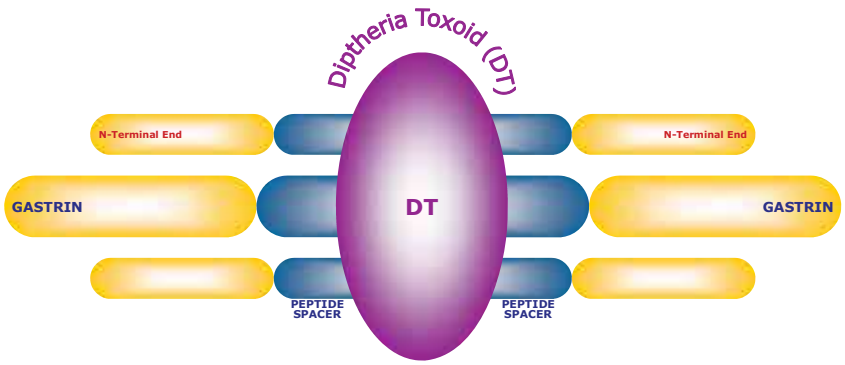


Fig 5.2 Diagrammatic representation of G17DT immunogen

In a phase II study of G17DT in gastric carcinoma 85% of patients achieved an antibody response of greater than 3 units with a median of 15.25 units at 12 weeks; this fell to a median of 1.0 unit prior to booster injections and increased to a median response of 7.7 four to five weeks post booster (Gilliam, Watson *et al*. 2004). In a

phase II study of patients with advanced pancreatic cancer given 250µg dose of G17DT 50% achieved antibody titres after 4 weeks and 72% by 6 weeks with peak antibody titre occurring at 8 weeks, titres then dropped at varying rates over the following weeks (Brett, Smith *et al*. 2002).

5.2.1 The effect of G17DT on cancer progression in INS-GAS mice

It was hypothesised that by immunising INS-GAS mice with the anti-gastrin immunogen G17DT, which binds to and neutralises G17 and gly-G17, that cancer progression could be slowed or even prevented in this model.

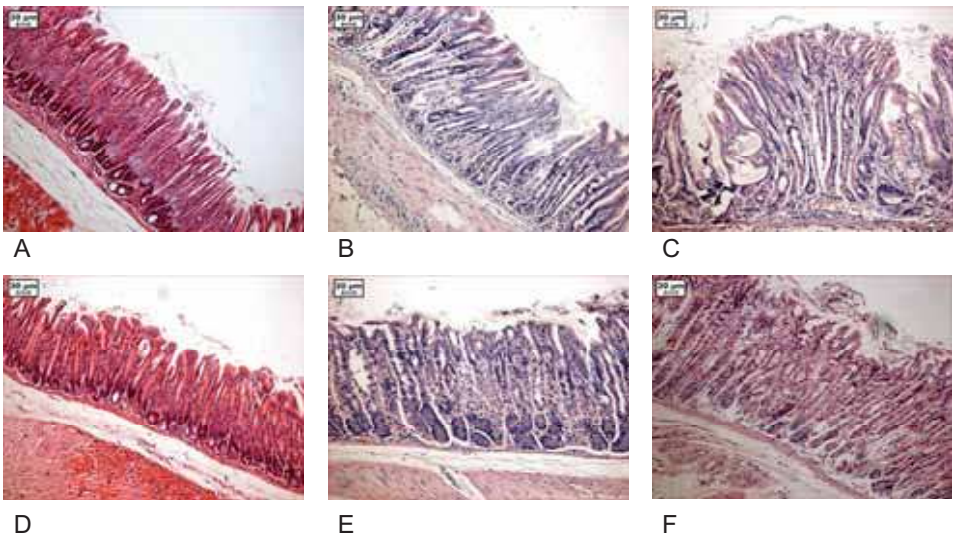


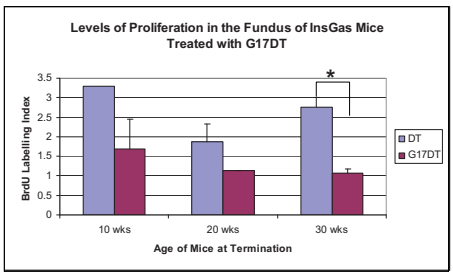
Fig 5.3 H and E stained sections of INS-GAS fundus from DT treated groups at 10, 20 and 30wks (A-C) and G17DT treated groups at 10, 20 and 30wks (D-F).

Fig. 5.3 shows the progression of the normal murine fundic mucosa from an organised structure made up of isthmus (gastric pit) containing the mucus cells, neck region

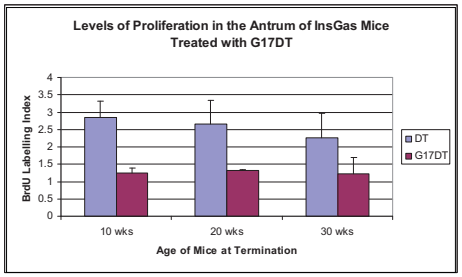
containing the parietal cells and the base containing the chief cells (A and D), to disorganised foveolar hyperplasia (C). These changes are more marked in the DT treated group, with a loss of parietal cells in the neck region at 20weeks (B) and a thickening of mucosal surface as a result at 30weeks (C). There is an increase in inflammatory cells at 20wks (E) in the G17DT group, followed by some thickening of the muscosa at 30weeks (F), but there is no loss of parietal cells in the neck region or gastric hypertrophy characterised by the thickened gastric folds seen in the DT treated group.

Proliferation

Over-proliferation of cells in the gastric mucosa is linked to gastric cancer progression and therefore a key area to investigate in order to assess the chemoprophylaxis potential of G17DT. Glands in the fundus and antrum were analysed separately to give BrdU labelling indices for both.

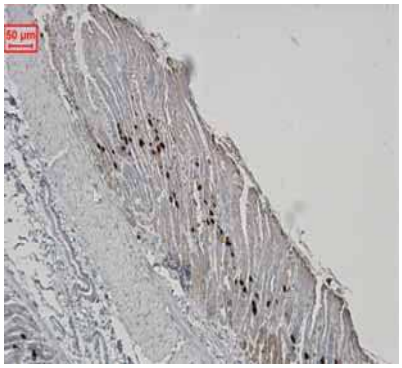


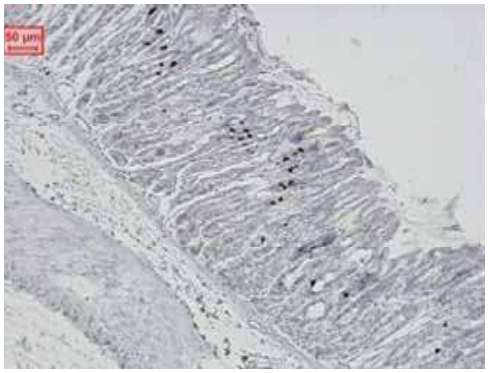
A



B

Fig 5.4 Comparison of the levels of proliferation at different time points when INS-GAS mice were treated with G17DT compared to DT vehicle in the fundus (A) and antrum (B). Mean results for 5 animals per group are shown along with standard error bars. * $p<0.05$ using one way ANOVA.





A B

Fig 5.5 Examples of BrdU staining of fundic glands in 30 week old INS-GAS mice treated with DT (A) and G17DT (B).

In the DT-treated group the BrdU labelling index remains around 2.5 for the length of the study in both the fundus and the antrum, with a slight dip at 20 weeks in the

fundus. In comparison the BrdU labelling index for the G17DT treated group is consistently lower at around 1.0. In the fundus at 30 weeks the level of proliferation is a significant 2.5-fold ($p<0.05$) lower in the G17DT treated group than the DT treated group (fig 5.4); this is at the point when hyperplasia starts to develop in the DT treated group (fig 5.3).

Fig 5.5 visually compares the level of staining of proliferating cells in the fundus of INS-GAS mice treated with G17DT relative to that in mice treated with DT and it is clear that there are fewer stained cells in the G17DT treated mouse, indicating less proliferation. Also of note is the location of these proliferating cells. In the G17DT treated mouse they are in the mid-region of the gland, which is normal; however, in the DT-treated mouse some proliferating cells can be seen slightly lower than the mid-region, which is considered abnormal and a possible indicator of an early step in the cancer carcinogenesis sequence.

Due to there being a significant decrease in the level of proliferation in the fundus of in the 30 week old mice treated with G17DT, it was decided to further analyse the epithelial cell proliferation in this group by examining proliferation in pre-cancerous lesions as well as normal glands. Fig. 5.6 shows that in both the normal glands and pre-cancerous lesions G17DT treatment resulted in significantly reduced proliferation of 3.9% in the normal glands and 7.6% in the pre-cancerous lesions ($p<0.005$ and $p<0.002$ respectively) compared to the DT control group.

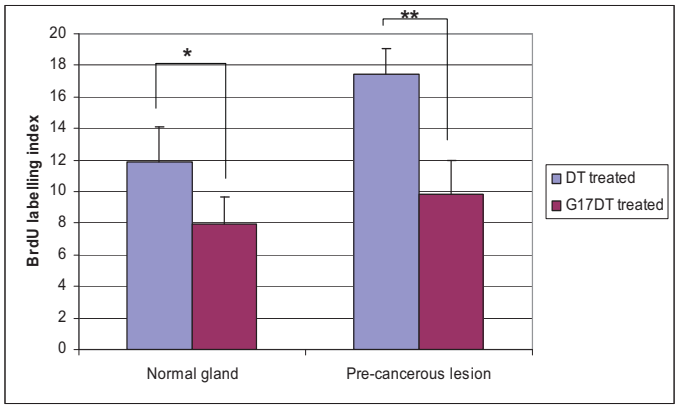


Fig. 5.6 Comparison of levels of proliferation in the fundus of 30 week old INS-GAS mice treated with G17DT or DT in normal glands and pre-cancerous lesions. Mean results for 5 animals per group are shown with standard error bars.

* $p<0.005$, ** $p<0.002$ using a one way ANOVA.

Blood Vessel Density (BVD)

Angiogenesis is necessary for cancer progression because tumours require a good blood supply to continue to grow (Rmali, Puntis *et al*. 2007). As a measure of angiogenesis, sections were stained with CD34, an antibody, which stains new blood vessels. The number of events per view was manually counted in 10 hotspots throughout the fundus of the section to give a mean BVD per section. BVD was used as a measure of angiogenesis, with a decrease in BVD being representative of reduced angiogenesis.

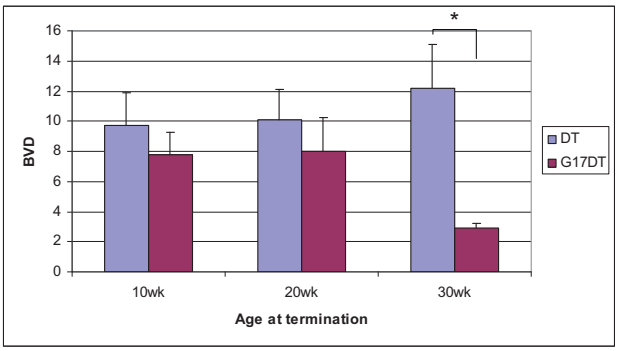
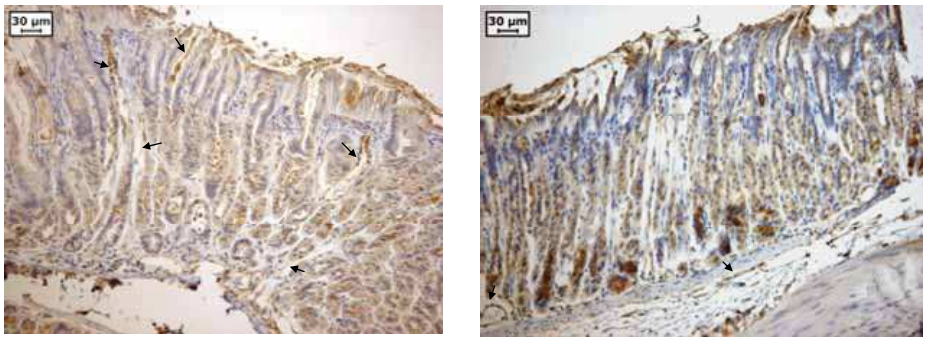


Fig. 5.7 Comparison of Blood Vessel Density (BVD) in the fundus of INS-GAS mice treated with either G17DT or DT. Mean results for 5 animals per group are shown along with standard error bars. * $p<0.01$ using a one way ANOVA.



A B

Fig 5.8 Example of CD34 staining blood vessels (arrows) in INS-GAS mice treated with DT (A) and G17DT (B).

Fig. 5.7 shows that there was a trend to a reduction in BVD, although not significant, in both the 10 and 20 week old INS-GAS mice treated with G17DT compared to DT. However in the 30 week old INS-GAS mice treated with G17DT there was a

significant 4.1 fold ($p<0.01$) reduction in BVD compared to DT. In the DT treated mice there tended to be numerous blood vessels between the glands compared to the DT treated mice where blood vessels were mainly in the submucosa (Fig 5.8). This suggests that immunisation with G17DT may reduce angiogenesis in this model.

COX-2 protein expression

COX-2 has been shown to be associated with tumour development and progression (Sinicrope and Gill 2004). Increased COX-2 expression has also been linked to increased levels of gastrin (Guo, Cheng *et al*. 2002; Konturek, Kania *et al*. 2003). Therefore, by lowering gastrin levels with G17DT treatment it was hypothesised that an effect on COX-2 levels might be observed. Sections were stained for COX-2 and 10 hotspots within the fundus analysed using the QWin software to give a mean H-score per section.

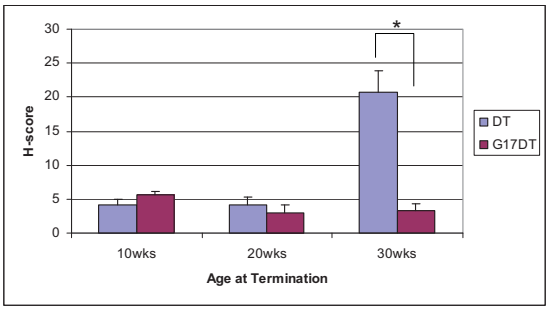


Fig. 5.9 comparison of COX-2 protein expression in the fundus of INS-GAS mice treated with G17DT or DT. Mean results for 5 animals per group are shown along with standard error bars. * p<0.001 using a one way ANOVA.

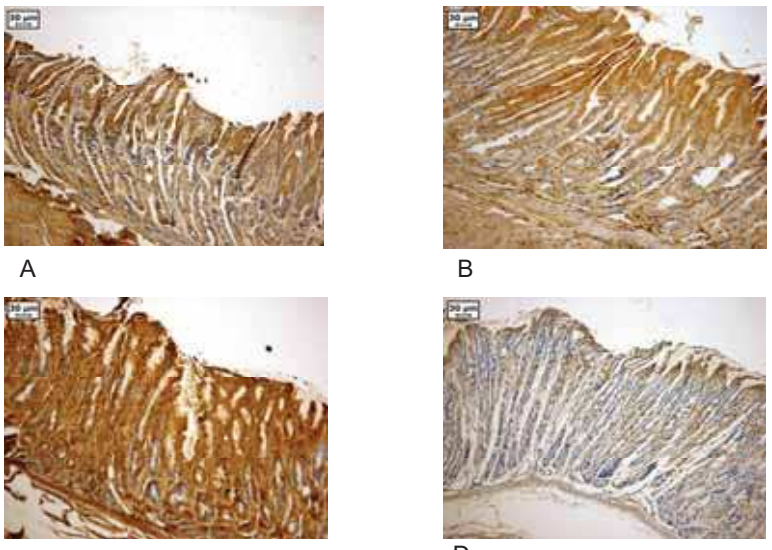


Fig. 5.10 Representative COX-2 staining in the fundus of 10 week old INS-GAS mice treated with DT (A) and G17DT (B), and 30 week old INS-GAS mice treated with DT (C) and G17DT (D).

Fig. 5.9 shows that at weeks 10 and 20 in the DT group the levels of COX-2 protein expression remain low with a sharp 4 fold increase at 30 weeks. In comparison the levels of COX-2 protein expression in the G17DT treated group are maintained at a

low level even at 30 weeks, resulting in significantly lower expression than that of the DT treated group at the 30 week time point. Fig 5.10A and B shows that at 10wks the level of staining is comparable between the DT and G17DT treated groups with the most intense staining in the pit. At 30 weeks fig. 5.10C and D clearly show that there is prominent staining of the superficial interstitial cells and co-localisation with epithelial cells compared to lower levels of superficial interstitial cell staining and co-localised epithelial cell staining in the G17DT treated

5.2.3 Discussion

Gastrin has been shown to stimulate the *in vitro* growth of the gastric cell line AGS (Szabo, Rumi *et al*. 2000). Specific inhibition of the gastrin gene with small interfering RNA (siRNA) has been shown to cause a reduction in cell survival in representative cell lines of gastric, colorectal and pancreatic adenocarcinoma (Grabowska, Hughes *et al*. 2007). Therefore, it was hypothesised that neutralisation of G17 and gly-G17 with the immunogen G17DT may slow or prevent cancer progression in mouse models of gastric.

Immunisation of INS-GAS mice with G17DT resulted in significant decreases in proliferation of both the normal gastric glands and pre-cancerous lesions. This further confirms the role of gastrin as a stimulator of proliferation in gastric cancer models and highlights the potential therapeutic role of G17DT.

Angiogenesis, the formation of new blood vessels, is a key stage in the progression of all cancers. G17 and gly-G17 have been shown to stimulate angiogenesis in HUVECs

to the same extent as VEGF through isoforms of the CCK-2 receptor and these pro-angiogenic effects are thought in part to be mediated by HB-EGF gene expression (Clarke, Dickson *et al*. 2006). In the fundus of the INS-GAS mouse G17DT significantly reduced BVD. Therefore, G17DTs' ability to reduce BVD compared to DT alone is further evidence of G17 and gly-G17s role in the angiogenic process of gastric cancer progression and that this may be true for other GI cancers.

G17DT maintained low COX-2 protein expression levels expected in the normal gastric mucosa through out the time course of the study compared to the DT treated group, which showed a rapid increase of COX-2 protein expression at 30 weeks. This sudden increase of COX-2 protein expression at 30 weeks in the DT treated group may be in response to inflammation and gastric mucosal damage caused by hypergastrinemia, demonstrating that G17DT immunisation may be protective against inflammation and mucosal damage in the stomach.

In human gastric cancer specimens COX-2 has been shown to positively correlate with angiogenesis and tumour metastasis to lymph nodes (Murata, Kawano *et al*. 1999; Mao, Wang *et al*. 2007). This would suggest that the reduction in BVD seen in the G17DT treated INS-GAS mice may be mediated through a reduction in COX-2. However, immunohistochemistry showed that COX-2 protein was expressed in a number of cell types not just ECL and parietal, which express the CCK-2 receptor, indicating that gastrin may also indirectly up-regulate COX-2 in the gastric mucosa.

5.3 The effect of COX inhibition on cancer progression in the INS-GAS mouse model

There are data to suggest that chronic use of general COX inhibitors like aspirin and specific COX-2 inhibitors like rofecoxib may reduce the risk of GI cancers, including gastric cancer (Jiang and Wong 2003; Leung, Ng *et al*. 2006). Therefore, it was hypothesised that treating INS-GAS mice with aspirin or celecoxib may slow or prevent cancer progression, by reducing proliferation and angiogenesis.

5.3.1 Proliferation

As explained in section 5.1.2.1 BrdU was used to stain proliferating cells and then the number of proliferating cells per gland assessed.

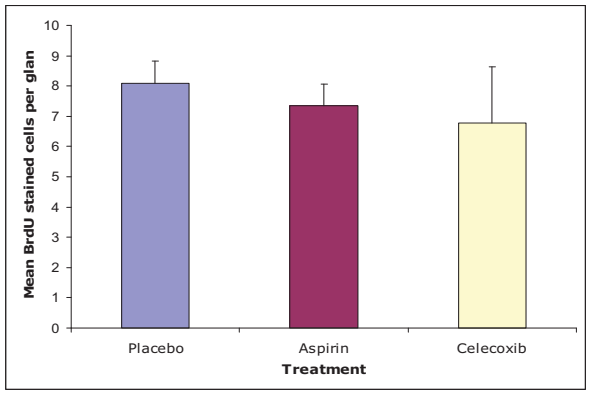


Fig. 5.11 The effect of COX inhibition on proliferation in the fundus of INS-GAS mice. Mean results for 5 animals per group shown with standard error bars.

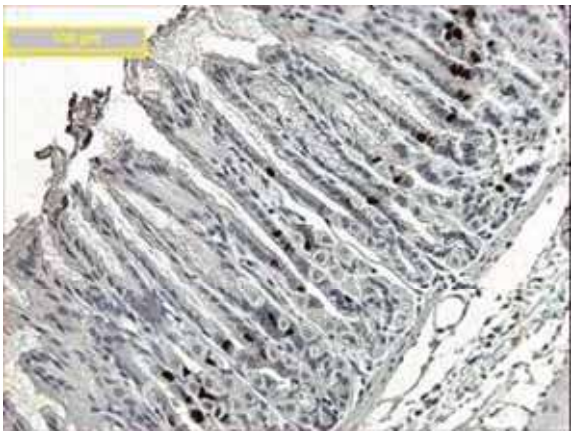


Fig. 5.12 Example of proliferating cells stained with BrdU from the fundus of an INS-GAS mouse

Fig.5.11 shows that although both aspirin and celecoxib reduced proliferation in the fundus compared to placebo this reduction was not significant. Fig. 5.12 shows staining of proliferating cells in the fundus region of the stomach. The expected region of proliferation in the fundic gland is the mid-region and as can been seen in fig. 5.12 some proliferating cells are present towards the base of the gland, due to the hyperproliferative state of the INS-GAS fundus as a result of hypergastrinimia, this was the case in both the placebo and the aspirin and celecoxib treated groups.

5.3.2 Blood vessel density

As previously described in section 5.1.2.2 CD34 staining was used to assess blood vessel density as a measure of angiogenesis.

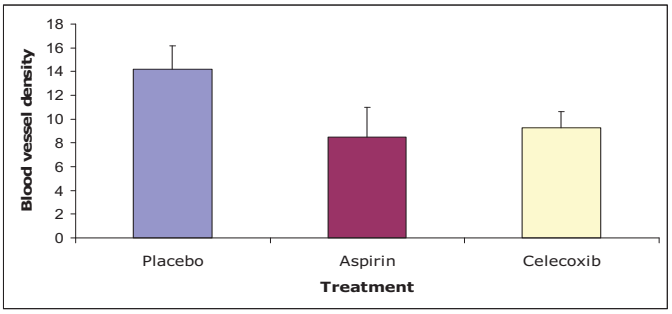


Fig. 5.13 The effect of COX inhibition on angiogenesis in the fundus of INS-GAS mice. Mean results for 5 animals per group shown with standard error bars.

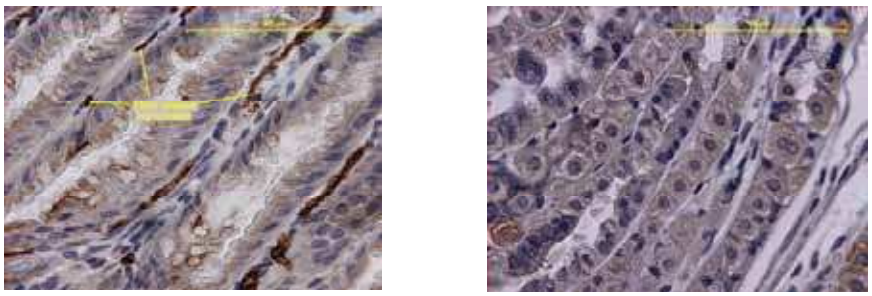


Fig. 5.14 Example of CD34 staining of blood vessels in the fundus of an INS-GAS mouse (A). Example of negative (B)

Both aspirin and celecoxib reduced blood vessel density in the fundus of INS-GAS mice, but it was not significant (fig. 5.13). An example of the type of blood vessel staining seen with CD34 is shown in fig 5.14; the blood vessels are present between the gastric glands.

5.3.3 Discussion

In the INS-GAS model, neither aspirin nor celecoxib had an effect on proliferation and although blood vessel density was reduced by both agents, neither did so significantly. These results support the theory that it is G17 that is driving the increase in COX-2 seen in this model and that in a hypergastrinemic environment G17DT immunisation is more successful in reducing the effects of COX-2 over-expression than the COX inhibitors, aspirin and celecoxib.

5.1.1 Study to determine the effect of *H. felis* in INS-GAS mouse model (short term)

Mice were given either 1 x 10^8 cfu/ml of the relevant strain of bacteria in brucella broth or an equivalent volume of brucella broth as described in section 2.7 on days 1, 3 and 5 of the study. Mice were terminated after 4 weeks and their stomachs removed and sectioned (section 2.5.1). *H. felis* (ATCC49179), which has no *cag* pathogenicity island (Viala, Chaput *et al*. 2004) and has been well-documented as an accelerant in cancer progression in INS-GAS mice (Wang, Dangler *et al*. 2000) was the strain used.

Gastric unit length

Image analysis software was used to measure gastric unit length as a crude assessment of proliferation.

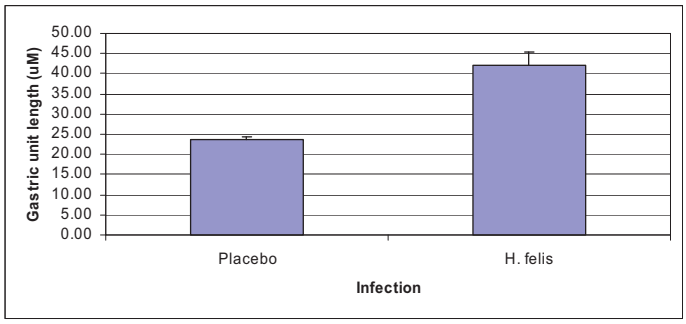


Fig. 5.15 The effect of *H. felis* infection on gastric unit length in 4wk old INS-GAS mice compared to placebo. Mean results for 5 animals per group are shown along with standard error bars.

Fig. 5.15 shows that infection of INS-GAS mice with *H. felis* resulted in a 1.7-fold increase in gastric unit length compared to placebo.

Serum gastrin levels

Wang *et al* (Wang, Dangler *et al*. 2000) showed that hypergastrinemic mice (INS-GAS) have increased parietal cell number and acid secretion up to approximately 4 months. Thereafter, both parietal cell number and acid secretion gradually decrease and there is an increase in expression of transforming growth factor-α (TGF–α) and a progression towards gastric cancer. This sequence of events is exacerbated by *Helicobacter* infection.

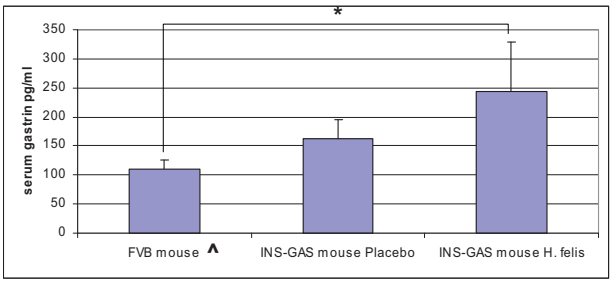


Fig. 5.16. Comparison of serum gastrin (G17) levels in FVB mice and 4 week old INS-GAS mice infected with either placebo or *H. felis*. Mean results for 5 animals per group are shown along with standard error bars. *p<0.005 using a one way ANOVA.

^ Data from thesis by Mo El-Zaatari 2008

Serum gastrin levels were measured in the FVB (the background for INS-GAS mice) and INS-GAS mice either infected with *H. felis* or placebo. Infection resulted in a significant ($p<0.05$) increase in serum gastrin levels in INS-GAS mice compared to FVB, and there was an increase although not significant between the infected INS-GAS and the placebo. this may be because this model already has elevated serum gastrin (Fig. 5.16).

5.1.2 Long term infection with *H. felis*

Based on the results obtained in section 5.1.1, a long term 9 month study was set up using *H. felis* to infect INS-GAS mice.

Due to work previously published (Wang, Dangler *et al*. 2000) it was decided to concentrate on analysis in the fundus region of the stomach as lesions have only been shown there and not in the antrum.

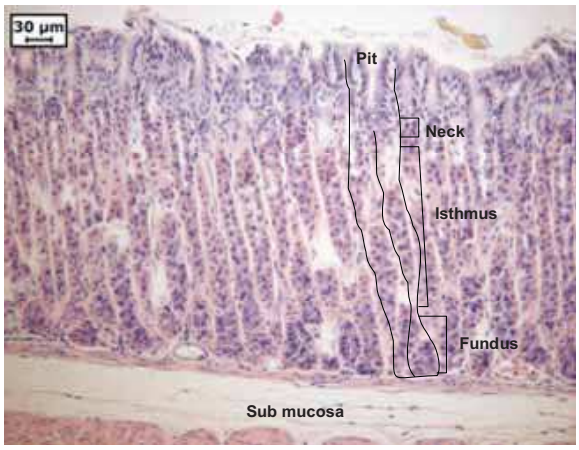


Fig. 5.17 H and E staining of the fundic region of a normal (FVB) mouse.

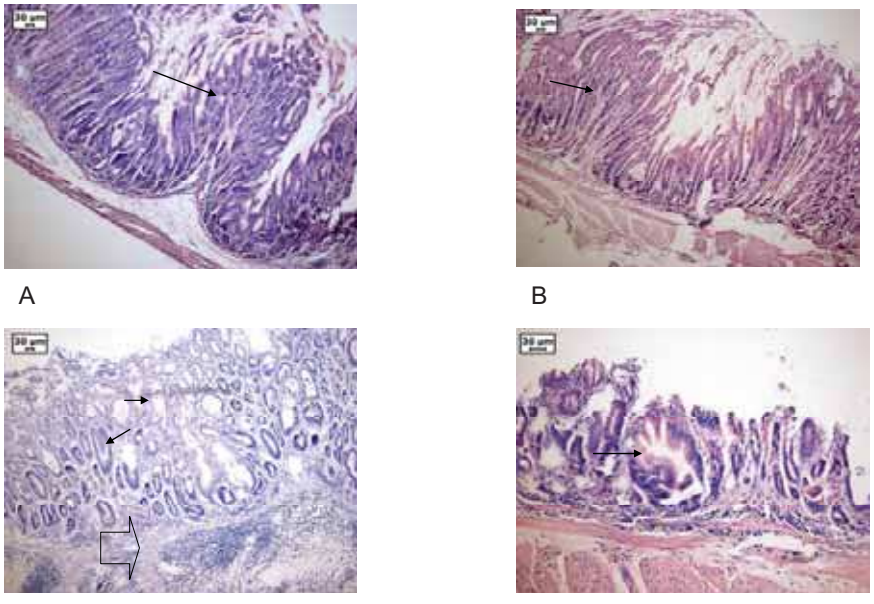
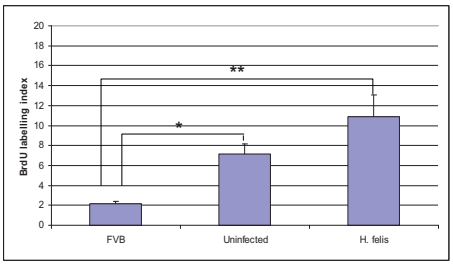


Fig. 5.18 H and E staining showing examples of the fundus from 9 month old INS-GAS mice (A and B) and the fundus from INS-GAS mice infected with *H.felis* (C and D).

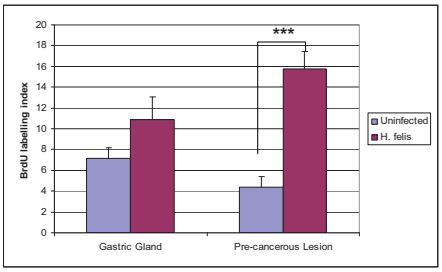
The fundic region of a normal (FVB) mouse stomach is comprised of organised fundic (gastric) glands. The fundic glands are simple, branched, tubular glands that extend from the bottom of the gastric pit to the muscularis mucosa. In a normal stomach the glands are of a uniform length (fig 5.17). However, hypergastrinemia causes fundic hypertrophy with thickened folds (fig. 5.18A and B arrow) and loss of parietal cells in the upper two thirds of the gland. Infection with *Helicobacter* species such as *H. felis* in the INS-GAS mouse causes increased disruption to the glands resulting in irregular tubules and lesions (microacini formation) (fig. 5.18C and D arrow), and inflammatory infiltrate (fig. 5.18C open arrow).

Proliferation

In order to assess cellular proliferation, 1 hour before termination mice were given BrdU by intravenous injection. BrdU is taken up by cells, which are in the S-phase of mitosis, and gives a snap-shot of the number of proliferating cells at termination (Boswald, Harasim *et al*. 1990). Immunohistochemistry with a specific BrdU antibody was then used to visualise the cells, which had taken up the BrdU. For each field of view the number of cells per gland and the number of stained cells in each gland were counted, in order to calculate a percentage of stained cells per gland. For each section up to 10 fields of view were analysed dependent on sample size and the mean value taken. The same was done for pre-cancerous lesions. These reading were then reported as the BrdU labelling index i.e. the mean percentage of proliferating cells per gland/lesion, data is summarised in fig 5.19.



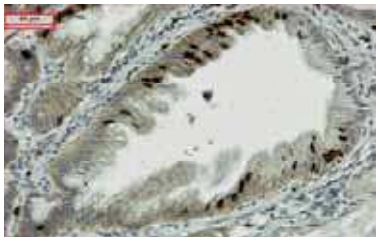
A



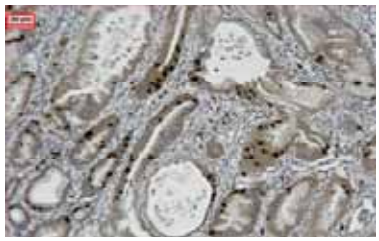
B

Fig. 5.19. The effect of *H. felis* infection on proliferation. Fig. A shows the effect of *H. felis* infection on the proliferation of the gastric glands in INS-GAS mice compared to uninfected INS-GAS mice and FVB mice. Fig. B shows the effect of *H. felis* infection in INS-GAS mice compared to uninfected INS-GAS mice in the normal gastric gland and in pre-cancerous lesions. Mean results for 5 animals per group are shown along with standard error bars. *p<0.05, **p<0.001, ***p<0.001 using a one-way ANOVA.

A. Pre-cancerous lesion in an uninfected INS-GAS mouse (x20)



B. Pre-cancerous lesion in an *H. felis* infected INS-GAS mouse (x20)



C. Pre-cancerous lesions in an *H. felis* infected INS-GAS mouse (x10)

Fig 5.20 Proliferation in pre-cancerous lesions of 9 month old INS-GAS mice with and without *H. felis* infection.

Fig. 5.19A shows that, compared to Friend virus B-type (FVB) mice, INS-GAS mice have a significantly higher BrdU index in the normal gastric gland ($p<0.05$) equating

to greater proliferation. Proliferation is further increased in the INS-GAS model compared to the FVB model by infection with *H. felis* ($p<0.001$). There is also an increase, although not significant, in proliferation of *H. felis* infected INS-GAS mice compared to uninfected INS-GAS mice. Infection of INS-GAS mice with *H. felis* also resulted in a significant increase in proliferation in pre-cancerous lesions ($p<0.001$). Fig 5.20 shows that the proliferating cells are mainly confined to the pre-cancerous lesions of *H. pylori* infected and uninfected INS-GAS mice, with a greater number of proliferating cells comprising the lesions of *H. pylori* infected mice. The pre-cancerous lesions are recognisable by their lack of glandular structure and the increased size of the cell nuclei.

Apoptosis

An antibody to Bcl-2 was used as a marker of apoptosis. The Bcl-2 protein prolongs cell survival and is therefore considered to be a suppressor of apoptosis. There is evidence to suggest that Bcl-2 is up-regulated in gastric pre-malignant lesions and down-regulated after malignant change (Anagnostopoulos, Stefanou *et al*. 2005).

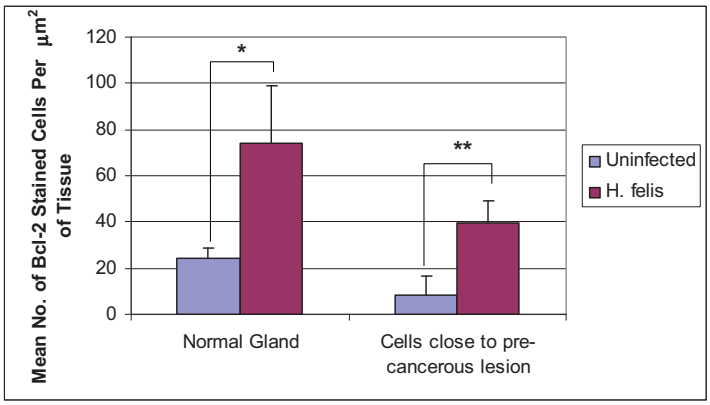
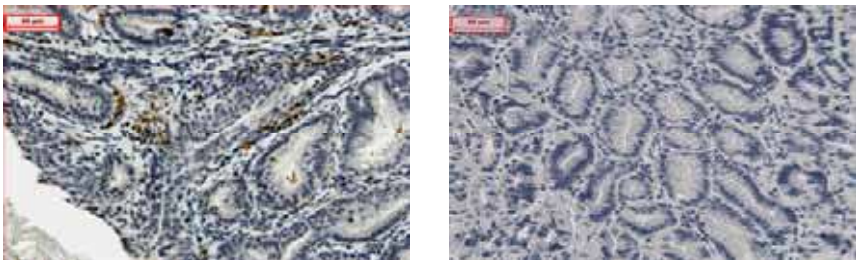


Fig. 5.21 Bcl-2 staining in the normal glands and close to the pre-cancerous lesions in the infected mice compared to uninfected. Mean results for 5 animals per group are shown along with standard error bars. *p<0.01, **p<0.05, using a one-way ANOVA.



A. *H. felis* infected INS-GAS mouse stomach

B. Uninfected INS-GAS mouse stomach

Fig. 5.22 Bcl-2 staining in the normal gastric glands and the interstitial tissue of pre-cancerous lesions in INS-GAS mice either infected with *H. felis* (A) or uninfected (B).

Results are summarised in fig 5.21 and show that in both the normal gastric glands and in the interstitial tissue surrounding the precancerous lesions, infection with *H.*

felis resulted in an increase of Bcl-2 staining correlating to a significant reduction in apoptosis compared to uninfected controls ($p<0.05$). This fits with the increased proliferation shown in the precancerous lesions of infected mice in fig. 5.6, because if cells are proliferating then they will not be undergoing apoptosis. No staining was observed within the pre-malignant lesions but staining was present in the interstitial tissue, comprising of stromal and inflammatory cells, next to the lesions (fig. 5.22A).

5.1.3 Discussion

There are few examples of spontaneous gastric adenocarcinomas in rodents. For these reasons a number of chemical carcinogens have been used to induce carcinogenesis including; polycyclic aromatic hydrocarbons and *N*-nitroso (Pritchard and Przemeck 2004).

With the WHOs classification of *H. pylori* as a gastric carcinogen in 1994 a number of animal models were set up to investigate this phenomenon. Studies in Mongolian gerbils showed the induction of gastric carcinoma in the absence of other stimuli (Hirayama, Takagi *et al*. 1996; Watanabe, Tada *et al*. 1998), but in mice there were mixed results with best infection rates seen with *H. pylori* Sydney Strain1 (SS1) which does not initiate a strong immune response and *H. felis* which does not infect humans (Court, Robinson *et al*. 2002).

In this work I have confirmed these findings showing short term infection with *H. felis* causes increased gastric unit length and serum gastrin levels in INS-GAS mice compared to uninfected controls and that longer term *H. felis* infection results in a

significant increase in proliferation and decrease in apoptosis in pre-malignant lesions of INS-GAS mice.

Chapter 6

Therapeutic intervention in the *Apc*$^{Min/+}$ mouse model

6.0 The *Apc*$^{Min/+}$ mouse as a model of therapeutic intervention in intestinal cancer progression

Due to rodents, such as rats, having almost no spontaneous colon cancer, a number of rat models have been developed whereby rats are given carcinogens to promote colon tumours. The most commonly used carcinogens are dimethylhydraize derivatives. Dimethylhydraize is metabolised to azoxymethane (AOM) and methylazoxymethanol in rats, which induces tumours that share many histopathological characteristics of human tumours (Corpet and Pierre 2003). Shared characteristics include; mutated *K-ras* and β-catenin genes (Dashwood, Suzui *et al*. 1998). They also show microsatellite instability, but unlike human tumours they rarely have a mutated *Apc* gene and never p53 mutations (De Filippo, Caderni *et al*. 1998) added to this they have a low tendency to metastasize. Other colon carcinogens used in rat models include specific nitrosamines and hetercyclic amines, such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), which induce *Apc* mutations and microsatellite instability (Canzian, Ushijima *et al*. 1994), but no *K-ras* or p53 mutations (Kakiuchi, Ushijima *et al*. 1993) (Makino, Ushijima *et al*. 1994). However, AOM is favoured as it is the least expensive, most potent and easiest to use. In a carcinogen induced tumour model the chemopreventative agent can be given prior to carcinogen exposure and during the initiation phase or, during the promotion-progression phase or through both phases. The main end point in most rat studies is development of colon tumours (Corpet and Pierre 2003).

In 1992 a mutant mouse, Min, was shown to have multiple intestinal neoplasia (Moser, Pitot *et al*. 1990). This mouse was subsequently shown to have a mutated

Apc gene, similar to that seen in patients with FAP and many forms of sporadic cancers. This model was seen as promising due to its mimicry of the rapid development of adenomatous polyps that affect humans with the germline inactivation of one *Apc* gene (Corpet and Pierre 2003). However, unlike humans the Min model seldom shows signs of adenocarcinomas, and no typical aberrant crypts foci (ACF) arise from the intestinal mucosa and therefore the ACF to carcinoma progression is not established. Another difference is that *K-ras* mutations are not observed in Min mouse polyps, whereas they are in many human tumours (Shoemaker, Luongo *et al*. 1997), and p53 inactivation, which is frequent in human cancers do not increase tumour burden in Min mice (Fazeli, Steen *et al*. 1997).

The Min mouse model is favoured over the AOM rat model as a way of evaluating the effects of chemoprevention and diet as it avoids carcinogen handling and has a shorter assay time; however, it is more expensive. Treatments are typically given at 4-5 weeks of age when tumours may already be present; the timing mimics that of clinical intervention trials. The major drawback of the Min model as a model of human colon cancer is that their tumours occur predominately in the small intestine not the colon (Corpet and Pierre 2003).

The $Apc^{Min/+}$ mouse is one of the most commonly used mouse models for investigating intestinal tumorigenesis. Adenomatous Polyposis Coli (APC) is classed as a tumour suppressor gene due to its ability to regulate the cellular levels of the protooncogene β-catenin. Other functions of the *APC* gene are thought to include, microtubule dynamics, cell cycle control, cell adhesion and chromosomal stability.

The mouse APC protein exhibits 90% amino acid homology to that of the human APC protein.

There are several variations of the *Apc*$^{min/+}$ mouse model; each has different truncating mutations of the *APC* gene**.** The variation used for this work was a fully penetrant dominant mutation induced in a C57BL/6J mouse by ethylnitrosourea, which causes a base substitution mutation in the *Apc* gene, which creates a stop codon truncating the polypeptide at J:830. These mice develop multiple polyps in their small intestine and some in their large intestine; these polyps bleed, which is exhibited as anaemia resulting in pale feet and snout areas (fig 6.1) at the end point of the study.

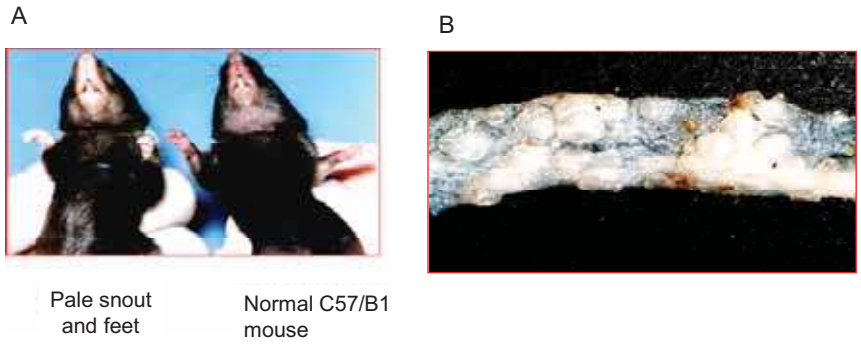


Fig. 6.1 Example of an *Apc*^{min} mouse with pale snout and feet and a normal C57/B1 mouse with pink snout and feet (A). Small intestine of a *Apc*^{min} mouse with multiple polyps (B).

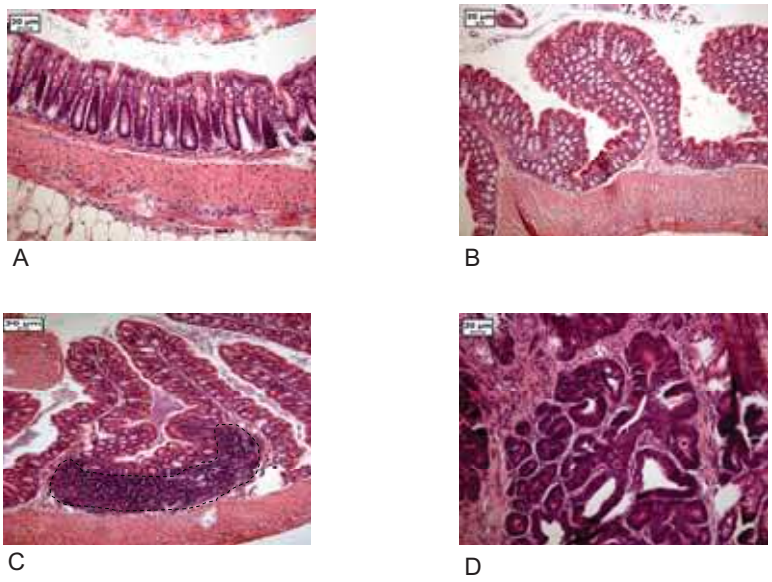


Fig.6.2 H and E sections showing the progression from normal large intestine through polyp development to adenoma in APC^{min} mice (A-D represent mice at 4, 8, 12 and 16wks respectively).

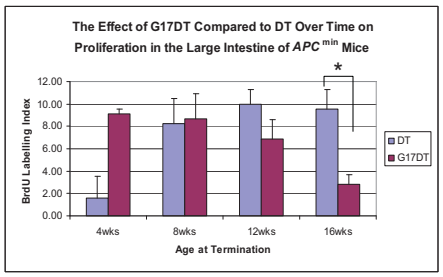
The normal large intestine is made up of straight tubular glands termed crypts of Liebrakuhn, which extend down from the lumenal surface to the muscularis mucosa. The crypts are comprised of simple columnar absorptive cells, mucus secreting goblet cells, stem cells at the base and endocrine cells (fig 6.2.A). Mutation in the *Apc* gene causes hyperproliferation in the crypts, which is the first step in the development of polyps, with the loss of uniform structure (fig 6.2. B and C). Fig. 6.2C shows a large lymphoid follicle an indictor of an immune response. Finally, polyps can further progress to adenomatous polyps with greater loss of structure and reduced cytoplasm within the epithelial cells (fig 6.2.D).

6.1 The effect of G17DT on cancer progression in *Apc*$^{Min/+}$ mice

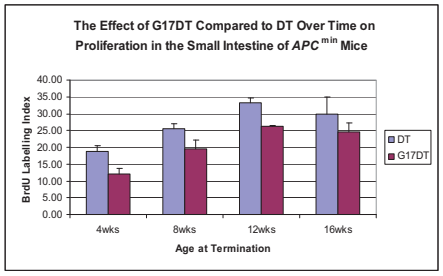
It was hypothesised that by immunising *Apc*$^{Min/+}$ mice with the anti-gastrin immunogen G17DT, which binds to and neutralises G17 and gly-G17, that cancer progression could be slowed or even prevented in this model.

6.1.1 Proliferation

Proliferation was measured using BrdU as previously described in section 5.1.1.1. For each field of view the numbers of stained cells and the total number of cells were counted per crypt and the percentage of stained cells per crypt calculated. Also in the polyps the percentage of stained cells were calculated.

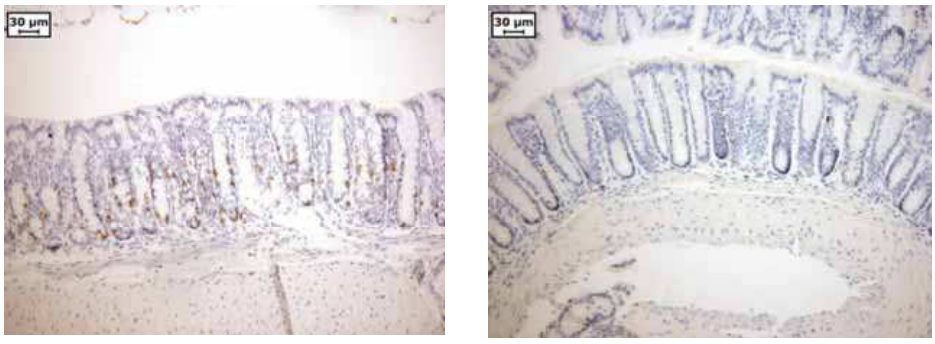


A



B

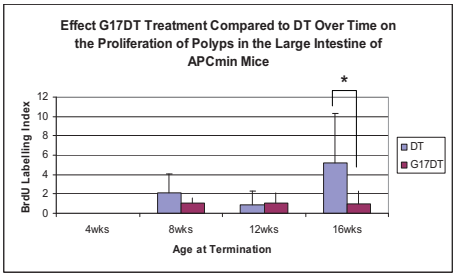
Fig. 6.3 Comparison of the levels of proliferation in the crypts of the large (A) and small (B) intestine of *Apc*^{min} mice when treated with G17DT. Mean results for 5 animals per group are shown along with standard error bars. *p<0.005 using a one way ANOVA.



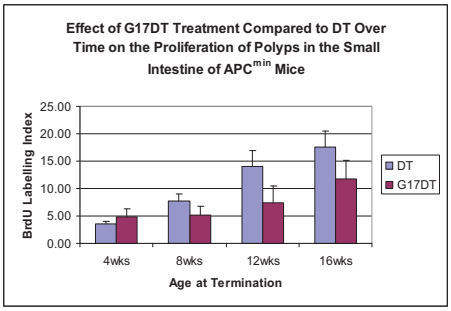
A B

Fig 6.4 Example of BrdU stained proliferating cells in colonic crypts of APC^{min} mice treated with DT (A) and G17DT (B).

In the crypts of the large intestine (Fig. 6.3A) the proliferation in the DT treated group remained fairly constant from 8-16 weeks with a decrease in the proliferation of the G17DT group at 12 weeks (0.5 fold) and a significant decrease at 16 weeks (3 fold $p<0.005$). Fig. 6.3B shows that there was a decrease in proliferation in the crypts of the small intestine of the G17DT treated groups at all time points compared to DT groups. Although there was not a significant difference between the groups at individual time points, when the data was considered as a set there was a significant decrease in the G17DT group compared to the DT over time ($p<0.001$ using 2-way ANOVA). Fig. 6.4 shows there were less BrdU labelled cells in the DT-treated sample compared to the G17DT treated and that in the DT treated sample there were proliferating cells above the bottom third of the crypt, the area were proliferation is normally observed.

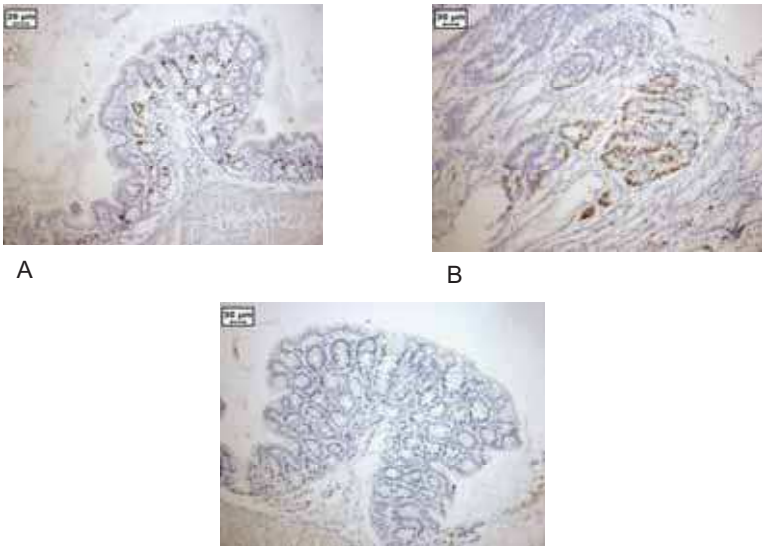


A



B

Fig. 6.5 Comparison of the levels of proliferation in the polyps of the large (A) and small (B) intestine of *Apc*^{min} mice when treated with G17DT. Mean results for 5 animals per group are shown along with standard error bars. There were no polyps present at 4wks in the large intestine. *p<0.005 using one way ANOVA.



A B C

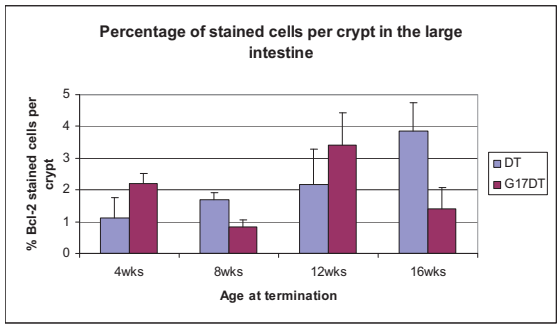
Fig 6.6 Examples of proliferating cells stained with BrdU in polyps in the large intestine of 16 week old APC^{min} mice treated with DT (A and B) and G17DT (C).

At 4 weeks there were no polyps in the large intestine of either group. As shown in fig 6.5A at 8 and 12weeks the level of proliferation in the polyps was similar in the

G17DT compared to the DT, and then at 16 weeks there was an increase in proliferation in the DT group, but proliferation in the G17DT group remained similar to that of 8 and 12 weeks. There was a significant 5.3-fold increase ($p<0.005$) in proliferation at 16 weeks in the DT group compared to G17DT. The polyps in the small intestine (Fig 6.5B) showed increased proliferation over time in both groups with slightly less in the G17DT groups, except at 4 weeks when proliferation was slightly higher in the G17DT group compared to DT. There was no significant reduction in proliferation at any of the time points, but when the data was considered as a set there was a significant decrease in the G17DT group compared to DT over time ($p<0.001$ using 2-way ANOVA). A visual comparison of proliferation in the polyps at 16 weeks is shown in fig. 6.6.

6.1.2 Apoptosis

As described in section 5.1.2.2 Bcl-2 was used as a marker of apoptosis.



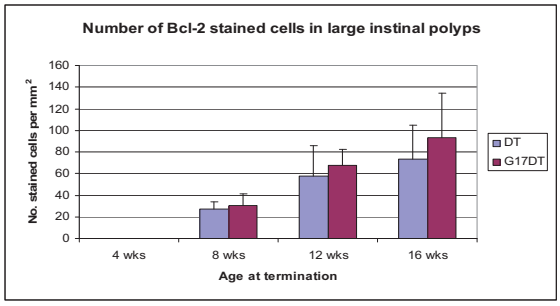


Fig. 6.7 The level of Bcl-2 protein expression in the large intestinal crypts and polyps of APC^{min} mice. Mean results shown for 5 animals per group along with standard error bars.

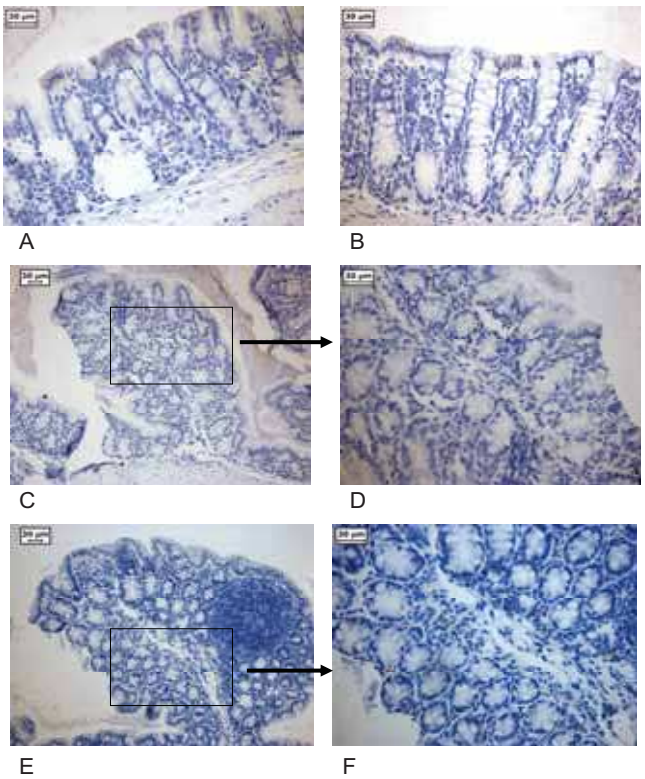
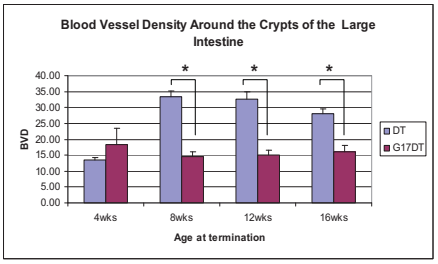


Fig. 6.8 Examples of Bcl-2 staining in crypts of APC^{min} mice treated with DT (A) and G17DT (B) and polyps treated with DT (C and D) and G17DT (E and F).

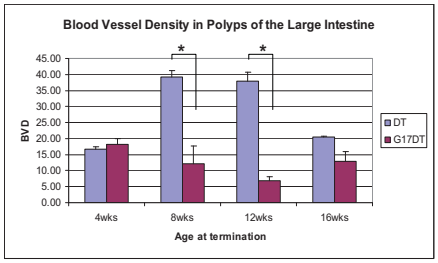
In the DT treated *Apc*$^{Min/+}$ mice the level of Bcl-2 staining steadily increased over the time course of the study in both the crypts and the polyps of the large intestine. This was also the case in the polyps of G17DT treated mice, which remained slightly higher than in the DT treated groups. In comparison, in the crypts the level of Bcl-2 staining was higher in the G17DT treated mice at 4 and 12 weeks and lower at weeks 8 and 16 ($p=0.061$) (fig 6.7). In the crypts, staining was mainly in the cells close to the luminal surface and in the polyps stained cells tended to be present in small clusters, but with no clear defined position within the polyp (fig 6.8).

6.1.3 BVD

BVD was assessed as previously described using CD34 staining.

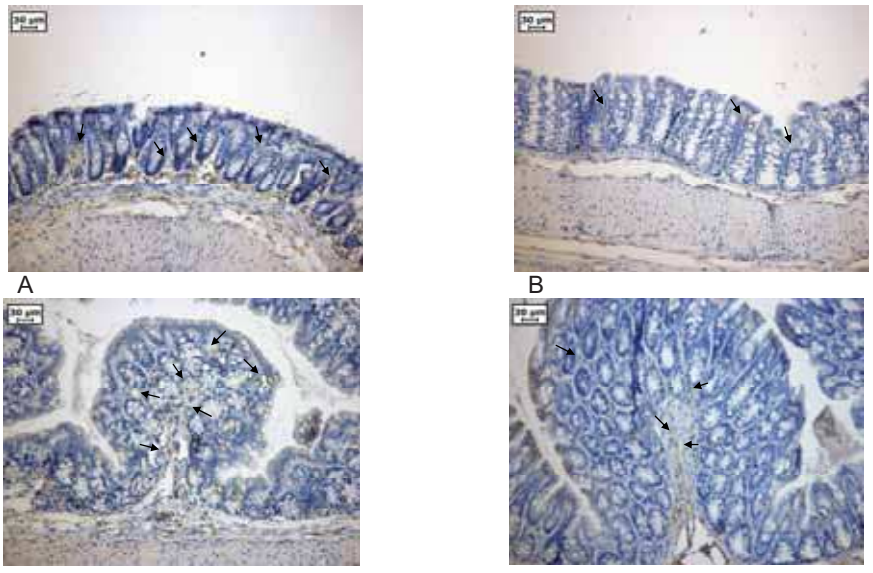


A



B

Fig. 6.9 The effect of G17DT immunisation on BVD around the crypts (A) and within polyps (B) of the large intestine of *Apc*^{min}. Mean results for 5 animals per group are shown along with standard error bars. *p<0.001 using a one-way ANOVA.



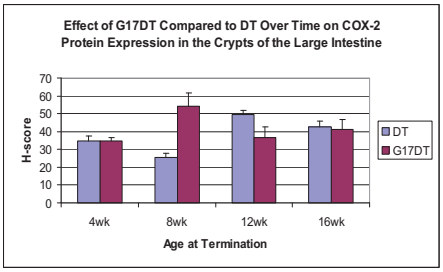
A B C D

Fig. 6.10 Examples of CD34 staining of blood vessels (arrows) in the large intestine of 16 week old APC^{min} mice. Normal crypts treated with DT (A) and G17DT (B) and polyps treated with DT (C) and G17DT (D).

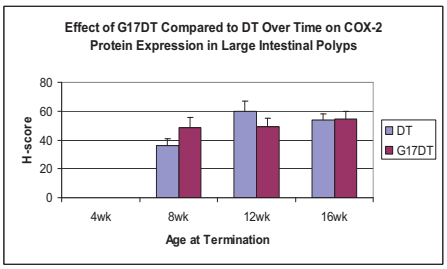
In the DT treated animals the BVD in the crypts and the polyps was around 15 at the first time point, increased to 30 in the crypts at the remaining time points, and to 35 in the polyps at weeks 8 and 12 with a decrease back to levels similar to those at week 4, at week 16. G17DT significantly ($p<0.001$) reduced and maintained BVD at the initial value of 15 for the duration of the study in the crypts and when taken as a set there was a significant ($p<0.005$ using 2-way ANOVA) decrease in the G17DT group compared to DT. In the polyps, G17DT significantly ($p<0.001$) reduced BVD compared to DT at weeks 8 and 12 and maintained this level at week 16, but this was not significant due to the reduction seen in the DT group, over time when the data was taken as a set there was a significant ($p<0.005$ using 2-way ANOVA) decrease in the G17DT treated groups compared to DT (summarised in fig 6.9). Examples of blood vessel staining are shown in fig.6.10. The pattern of blood vessel distribution is similar in both groups but there are visibly less blood vessels in the G17DT group compared to the DT. In the sections of the large intestine without polyps blood vessels are present between the crypts and in polyps they are most dense in the central stalk.

6.1.4 COX-2 protein expression

Due to the results seen for proliferation and BVD in the large intestine and this being the area that G17DT is known to have more of an effect, it was decided to concentrate on the COX-2 expression in the large intestine.

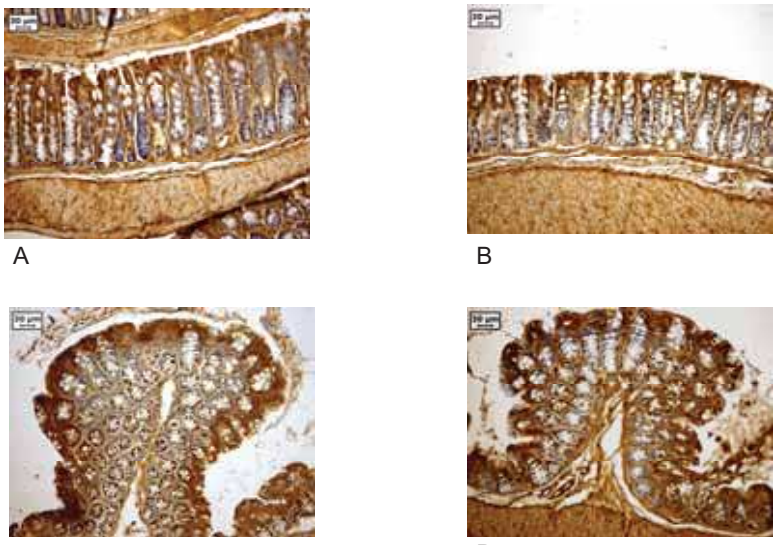


A



B

Fig. 6.11 Comparison of COX-2 protein expression in normal crypts (A) and polyps (B) of the large intestine of *Apc*^{min} mice treated with G17DT or DT. Mean results for 5 animals are shown along with standard error bars.



A B C D

Fig 6.12 Examples of COX-staining in the large intestine of 16 week old APC^{min} mice. Normal crypts treated with DT (A) and G17DT (B) and polyps treated with DT (C) and G17DT (D).

There was no significant decrease in COX-2 protein expression at any of the time points, in either the crypts (fig. 6.11) or polyps (fig 6.11B) of the large intestine when treated with G17DT compared to DT. COX-2 staining was most intense in the pit

region of both the crypts and the polyps with diffuse interstitial staining; there was also occasional nuclear staining (see fig 6.12).

6.1.5 Discussion

Previous work within our group has shown that when hypergastrinemia was induced by administration of omeprazole, a protein pump inhibitor, the effects could be reversed with G17DT immunisation (Watson and Smith 2001). Polyps of both the large and small intestine of *Apc*$^{Min/+}$ mice have been shown to express the gastrin gene, progastrin and gly-gastrin (Watson S.A 1998). Therefore this model offered an interesting environment in which to test the role of G17DT, since it inhibits gly-G17, and could potentially inhibit autocrine pathways established by these tumours.

In contrast with gastric adenocarcinomas where G17 and G34 are linked to proliferation and tumour development and progastrin is expressed at low levels (Henwood, Clarke *et al*. 2001), in colorectal carcinogenesis progastrin plays a more active role and therefore, although G17DT neutralises gly-G17, which is important in colorectal cancer progression (Siddheshwar, Gray *et al*. 2001) its inability to affect progastrin can be seen as a weakness in it effectiveness for treating colorectal cancer progression and may explain why less significant results were obtained in the *Apc*$^{Min/+}$ model compared to the INS-GAS model.

As previously discussed angiogenesis is a key process in carcinogenesis and therefore the ability of G17DT to significantly reduced BVD in the large intestine of the

Apc$^{Min/+}$ mouse compared to DT is further evidence of G17 and gly-G17s role in the angiogenic process of colorectal cancer progression.

Mutations in the *APC* gene are considered to be initiators in the colorectal carcinogenesis sequence, which leads to induction of COX-2 expression. Oshima *et al* (Oshima, Dinchuk *et al*. 1996) showed a 86% decrease in polyp burden in *Apc*$^{Min/+}$ mice with a disrupted *COX-2* gene. Currently the molecular mechanisms, which lead to COX-2 over-expression in colorectal cells are not fully understood.

Studies have shown that gastrin and the CCK-2 receptor are linked to increased COX-2 levels in colon carcinoma cells (Williams, Shattuck-Brandt *et al*. 1999; Guo, Cheng *et al*. 2002) and hypergastrinaemia, due to *H. pylori* infection, has been linked to increased COX-2 expression in colon tissue suggesting that gastrin may regulate COX-2 expression in colorectal cancer. However, in comparison with the results seen in the INS-GAS mice treated with G17DT no reduction in COX-2 protein expression was seen in the *Apc*$^{Min/+}$ mice treated with G17DT compared to DT treated. G17 has been shown to transactivate the COX-2 promoter through the CCK-2i4sv receptor in human colon cancer cell lines Colo320 and COS-7 (Huang, Ansorge *et al*. 2007). A possible explanation for the lack of reduction of COX-2 protein expression in this work could be that it is progastrin that is involved in the up-regulation of COX-2 in colorectal cancer progression and not G17 or gly-G17 in the *Apc*$^{Min/+}$ mouse model. This theory fits with the work by Konturek *et al* (Konturek, Rembiasz *et al*. 2006) that showed colorectal cancer patients exhibited significantly higher serum progastrin levels than healthy individuals and that this hyperprogastrinemia decreased after COX-2 inhibition with celecoxib treatment, also implying that it is gastrin expression

which is up-regulated by COX-2 and not the other way round. There is also evidence that colorectal cancer cells are capable of producing and releasing large quantities of progastrin fragments since plasma concentrations of progastrin dramatically reduce when the cancer is removed but concentrations of G17 and G-34 do not (Konturek, Bielanski *et al*. 2002).

Since G17DT decreased angiogenesis in the *Apc*$^{Min/+}$ mouse model, but did not effect COX-2 protein expression, unlike in the INS-GAS mouse model, it would suggest that the decrease in angiogenesis may not be a direct consequence of a reduction in COX-2.

6.2 The effect of COX inhibition on cancer progression in the *Apc*$^{Min/+}$ mouse model

It was hypothesised that the use of a general COX inhibitor, such as aspirin, and a specific COX-2 inhibitor, such as celecoxib, could slow and even prevent polyp/tumour progression in this model.

6.2.1 Polyp burden in the small and large intestine

At termination the number of polyps in the small and large intestine were assessed using a dissecting microscope, results shown in fig. 6.13.

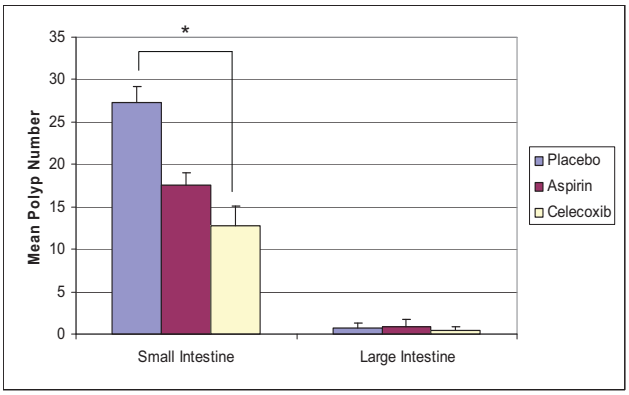


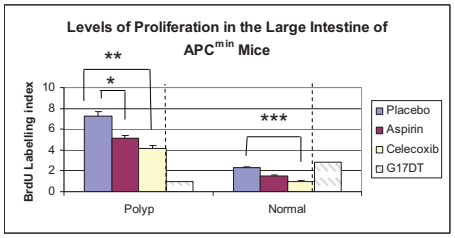
Fig. 6.13 Comparison of polyp burden in the small and large intestine of *APC*^{min} mice when treated with COX inhibitors compared to placebo. Mean results for 5 animals per group are shown along with standard error bars. *p<0.001 using one way ANOVA.

In the small intestine there was a decrease in polyp burden in both the aspirin and celecoxib treated groups, with a significant reduction ($p<0.001$) of just over 50% in

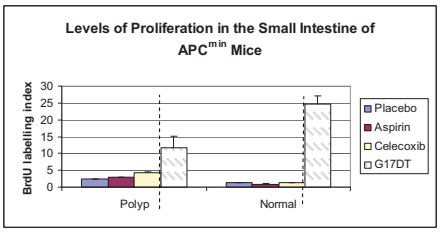
the celecoxib group compared to placebo. In the large intestine the polyp burden was very low and might explain why no significant reduction was observed.

6.2.2 Proliferation

Proliferation was measured using BrdU as previously described in section 3.1.1.1. The percentage staining was assessed to give a labelling index separately in the polyps and the normal intestinal tissue.

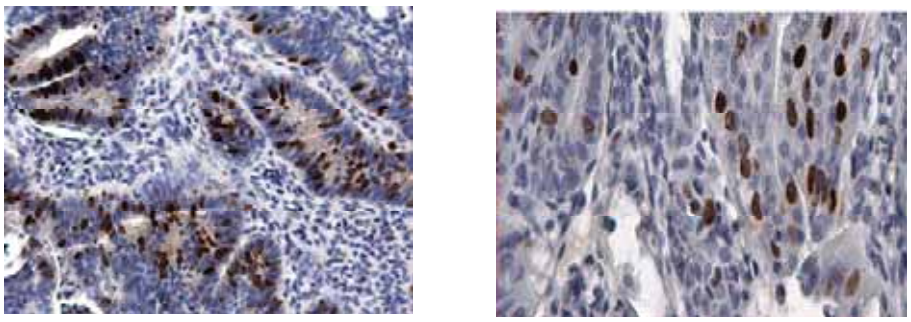


A



B

Fig. 6.14 The effect of treating APC^{min} mice with aspirin and celecoxib on proliferation in the intestine. Fig. A shows the effect in the large intestine and fig. B in the small intestine. Mean results for 5 animals per group are shown along with standard error bars. *p<0.01, **p<0.005, ***p<0.01 using one way ANOVA. G17DT data taken from section 6.1.1.

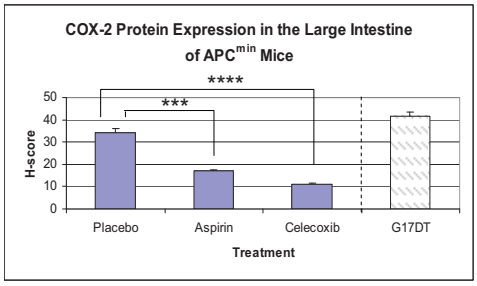


A B

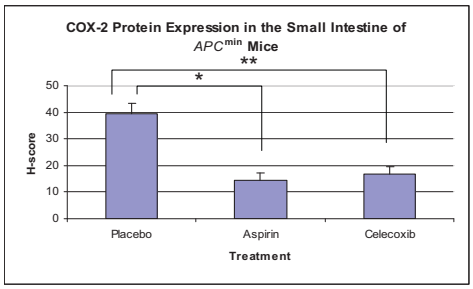
Fig 6.15 BrdU staining of proliferating cells in the large intestine in *APC*^{min} mice given placebo (x10) (A) or celecoxib (x20)(B).

In the large intestine (fig. 6.14A) there was a significant decrease in proliferation in the polyps in the aspirin ($p<0.01$) and celecoxib ($p<0.005$) treated groups and celecoxib also caused a significant ($p<0.01$) decrease in the proliferation of normal large intestinal tissue. In the small intestine (Fig. 6.14B) there was a slight increase in proliferation in the polyps with both treatments and no effect was seen in the normal tissue. Example of BrdU staining in the large intestine can be seen in fig 6.15.

6.2.3 Cox-2 protein expression

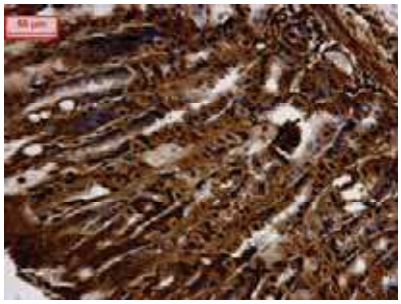


A

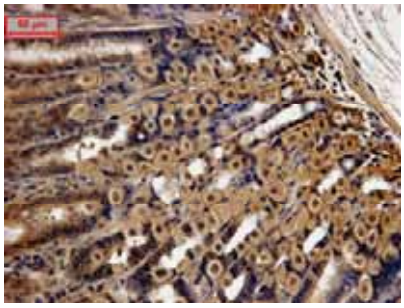


B

Fig. 6.16 The effect of Aspirin and Celecoxib on COX-2 protein expression in the large (A) and small (B) intestine of *APC*min mice. Mean results shown for 5 animals per group along with standard error bars. *p<0.001,**p<0.005, ***p<0.01, ****p<0.01 using one way ANOVA. G17DT data taken from section 6.1.4.



A



B

Fig. 6.17 Example of COX-2 protein staining in the large intestine of APC^{min} mice treated with placebo (A) or celecoxib (B).

COX-2 protein expression was significantly reduced in both the small and large intestine by aspirin ($p<0.001$ and $p<0.01$ respectively) and celecoxib ($p<0.005$ and $p<0.01$ respectively) as illustrated in fig. 6.16. An example of COX-2 protein staining in the large intestine was shown in fig. 6.17. In both the placebo and the celecoxib treated mice the pattern of staining is similar, but the staining was less intense in the celecoxib treated mouse.

6.2.4 Blood vessel density

As previously described CD34 was used to stain and assess blood vessel density as a measure of angiogenesis.

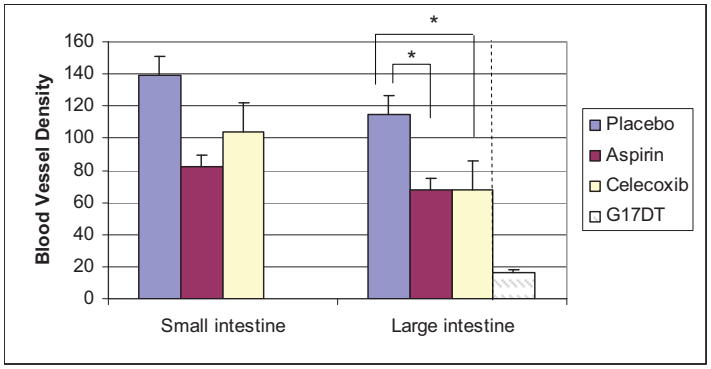
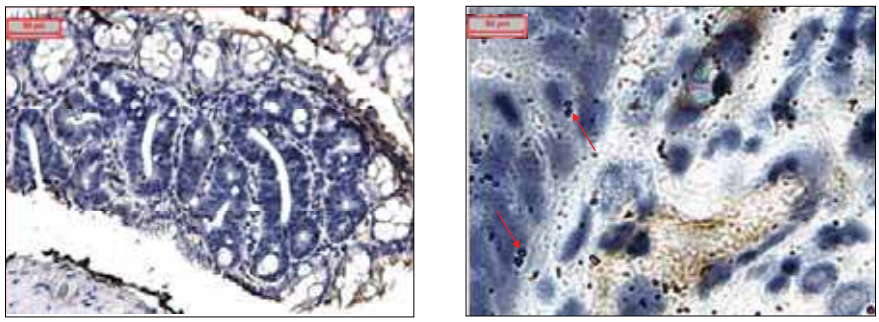


Fig.6.18 The effect of treating APC^{min} mice with Aspirin and Celecoxib on blood vessel density in colonic crypts. Mean results shown for 5 animals per group along with standard error bars. *$p<0.001$ using one way ANOVA. G17DT data taken from section 6.1.3.



A B

Fig. 6.19 Example of CD34 staining of blood vessels in the intestine of a APC^{min} mouse at x20 (A) and x40 (B). Arrows indicate blood vessels, transverse vessels appear as circles and longitudinal vessels appear as tubes.

Blood vessel density was decreased in the small intestine by both aspirin and celecoxib, but not significantly. However, the decrease in the large intestine was significant for both treatments ($p<0.001$), although not to the same extent as seen in the previous study with G17DT (fig. 6.18). Fig.6.19 shows examples of blood vessel staining in *Apc*$^{Min/+}$ mice.

6.2.5 Discussion

As in the INS-GAS mouse model we used COX inhibition in the *Apc*$^{Min/+}$ model to further explore the relationship between COX expression and colorectal cancer progression using aspirin and celecoxib treatment..

In contrast to the INS-Gas model, in the *Apc*$^{Min/+}$ mouse both celecoxib and aspirin significantly reduced proliferation in the polyps, BVD in the large intestine and COX-

2 protein expression in the large and small intestine. Also celecoxib reduced the polyp burden in the small intestine, no reduction was seen in the polyp burden of the large intestine but this is likely to be due to the low number of polyps that form in the large intestine of this model. Aspirin also reduced proliferation in the normal crypts of the large intestine.

COX inhibitors work by blocking the conversion of arachadonic acid to prostaglandins by COX enzymes, COX-1 and -2. Although the enzymes are not used up during the reaction they do need replenishing after use and therefore if they are not used then more is not produced, which explains why COX inhibitors also causes a reduction in COX protein.

The results achieved in the *Apc*$^{Min/+}$ model with COX inhibition would further suggest that G17 is not significantly involved in the progression of colorectal cancer in this model. Also of note is the fact that only aspirin reduced proliferation in the normal crypts suggesting a role for COX-1 in colorectal cancer progression since aspirin has been shown to be more effective at inhibiting COX-1 than COX-2 (Vane and Botting 1998). COX-1 and not COX-2 have been shown to be expressed in the normal crypts of *Apc*$^{Min/+}$ mice by Chulada *et al* (Chulada, Thompson *et al*. 2000) and studies have indicated that COX-1 can metabolically activate procarcinogens to mutagenic intermediates, an activation, which can be inhibited by aspirin (Marnett 1992).

Chapter 7

DISCUSSION

7.1 Gastrin as a multi modal growth factor

Gastrin impacts on a number of biological functions in addition to its central role in acid secretion including pro-proliferative and anti-apoptotic activities. It is these extended functions which have led to it being investigated in the malignant setting. In a recent review, gastrin-mediated carcinogenic pathways were detailed (see below for summary diagram fig.7.1). Included in these activities was the pro-proliferative, anti-apoptotic and pro-angiogenic pathways that were investigated in a variety of models and tissue types in the present thesis.

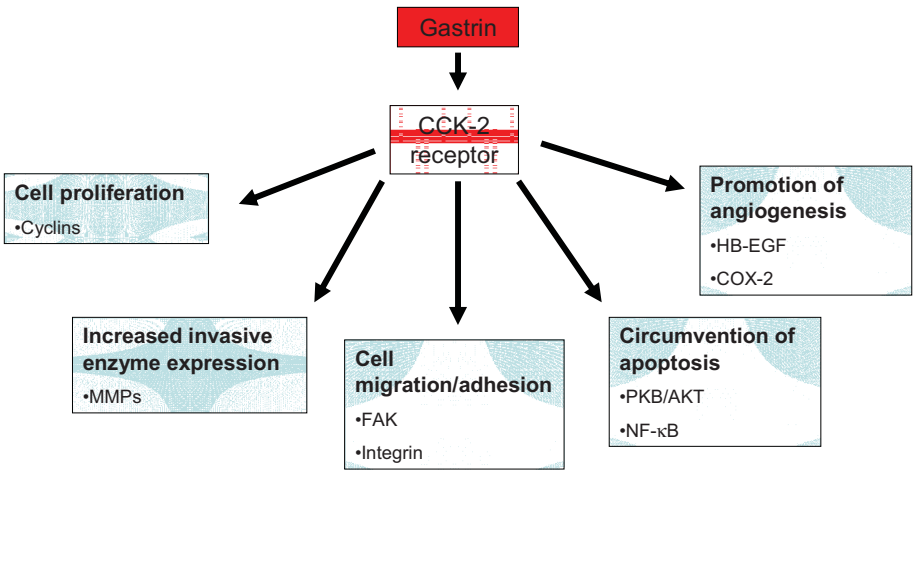


Fig. 7.1 Gastrin mediated carcinogenic pathways. Gastrin mediates gene expression which is associated with cell division, invasion, angiogenesis and anti-apoptotic activity. FAK=focal adhesion kinase. Adapted from Watson *et al* 2006.

The present studies have addressed the prognostic role of gastrin and CCK-2R in patient specimens and examined the therapeutic role of gastrin neutralisation in transgenic models of GI carcinogenesis, dissecting out the role of the closely associated carcinogenic enzyme, COX-2. The latter is particularly timely due to the

side effect profile associated with a number of COX-2 inhibitors, which appeared to be key therapeutics as GI chemopreventative agents, particularly for neoplasia arising within the colon.

7.2 Choice of models and tissue to investigate the role of gastrin in GI carcinogenesis

(i) Model of Gastric carcinogenesis and *Helicobacter* interactions in this process.

With the WHOs classification of *H. pylori* as a gastric carcinogen in 1994 a number of animal models were set up to investigate its role in carcinogenesis. Studies in Mongolian gerbils showed the induction of gastric carcinoma in the absence of other stimuli (Hirayama, Takagi *et al*. 1996; Watanabe, Tada *et al*. 1998), but in mice there were mixed results with best infection rates seen with *H. pylori* Sydney Strain1 (SS1) which does not initiate a strong immune response and *H. felis* which does not infect humans (Court, Robinson *et al*. 2002).

The Division of Pre-Clinical Oncology's *in vivo* unit have a breeding colony of INS-GAS mice which offered the opportunity to further investigate the down-stream pathways associated with hyper-gastrinaemic induction of neoplasia, to define the role of anti-gastrin therapy (G17DT) in malignant progression of this model and to also further determine the role of *Helicobacter* infection in the hypergastrinaemic environment.

In humans *H. pylori* infection has been shown to cause hypergastrinaemia, either by cytokine induced stimulation of G-cells to release gastrin or as a result of a feed-back loop due to parietal cell loss and hypochlorhydria (Weigert, Schaffer *et al*. 1996). Various groups have demonstrated that when this model is infected with *H. felis* the process of gastric carcinogenesis is accelerated, with infected mice showing marked gastric hypertrophy and invasive carcinomas of the fundus at 9 months compared to uninfected mice, which do not show these changes until 20 months (Fox, Rogers *et al*. 2003; Fox, Wang *et al*. 2003). A finding which is supported by results presented in this work, which showed long term infection with *H. felis* caused a significant increase in proliferation and decrease in apoptosis in INS-GAS mice compared to uninfected controls.

(ii) Model of colon carcinogenesis

As discussed in section 6.0 the $Apc^{Min/+}$ mouse model is the favoured model for determining chemo-preventative effects in colorectal carcinogenesis and this is also a model that I had access to through the Division of Pre-Clinical Oncology's *in vivo* unit. Previous work within our group has shown that when hypergastrinemia was induced by administration of omeprazole, a protein pump inhibitor, the effects could be reversed with G17DT immunisation (Watson and Smith 2001). Polyps of both the large and small intestine of $Apc^{Min/+}$ mice have been shown to express the gastrin gene, progastrin and gly-gastrin (Watson S.A 1998). Therefore this model offered an interesting environment in which to test the role of G17DT, since it inhibits gly-G17, and could potentially inhibit autocrine pathways established by these tumours.

(iii) Oesophageal carcinogenesis tissue

Although there are animal models available for oesophageal cancer, such as, Wistar rats which have undergone surgery and exhibit gastric reflux (Kumagai, Mukaisho *et al*. 2003), I did not have access to these models through my department. However, as a result of collaboration with Dr. Ragunath in Gastroenterology, Division of Medicine, University of Nottingham, oesophageal pinch biopsies and serum were obtained from patients attending an endoscopy clinic for Barrett's oesophagus. This then allowed the investigation of a number of parameters including anti-apoptotic and pro-angiogenic markers in relation to serum gastrin and CCK-2 receptor levels.

(iv) Pancreatic cancer tissue

As with oesophageal cancer, there are model of pancreatic cancer, such as, the hamster-BOP model, Syrian hamsters that have pancreatic adenocarcinomas induced by the injection of the carcinogen N-nitrosobis-2-oxopropylamine (Takahashi, Furukawa *et al*. 1990), but again I did not have access to this. Instead, in collaboration with Mr. Dileep Lobo in GI Surgery, Division of Surgery, University of Nottingham, I was able to obtain human pancreatic TMA's containing normal pancreas, negative lymph nodes, positive lymph nodes, pancreatitis and pancreatic cancer tissue for which survival data was also available. This offered the ideal opportunity to evaluate a number of factors including; CCK-2 receptor expression, anti-apoptotic factors and COX-2 and explore correlations.

7.3 Gastrin isoform sensitivity through the GI tract and activation of down-stream pathways

Barrett's, gastric and pancreatic cancer have all been linked to the amidated form of gastrin; in addition glycine extended gastrin is thought to play a role in Barrett's and pancreatic cancer. This contrasts with colon cancer where the main forms of gastrin that are involved are progastrin and glycine extended gastrin (Wang, Dangler *et al*. 2000; Watson and Smith 2001; Ptak-Belowska, Pawlik *et al*. 2007; Beales and Ogunwobi 2009).

(i) Barrett's oesophagus

High levels of CCK-2 receptor have previously been reported in Barrett's tissue (Haigh, Attwood *et al*. 2003) and this has been confirmed in this work. However the high levels of circulating serum gastrin due to PPI treatment may have contributed to the down regulation of the receptor seen in IM and HGD compared to CLO.

This work has highlighted a role for the CCK-2 receptor in promoting angiogenesis through VEGF-R2, by the positive correlation between CCK-2 receptor and VEGF-R2, although no correlation was seen with another indicator of angiogenesis, HB-EGF, which our group has previously shown can be up-regulated by gastrin (Clarke, Dickson *et al*. 2006). VEGF-R2 has also been linked to tumour proliferation and cell survival possibly by inhibiting apoptosis through the up-regulation of Bcl-2 (Masood, Cai *et al*. 2001; Liang, Brekken *et al*. 2006).

The up-regulation of Bcl-2 in the progression of Barrett's metaplasia to dysplasia (Shimizu, Vallbohmer *et al*. 2006) has been further confirmed in this work along with the novel finding that XIAP is up-regulated in Barrett's progression and in individual patients there were positive correlations between Bcl-2 and XIAP expression. Another key finding was the positive correlation between serum gastrin and XIAP, and positive correlations between p-Akt and both serum gastrin and CCK-2 receptor, further strengthen the link between gastrin and its receptor and anti-apoptotic markers, supporting other work carried out in our group which showed activation of Akt by gastrin in Barrett's tissue (Harris, Clarke *et al*. 2004). It is therefore feasible to hypothesise that in Barrett's oesophagus high levels of gastrin acting through the CCK-2 receptor may increase VEGF-R2 expression which in turn up-regulates Bcl-2 and XIAP.

(ii) Gastric cancer

The normal regulation of gastric acid secretion in the stomach by gastrin is achieved through a cellular sequence whereby gastrin released by the antral G-cells stimulates ECL cells to secrete histamine, which in turn stimulates parietal cells to secrete hydrochloric acid (Waldum, Sandvik *et al*. 1991). Gastrin exerts its effects via the CCK-2 receptor which has been shown to be expressed on both ECL and parietal cells (Schmitz, Goke *et al*. 2001). As previously discussed the INS-GAS mouse has elevated levels of serum gastrin G17, which leads to increased gastric acid secretion and also increased numbers of parietal and ECL cells (Wang, Dangler *et al*. 2000).

In this model therapeutic intervention with G17DT, which neutralises G17 and gly-G17, significantly reduced proliferation and BVD as well as suppressing COX-2

protein expression over time. Other groups have shown that COX-2 positively correlates with angiogenesis in gastric cancer (Murata, Kawano *et al*. 1999; Mao, Wang *et al*. 2007). It is therefore reasonable to hypothesise that the reduction in BVD seen by G17DT intervention is mediated through a reduction in COX-2.

(iii) Pancreatic cancer

This work has confirmed the finding by Caplin *et al* (Caplin, Savage *et al*. 2000) that the CCK-2 receptor is expressed on pancreatic cancer cells and not normal pancreatic cells. A novel finding of this work was the expression of the CCK-2 receptor on endothelial cells and an increase in this expression in pancreatic cancer tissue compared to normal pancreas, along with a positive correlation between tumour recurrence and CCK-2 receptor expression both generally and localised to endothelial cells. In gastric cancer, gastrin-producing tumour cells have been linked to cancer recurrence (Stephens, Hopper *et al*. 2007), but there is currently no other data on this relating to pancreatic cancer. Also of importance was the finding that there was a negative correlation between CCK-2 receptor expression and survival.

These negative patient outcomes linked to CCK-2 receptor expression may in part be mediated by XIAP expression. In gastro-oesophageal cell lines gastrin has been shown to increase XIAP expression (Harris 2005) and my work has shown a positive correlation between CCK-2 receptor expression and XIAP. There was also a positive correlation between CCK-2 receptor expression on endothelial cells and XIAP which may impact on angiogenesis, possibly through VEGF-R2, since VEGF has been linked to up-regulation of XIAP (Tran, Rak *et al*. 1999) and my work in Barrett's has shown a correlation between VEGF-R2 and serum gastrin.

The other anti-apoptotic marker assessed, Bcl-2, did not show any correlation with the CCK-2 receptor parameters, but the levels and pattern shown in this study were inline with those published by Campani *et al* (Campani, Esposito *et al*. 2001).

(iv) Colon cancer

The *Apc*$^{Min/+}$ mouse has increased levels of progastrin and gly-G17, which is expressed in polyps of both the small and large intestine (Watson and Smith 2001); these unprocessed forms of gastrin have been shown to promote proliferation in the colon (Wang, Koh *et al*. 1996; Koh, Dockray *et al*. 1999) The MTI/G-Gly *Apc*$^{Min/+}$ mouse which expresses the glycine extended form of gastrin shows increased polyp size and number compared to the standard *Apc*$^{Min/+}$ mouse (Koh, Bulitta *et al*. 2000). The normal colon does not appear to express the classical CCK-2 receptor, suggesting that the actions of G-gly are modulated independently of the classical CCK-2 receptor possibly through pathways involving c-jun and/or PI-3 kinase (Todisco, Takeuchi *et al*. 1995; Kowalski-Chauvel, Pradayrol *et al*. 1997).

The main finding of this work in *Apc*$^{Min/+}$ mice was that therapeutic intervention with G17DT reduced BVD, highlighting a role for gly-G17 in angiogenesis and proliferation over the time course of the study. However, unlike the INS-GAS model there was no reduction in COX-2 protein expression.

The differences in the types of gastrin expressed and the receptors they work through, help to explain why G17DT intervention had greater potency in reducing markers of cancer progression in the INS-GAS mouse model than the *Apc*$^{Min/+}$ model. Since, immunisation with G17DT elicits the production of antibodies which cross-react with

and neutralise G17 and gly-G17, therefore preventing them stimulating cells and effectively the downstream targets of gastrin, G17DT immunisation does not neutralise progastrin (2003); therefore, this is a limiting factor in colonic but not gastric setting as progastrin plays a role in carcinogenesis in the former but not latter (Siddheshwar, Gray *et al*. 2001; Konturek, Bielanski *et al*. 2002)

7.4 Clinical relevance of gastrin in transgenic mouse models

This work has demonstrated the ability G17DT to significantly reduce indicators of carcinogenesis in animal models of gastric and colonic cancer that over express different forms of gastrin and follow similar progression to human cancers. The data achieved from the animal models in this work indicate that anti-gastrin treatment targeted at G17 is a feasible chemo-preventative route in gastric cancer, but that in colonic the inhibition of different forms of gastrin may be required.

7.5 Clinical relevance of gastrin in patient tissue samples

Barrett's oesophagus and pancreatic cancer progression are both linked to G17 and gly-G17, with G17 promoting growth of oesophageal and pancreatic cell lines *in vitro* (Haigh, Attwood *et al*. 2003) and gly-G17 inhibiting apoptosis (Todisco, Ramamoorthy *et al*. 2001; Beales and Ogunwobi 2009). In Barrett's oesophagus the inhibition of apoptosis by gly-G17 has been showed to be mediated through the JAK/STAT3 pathway, but not via the CCK-2 receptor (Beales and Ogunwobi 2009).

Also, unlike G17, gly-G17 does not appear to be involved in COX-2 expression in Barrett's (Abdalla, Lao-Sirieix *et al*. 2004). In pancreatic caner G17 stimulates tumour growth through the CCK-2 receptor, but as with Barrett's gly-G17 appears to work through a mechanism independent of the CCK-2 receptor (Seva, Dickinson *et al*. 1994).

The poor prognosis for Barrett's patients that progress to oesophageal adenocarcinoma is due to late diagnosis, by which point the cancer is advanced and metastasis has often occurred (Schlansky, Dimarino *et al*. 2006). It is for these reasons that individuals with Barrett's undergo regular endoscopy surveillance, as this is more sensitive than less invasive techniques such as barium swallow, faecal occult blood testing and brush cytology (Gerson and Triadafilopoulos 2002). While surveillance programmes have been effective in the prophylactic treatment of some cancers, such as cervical (Spitzer 2007), current research would suggest that it is unlikely to be the case with oesophageal adenocarcinoma progression from Barrett's oesophagus. Although new advances in endoscopy techniques, such as narrow band imaging (NBI), are offering a more reliable detection of dysplasia (Hamamoto, Endo *et al*. 2004), the way forward may be to also use biomarkers to classify Barrett's patients in conjunction with endoscopy assessment. Based on the data presented in this work a possible biomarker would be CCK-2 receptor protein levels, since Barrett's patients tend to be hypergastrinaemic and an increase in gastrin receptor leads to an increase in anti-apoptotic and proangiogenic pushing Barrett's to adenocarcinoma.

As previously mentioned the current mainstay of treatment for individuals with Barrett's oesophagus is long-term PPI therapy. This is mainly due to it being a condition that affects the elderly population, who are often unsuitable for invasive surgery (Krasner N. A 2005). Although the ability of PPIs to relieve symptoms is well-documented, there is no clear-cut evidence on their ability to prevent neoplastic change (Lanas 2005), and work presented in this study even suggests that hypergastrinaemia as a result of PPI therapy may induce expression of pro-angiogenic and anti-apoptotic factors, which drive Barrett's progression to oesophageal adenocarcinoma. For example this work has shown a positive correlation between serum gastrin levels and the anti-apoptotic markers XIAP and p-Akt along with positive correlations between the CCK-2 receptor and p-Akt and the pro-angiogenic marker VEGF-R2.

This study alone does not provide enough evidence to prohibit the current use of PPIs in the management of Barrett's oesophagus, especially since neoplastic change in Barrett's oesophagus can be driven by factors other than hypergastrinaemia. It does, however, imply that clinical trials are needed to further establish the role of PPI-induced hypergastrinaemia in oesophageal adenocarcinoma progression from Barrett's oesophagus, with a view to changing clinical management of Barrett's patients. A possible management route would be to test the CCK-2 receptor status and serum gastrin levels of Barrett's patients and give either CCK-2 receptor antagonists or immunise with G17DT to inhibit gastrin interactions on the oesophageal mucosa, which catalyse neoplastic changes but maintain the positive effect of PPIs on acid reflux. Further work is therefore also needed in relation to the effect of CCK-2 receptor antagonists and G17DT on the efficacy of PPIs.

The poor prognosis associated with pancreatic cancer and the current lack of effective treatment therapies makes it an area in need of a fresh approach. The data presented in this work supports the targeting of gastrin-induced cancer growth in the pancreas as a novel therapeutic target in a subset of patients that over-express the CCK-2 receptor. A randomised placebo-controlled study using G17DT showed marginal significant survival benefit and lower incidence of metastasis in the G17DT treated group (Gilliam and Watson 2007). However, when trialled in combination with gemcitabine, the standard chemotherapy agent for pancreatic cancer (Diaz-Rubio 2004), there was no survival advantage over gemcitabine alone, except on a subset analysis of patients with high antibody titres (Gilliam and Watson 2007). The use of a CCK-2 receptor antagonist Gastrazole in a randomised controlled trial also increased survival although not significantly compared to control. Survival was also not improved compared to 5-fluorouracil (Chau, Cunningham *et al*. 2006).

Although these initial trials do not offer an immediate answer, they are indicative that further work is required with respect to the involvement of different forms of both gastrin peptides and CCK-2 receptor isoforms to further establish their significance in the pancreatic cancer environment and that to be effective, treatments of pancreatic cancer need to be more targeted to individual patients dependent on their CCK-2 receptor status.

7.6 A comparison of gastrin neutralisation and inhibition of COX-2 in gastric and colonic carcinogenesis

Work *in vitro* has shown gastrin via the CCK-2 receptor significantly stimulates COX-2 expression possibly by the ERK5/MEF2 pathway and other MAPK dependent pathways (Guo, Cheng *et al*. 2002).

As discussed in section 1.6.3 COX-2 is involved in the synthesis of prostaglandin E_2 (PGE_2) from arachadonic acid. Prostaglandins play an important role in gastric mucosal integrity by helping protect the mucosa from excess acid, via an increase in the production of mucus as well as by directly inhibiting acid synthesis (Arakawa, Nakamura *et al*. 1990). Normally COX-2 is expressed at low levels in the stomach, but *in vivo* studies have shown increased expression in the gastric epithelium after acid-induced damage and *in vitro* after growth stimulation (Tsuji, Kawano *et al*. 1996; Sawaoka, Tsuji *et al*. 1997). Over-expression of COX-2 has also been reported in gastric lesions, and selective COX-2 inhibitors have been shown to delay gastric wound healing in rats, indicating a possible role in gastric ulcer healing (Sun, Tsuji *et al*. 2000).

In this work the reduction in BVD seen in the G17DT treated INS-GAS mice may be mediated through a reduction in COX-2 (fig 7.2). Since, immunohistochemistry showed that COX-2 protein was expressed in a number of cell types which do not express the CCK-2 receptor, gastrin may also indirectly up-regulate COX-2 in the gastric mucosa.

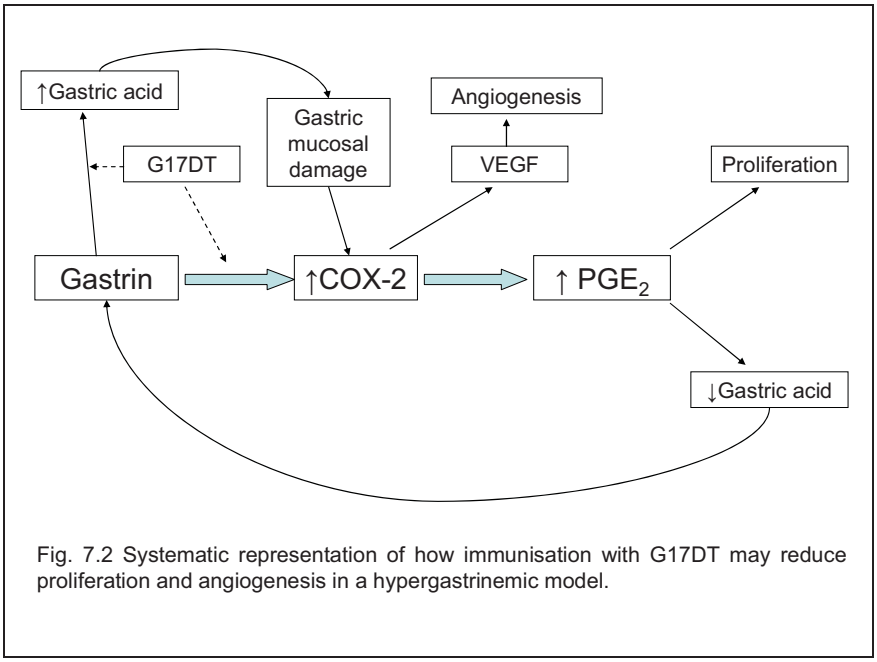


Fig. 7.2 Systematic representation of how immunisation with G17DT may reduce proliferation and angiogenesis in a hypergastrinemic model.

COX-2 has been shown to be up-regulated in human and rat intestinal tumours and is associated with an increase in metaplastic potential (Huang, Ansorge *et al*. 2007), which may be associated with up-regulation of the gastrin gene by β-catenin (fig. 7.3). A number of studies have shown a reduced risk of developing colorectal cancer in individuals who take NSAIDs In Australia in 1988 it was observed that there was a reduction in the incidence of colon cancer in individuals who regularly took aspirin (Kune, Kune *et al*. 1988). Since these initial observations several human studies have noted the protective effects inferred by NSAIDs (mainly aspirin), against colorectal cancer (Williams, Smalley *et al*. 1997). Studies using the NSAID sulindac have shown a significant reduction in tumour load and size in patients with the inherited autosomal dominant disease FAP (Williams, Smalley *et al*. 1997).

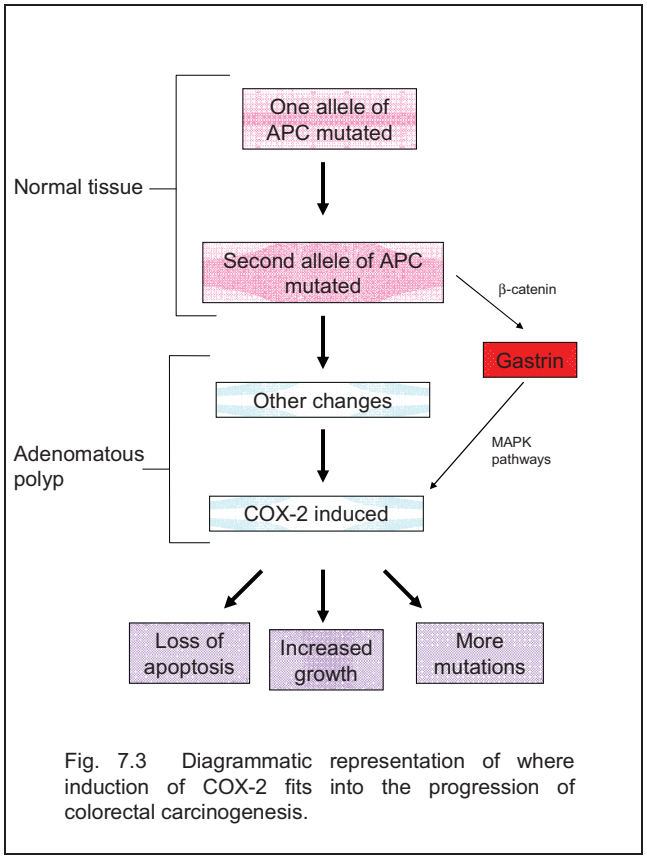


Fig. 7.3 Diagrammatic representation of where induction of COX-2 fits into the progression of colorectal carcinogenesis.

In the INS-GAS mouse study G17DT maintained low COX-2 protein expression levels expected in the normal gastric mucosa through out the time course of the study compared to the DT treated group, which showed a rapid increase of COX-2 protein expression at 30 weeks. This sudden increase of COX-2 protein expression at 30 weeks in the DT treated group may be in response to inflammation and gastric mucosal damage caused by hypergastrinemia, demonstrating that G17DT immunisation may be protective against inflammation and mucosal damage in the stomach.

7.7 The role of COX inhibitors in gastric and colonic cancer models

To further explore the relationship between COX expression and gastric and colorectal cancer progression the two mouse models were used to investigate the effects of aspirin and celecoxib treatment.

Aspirin was chosen as it inhibits both isoforms of COX, whereas celecoxib is a COX-2 specific inhibitor (coxib). Aspirin irreversibly inhibits COX-1 mediated synthesis of thromboxane A2 (TXA2), the major component of blood clots, and due to the anucleated state of platelets they can not regenerate COX-1, resulting in inhibition of platelet aggregation. This is in comparison to the reversible inhibition of COX-1 by other NSAIDs, which is dependent on the half life of the drug (Strand 2007). Coxibs were designed to overcome some of the side effects of NSAIDs, like gastric bleeding and ulcers, which are thought to be caused by the inhibition of COX-1.

In the INS-GAS model, neither aspirin nor celecoxib had an effect on proliferation and although BVD was reduced by both agents, neither did so significantly. These results support the theory that it is G17 that is driving the increase in COX-2 seen in this model and that in a hypergastrinemic environment G17DT immunisation is more successful in reducing the effects of COX-2 over-expression than the COX inhibitors, aspirin and celecoxib.

In comparison in the *Apc*$^{Min/+}$ mouse both celecoxib and aspirin significantly reduced proliferation in the polyps, BVD in the large intestine and COX-2 protein expression in the large and small intestine. Although celecoxib reduced the polyp burden in the

small intestine, no reduction was seen in the polyp burden of the large intestine but this is likely to be due to the low number of polyps that form in the large intestine of this model. Aspirin also reduced proliferation in the normal crypts of the large intestine.

The results achieved in the *Apc*$^{Min/+}$ model with COX inhibition would further suggest that G17 is not significantly involved in the progression of colorectal cancer in this model, although hypergastrinemia induced by PPIs has been shown to promote adenoma progression in this model (Watson and Smith 2001), which would suggest that levels of G17 need to be above a certain threshold to exert an effect. Also of note is the fact that only aspirin reduced proliferation in the normal crypts suggesting a role for COX-1 in colorectal cancer progression since aspirin has been shown to be more effective at inhibiting COX-1 than COX-2 (Vane and Botting 1998). COX-1 and not COX-2 have been shown to be expressed in the normal crypts of *Apc*$^{Min/+}$ mice by Chulada *et al* (Chulada, Thompson *et al*. 2000) and studies have indicated that COX-1 can metabolically activate procarcinogens to mutagenic intermediates, an activation, which can be inhibited by aspirin (Marnett 1992).

7.7.1 G17DT as a COX-2 inhibitor?

The first coxibs to be granted US Food and Drug administration (FDA) approval were celecoxib (Cerebrex) in December 1998 followed by rofecoxib (Vioxx) in May 1999 (Strand 2007). The publication of two large scale clinical trials, Celecoxib Long-term Arthritis Safety Study (CLASS), which ran from September 1998 – March 2000 (Silverstein, Faich *et al*. 2000) and the Vioxx Gastrointestinal Outcomes Research

(VIGOR), which concluded in 2000 (Bombardier, Laine *et al*. 2000), showed selective COX-2 inhibitors produced similarly efficacy, but fewer GI side effects than NSAIDs which led to the general acceptance of coxibs.

A number of studies have linked coxibs to an increased risk of cardiovascular events, which led to the relabeling of rofecoxib 14 months after its original approval to include potential cardiovascular risk (Strand 2007). Following the early termination of the Adenomatous Polyp Prevention On Vioxx study (APPROVe) due to a two-fold increase in the number of cardiovascular events in the rofecoxib group compared to placebo in August 2004 (Bresalier, Sandler *et al*. 2005), rofecoxib was withdrawn from the market (Strand 2007). It has since been discovered that some data on the occurrence of myocardial infarction was omitted from the VIGOR study (Curfman, Morrissey *et al*. 2005).

Increased risk of cardiovascular events has also been reported, with celecoxib in the 3 year Adenoma Prevention with Celecoxib (APC) (Solomon, McMurray *et al*. 2005) and the Prevention of Spontanous Adenomatous Polyp (Pre-SAP) as well as valdecoxib and parecoxib (Strand 2007).

There are currently three selective COX-2 inhibitors available (celecoxib, etoricoxib and lumiracoxib) all of which are considered safe in low doses, but further research is needed to determine cardiovascular risk at higher doses (Strand 2007).

With the loss of confidence in coxibs a gap is emerging in the clinical setting for an alternative method of COX-2 inhibition as a preventative of GI cancer progression.

Based on the data presented here and work by others, G17DT is a strong contender for this role in the case of gastric cancer, since it has been shown to inhibit a number of key systems linked to cancer progression such as proliferation and angiogenesis. Added to this, clinical trials have shown it is well tolerated in patients (Watson and Gilliam 2001). However, for colorectal cancer G17DT is unlikely to be the answer, since it does not appear to effect COX-2 expression in the colon unlike the gastric setting, although that is not to say that an alternative anti-gastrin treatment may not be effective, such as one targeted at progastrin. There is also the option of using G17DT or alternative anti-gastrin therapy in conjunction with low dose coxibs, as a reduction in the dose of the coxib is also likely to reduce the risk of myocardial events and any loss in efficacy at inhibiting COX-2 would be countered by the anti-gastrin therapy. Studies in the gastric cell line MKN-45 have shown a synergistic effect on reducing proliferation and increasing apoptosis with coxib, NS-398 and CCK-2 receptor antagonist, AG-041R (Sun, Zhu *et al*. 2008)

7.8 Gastrin neutralisation in a chemo-preventative setting

The role of gastrin in GI cancers is a crucial yet diverse one, where although there are similarities between the different types of cancers it is not a case of one theory fits all. For these reasons it is important to explore different methods of gastrin neutralisation in a chemo-preventative setting. The current options available are; monoclonal antibodies, CCK-2 receptor antagonists or a gastrin vaccine.

Monoclonal antibodies have been used in a number of cancer treatments including blocking growth signalling of EGF and as delivery systems for radio and chemo therapy (Gleissner, Schlenk *et al*. 2007; Omura 2008; Ulaner, Colletti *et al*. 2008).

Barderas *et al* (Barderas, Shochat *et al*. 2008) have shown that monoclonal antibodies which block G17 from binding to the CCK-2 receptor can reduce proliferation in Colo 320 WT, a colorectal cancer cell line that over-expresses CCK-2R, and also monoclonal antibodies directed against the N-terminal of G17 were more effective than those against the C-terminal. The high affinity human anti-gastrin monoclonal antibody XPA067.06 has been shown to neutralise human gastrin *in vivo* quickly and over a prolonged time course (Hsu, Patel *et al*. 2008).

The main draw back to monoclonal antibody therapy is that it requires constant administration over time, which leads to a number of side effects from the common such as, flu like symptoms, rashes, nausea and diarrhoea to rare yet more serious ones such as, infusion reactions, extremely low blood cell counts, heart failure and bleeding. Therefore in its current form monoclonal antibody therapy, while useful in the treatment of terminal patients is not ideal for long term treatment due to the constant administration and side effects.

Receptor antagonists are receptor ligands or drugs that bind to a receptor and do not provoke a biological response, but block or dampen an agonist mediated response. Therefore receptor antagonists have an affinity, but no efficacy for their receptors. To block the effects of gastrin the antagonist needs to be targeted against isoforms of the CCK-2 receptor.

The CCK-2 antagonist Gastrazole has been shown to prolong survival in patients with advanced pancreatic carcinoma (Chau, Cunningham *et al*. 2006), but its administration by protracted venous infusion is likely to mean its clinical use is limited. More promising are potent orally activated CCK-2 receptor antagonists, such as Z-360, which can reduce tumour size in MiaPaCa2 xenografts in nude mice and in PANC-1 xenografts in combination with gemcitabine (Kawasaki, Emori *et al*. 2008). In summary, while CCK-2 receptor antagonists, which require protracted venous infusion are not desirable in a clinical setting, those which are administered orally offer a favourable treatment route, although compliance with ongoing administration would need to be assured.

Endocrine therapy, where a synthetic hormone or drug is given to block the body's natural hormones, is one of the oldest methods of cancer treatment (Gonzalez and Lage 2007). A vaccine such as G17DT that raises antibodies that neutralises gastrin species is a viable option for chemo-prevention of GI cancers, which have been shown to be stimulated by gastrin.

In vivo, G17DT has been shown to produce strong antibody titres in rats injected with colon cancer DHDK12 cells, with titres in excess of those needed to neutralise serum G17, which may mean that these excess antibodies are available to neutralise cell-associated G17 peptides (Watson, Michaeli *et al*. 1996). G17DT has also been shown to be well tolerated in patients with gastric carcinomas, with no autoimmune reaction or immunological tolerance displayed (Gilliam, Watson *et al*. 2004).

Some of the draw backs to G17DT include the repeated injections required, which can lead to adverse reactions at the injection site and also because it's an immunogen patients need to have a functioning immune system to generate a response.

In severe combined immunodeficiency (SCID) mice injected with MGLVA1asc cells, a gastric ascities cell line derived from the human gastric carcinoma cell line MKN45G, G17DT had greater therapeutic effect than CI-988, a gastrin/CCK2 receptor antagonist (Watson, Michaeli *et al*. 1999). Possible reasons for this are that the antagonist has a lower affinity for gly-G17 than G17DT and that gly-G17 is mediated through an alternative receptor to the CCK-2 receptor and is therefore not blocked by this antagonist (Singh, Rae-Venter *et al*. 1985).

In conclusion, based on the data presented in this report and the current literature the neutralisation of gastrin peptides and/or the blocking of its receptor offers viable therapeutic modalities for the treatment of GI cancers in a subset of patients that over-express gastrin and/or its receptor. Neutralisation of gastrin peptides in these individuals would result in the reduction of COX-2, proliferation, angiogenesis and XIAP and therefore slow or even prevent cancer progression. Therefore, the research presented in this thesis has lead to the conclusion that the best way forward is to offer a targeted approach dependent on gastrin and CCK-2 receptor status. Currently CCK-2 receptor antagonists look like the optimal modality for achieving this, due to their low toxicity, oral administration and high activity.

7.9 Future work

This work has added more weight to the importance of gastrin in a number of GI cancers, and the possible role of gastrin and /or its receptor as a therapeutic target. More work is need to establish the different pathways in which gastrin is involved within the different cancer types, especially the relationship between gastrin and COX-2. To help understand this relationship I would propose mechanistic studies using relevant GI cell lines and treating with G17DT, COX inhibitors and combinations of G17DT and COX inhibitors and use western blot and RT-PCR to measure protein and gene expression levels of various markers including Bcl-2, Bax, PPARγ, COX-2 and gastrin (Chang, Song *et al*. 2006; Ptak-Belowska, Pawlik *et al*. 2007), as well as to determine the relationship between VEGF-R2, Bcl-2, XIAP and CCK-2 receptor. Also I feel it would be interesting to establish the mechanism by which gly-G17 reduces BVD in colonic cancer; this may be dependent on determining the receptor through which gly-G17 acts in the colon.

For the Barrett's work it could be beneficial if biopsies were available from subsequent endoscopies of the patients included in this study in order to observe possible progression to adenocarcinoma and relate this to parameters assessed in this thesis, to further strengthen the link between gastrin parameters and Barrett's progression. Another idea would be to obtain biopsies from individuals with oesophageal cancer and make a comparison betweens the ones that have progressed from Barrett's and those that have not to compare the different environments.

At a clinical level I would propose screening of Barrett's and pancreatic cancer patients for their CCK-2 receptor status and serum gastrin levels and considering treatment which included inhibition of the receptor probably as discussed earlier by a receptor antagonist.

Chapter 8

References

(1994). "Schistosomes, liver flukes and Helicobacter pylori. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994." IARC Monogr Eval Carcinog Risks Hum **61**: 1-241.

(2003). "Gastrin 17 vaccine--Aphton: Anti-gastrin 17 immunogen, G17DT." BioDrugs **17**(3): 223-5.

Abdalla, S. I., P. Lao-Sirieix*, et al.* (2004). "Gastrin-induced cyclooxygenase-2 expression in Barrett's carcinogenesis." Clin Cancer Res **10**(14): 4784-92.

Abnet, C. C., N. D. Freedman*, et al.* (2008). "A prospective study of BMI and risk of oesophageal and gastric adenocarcinoma." Eur J Cancer.

Alvarez, A., S. Ibiza*, et al.* (2006). "Gastrin induces leukocyte-endothelial cell interactions in vivo and contributes to the inflammation caused by Helicobacter pylori." Faseb J **20**(13): 2396-8.

Aly, A., A. Shulkes*, et al.* (2004). "Gastrins, cholecystokinins and gastrointestinal cancer." Biochim Biophys Acta **1704**(1): 1-10.

Anagnostopoulos, G. K., D. Stefanou*, et al.* (2005). "Bax and Bcl-2 protein expression in gastric precancerous lesions: immunohistochemical study." J Gastroenterol Hepatol **20**(11): 1674-8.

Arakawa, T., A. Nakamura*, et al.* (1990). "In vitro adaptive cytoprotection in gastric cells isolated from rat stomach." J Clin Gastroenterol **12 Suppl 1**: S32-8.

Arlt, A., A. Gehrz*, et al.* (2003). "Role of NF-kappaB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death." Oncogene **22**(21): 3243-51.

Atherton, J. C., P. Cao*, et al.* (1995). "Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration." J Biol Chem **270**(30): 17771-7.

Atherton, J. C., R. M. Peek, Jr.*, et al.* (1997). "Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori." Gastroenterology **112**(1): 92-9.

Bakke, I., G. Qvigstad*, et al.* (2001). "The CCK-2 receptor is located on the ECL cell, but not on the parietal cell." Scand J Gastroenterol **36**(11): 1128-33.

Balkwill, F. and A. Mantovani (2001). "Inflammation and cancer: back to Virchow?" Lancet **357**(9255): 539-45.

Barderas, R., S. Shochat*, et al.* (2008). "Designing antibodies for the inhibition of gastrin activity in tumoral cell lines." Int J Cancer.

Barrett, N. R. (1950). "Chronic peptic ulcer of the oesophagus and 'oesophagitis'." Br J Surg **38**(150): 175-82.

Bauerfeind, P., R. Garner*, et al.* (1997). "Synthesis and activity of Helicobacter pylori urease and catalase at low pH." Gut **40**(1): 25-30.

Beales, I. L., O. Ogunwobi*, et al.* (2007). "Activation of Akt is increased in the dysplasia-carcinoma sequence in Barrett's oesophagus and contributes to increased proliferation and inhibition of apoptosis: a histopathological and functional study." BMC Cancer **7**: 97.

Beales, I. L. and O. O. Ogunwobi (2009). "Glycine-extended gastrin inhibits apoptosis in Barrett's oesophageal and oesophageal adenocarcinoma cells through JAK2/STAT3 activation." J Mol Endocrinol **42**(4): 305-18.

Bold, R. J., J. Chandra*, et al.* (1999). "Gemcitabine-induced programmed cell death (apoptosis) of human pancreatic carcinoma is determined by Bcl-2 content." Ann Surg Oncol **6**(3): 279-85.

Bombardier, C., L. Laine*, et al.* (2000). "Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group." N Engl J Med **343**(21): 1520-8, 2 p following 1528.

Bond, J. H. (1993). "Polyp guideline: diagnosis, treatment, and surveillance for patients with nonfamilial colorectal polyps. The Practice Parameters Committee of the American College of Gastroenterology." Ann Intern Med **119**(8): 836-43.

Bouch, N., V. Stellmach *et al,* (1996). "How tumours become angiogenic." Adv. Can. Res. **69**: 135-174.

Bos, J. L., E. R. Fearon*, et al.* (1987). "Prevalence of ras gene mutations in human colorectal cancers." Nature **327**(6120): 293-7.

Boswald, M., S. Harasim*, et al.* (1990). "Tracer dose and availability time of thymidine and bromodeoxyuridine: application of bromodeoxyuridine in cell kinetic studies." Cell Tissue Kinet **23**(3): 169-81.

Boxer, L. M. and C. V. Dang (2001). "Translocations involving c-myc and c-myc function." Oncogene **20**(40): 5595-610.

Brand, S. J. and P. J. Fuller (1988). "Differential gastrin gene expression in rat gastrointestinal tract and pancreas during neonatal development." J Biol Chem **263**(11): 5341-7.

Bresalier, R. S., R. S. Sandler*, et al.* (2005). "Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial." N Engl J Med **352**(11): 1092-102.

Brett, B. T., S. C. Smith*, et al.* (2002). "Phase II study of anti-gastrin-17 antibodies, raised to G17DT, in advanced pancreatic cancer." J Clin Oncol **20**(20): 4225-31.

Brigati, C., D. M. Noonan*, et al.* (2002). "Tumors and inflammatory infiltrates: friends or foes?" Clin Exp Metastasis **19**(3): 247-58.

Brockman, J. A., D. C. Scherer*, et al.* (1995). "Coupling of a signal response domain in I kappa B alpha to multiple pathways for NF-kappa B activation." Mol Cell Biol **15**(5): 2809-18.

Brunner, G., M. Hell*, et al.* (1995). "Influence of lansoprazole on intragastric 24-hour pH, meal-stimulated gastric acid secretion, and concentrations of gastrointestinal hormones and enzymes in serum and gastric juice in healthy volunteers." Digestion **56**(2): 137-44.

Buchler, M., H. Friess*, et al.* (1991). "A randomized controlled trial of adjuvant immunotherapy (murine monoclonal antibody 494/32) in resectable pancreatic cancer." Cancer **68**(7): 1507-12.

Burris, H. A., 3rd, M. J. Moore*, et al.* (1997). "Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial." J Clin Oncol **15**(6): 2403-13.

Busler, V. J., V. J. Torres*, et al.* (2006). "Protein-protein interactions among Helicobacter pylori cag proteins." J Bacteriol **188**(13): 4787-800.

Calvert, P. M. and H. Frucht (2002). "The genetics of colorectal cancer." Ann Intern Med **137**(7): 603-12.

Campani, D., I. Esposito*, et al.* (2001). "Bcl-2 expression in pancreas development and pancreatic cancer progression." J Pathol **194**(4): 444-50.

Canzian, F., T. Ushijima*, et al.* (1994). "Instability of microsatellites in rat colon tumors induced by heterocyclic amines." Cancer Res **54**(24): 6315-7.

Carrillo-Infante C, G. Abbadessa *et al* (2007). "Viral infections as a cause of cancer (review)**Int. J. Oncol**. **30** (6): 1521–8.

Caplin, M., K. Savage*, et al.* (2000). "Expression and processing of gastrin in pancreatic adenocarcinoma." Br J Surg **87**(8): 1035-40.

Censini, S., C. Lange*, et al.* (1996). "cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors." Proc Natl Acad Sci U S A **93**(25): 14648-53.

Chandrasekharan, N. V., H. Dai*, et al.* (2002). "COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression." Proc Natl Acad Sci U S A **99**(21): 13926-31.

Chang, A. J., D. H. Song*, et al.* (2006). "Attenuation of peroxisome proliferator-activated receptor gamma (PPARgamma) mediates gastrin-stimulated colorectal cancer cell proliferation." J Biol Chem **281**(21): 14700-10.

Chang, J. T. and D. A. Katzka (2004). "Gastroesophageal reflux disease, Barrett esophagus, and esophageal adenocarcinoma." Arch Intern Med **164**(14): 1482-8.

Chau, I., D. Cunningham*, et al.* (2006). "Gastrazole (JB95008), a novel CCK2/gastrin receptor antagonist, in the treatment of advanced pancreatic cancer: results from two randomised controlled trials." Br J Cancer **94**(8): 1107-15.

Chen, J. J., P. L. Yao*, et al.* (2003). "Up-regulation of tumor interleukin-8 expression by infiltrating macrophages: its correlation with tumor angiogenesis and patient survival in non-small cell lung cancer." Clin Cancer Res **9**(2): 729-37.

Chulada, P. C., M. B. Thompson*, et al.* (2000). "Genetic disruption of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice." Cancer Res **60**(17): 4705-8.

Ciccotosto, G. D., A. McLeish*, et al.* (1995). "Expression, processing, and secretion of gastrin in patients with colorectal carcinoma." Gastroenterology **109**(4): 1142-53.

Clarke, P. A., J. H. Dickson*, et al.* (2006). "Gastrin enhances the angiogenic potential of endothelial cells via modulation of heparin-binding epidermal-like growth factor." Cancer Res **66**(7): 3504-12.

Clerc, P., M. Dufresne*, et al.* (1997). "Differential expression of the CCK-A and CCK-B/gastrin receptor genes in human cancers of the esophagus, stomach and colon." Int J Cancer **72**(6): 931-6.

Clerc, P., S. Leung-Theung-Long*, et al.* (2002). "Expression of CCK2 receptors in the murine pancreas: proliferation, transdifferentiation of acinar cells, and neoplasia." Gastroenterology **122**(2): 428-37.

Cong, B., S. J. Li*, et al.* (2003). "Expression and cell-specific localization of cholecystokinin receptors in rat lung." World J Gastroenterol **9**(6): 1273-7.

Corleto, V. D., S. U. Goebel*, et al.* (2003). "Co-existence of hyperparathyroidism, hypergastrinaemia and multiple gastric carcinoids is not always due to incomplete expression of the MEN-1 syndrome." Dig Liver Dis **35**(8): 585-9.

Corpet, D. E. and F. Pierre (2003). "Point: From animal models to prevention of colon cancer. Systematic review of chemoprevention in min mice and choice of the model system." Cancer Epidemiol Biomarkers Prev **12**(5): 391-400.

Correa, P. (1992). "Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention." Cancer Res **52**(24): 6735-40.

Court, M., P. A. Robinson*, et al.* (2002). "Gastric Helicobacter species infection in murine and gerbil models: comparative analysis of effects of H. pylori and H. felis on gastric epithelial cell proliferation." J Infect Dis **186**(9): 1348-52.

Coussens, L. M. and Z. Werb (2002). "Inflammation and cancer." Nature **420**(6917): 860-7.

Crabtree, J. E., J. I. Wyatt*, et al.* (1993). "Systemic and mucosal humoral responses to Helicobacter pylori in gastric cancer." Gut **34**(10): 1339-43.

Crean, G. P., M. W. Marshall*, et al.* (1969). "Parietal cell hyperplasia induced by the administration of pentagastrin (ICI 50,123) to rats." Gastroenterology **57**(2): 147-55.

Cross, A. J., M. F. Leitzmann*, et al.* (2007). "A prospective study of red and processed meat intake in relation to cancer risk." PLoS Med **4**(12): e325.

Cui, G., T. J. Koh*, et al.* (2004). "Overexpression of glycine-extended gastrin inhibits parietal cell loss and atrophy in the mouse stomach." Cancer Res **64**(22): 8160-6.

Curfman, G. D., S. Morrissey*, et al.* (2005). "Expression of concern: Bombardier *et al*., "Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis," N Engl J Med 2000;343:1520-8." N Engl J Med **353**(26): 2813-4.

Danesh, J. (1999). "Helicobacter pylori infection and gastric cancer: systematic review of the epidemiological studies." Aliment Pharmacol Ther **13**(7): 851-6.

Dannenberg, A. J., N. K. Altorki*, et al.* (2001). "Cyclo-oxygenase 2: a pharmacological target for the prevention of cancer." Lancet Oncol **2**(9): 544-51.

Dashwood, R. H., M. Suzui*, et al.* (1998). "High frequency of beta-catenin (ctnnb1) mutations in the colon tumors induced by two heterocyclic amines in the F344 rat." Cancer Res **58**(6): 1127-9.

De Filippo, C., G. Caderni*, et al.* (1998). "Mutations of the Apc gene in experimental colorectal carcinogenesis induced by azoxymethane in F344 rats." Br J Cancer **77**(12): 2148-51.

de la Chapelle, A. (2004). "Genetic predisposition to colorectal cancer." Nat Rev Cancer **4**(10): 769-80.

de Weerth, A., T. von Schrenck*, et al.* (1999). "Human pancreatic cancer cell lines express the CCKB receptor." Hepatogastroenterology **46**(25): 472-8.

DeVault, K. R. and D. O. Castell (2005). "Updated guidelines for the diagnosis and treatment of gastroesophageal reflux disease." Am J Gastroenterol **100**(1): 190-200.

Diaz-Rubio, E. (2004). "New chemotherapeutic advances in pancreatic, colorectal, and gastric cancers." Oncologist **9**(3): 282-94.

Ding, W. Q., S. M. Kuntz*, et al.* (2002). "A misspliced form of the cholecystokinin-B/gastrin receptor in pancreatic carcinoma: role of reduced sellular U2AF35 and a suboptimal 3'-splicing site leading to retention of the fourth intron." Cancer Res **62**(3): 947-52.

Ding, X. Z., W. G. Tong*, et al.* (2001). "Cyclooxygenases and lipoxygenases as potential targets for treatment of pancreatic cancer." Pancreatology **1**(4): 291-9.

Dockray, G. J., A. Varro*, et al.* (2001). "The gastrins: their production and biological activities." Annu Rev Physiol **63**: 119-39.

Dolcet, X., D. Llobet*, et al.* (2005). "NF-kB in development and progression of human cancer." Virchows Arch **446**(5): 475-82.

Donehower, L. A., M. Harvey*, et al.* (1992). "Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours." Nature **356**(6366): 215-21.

Dvorak, H. F. (1986). "Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing." N Engl J Med **315**(26): 1650-9.

Eaton, K. A., C. L. Brooks*, et al.* (1991). "Essential role of urease in pathogenesis of gastritis induced by Helicobacter pylori in gnotobiotic piglets." Infect Immun **59**(7): 2470-5.

Eckelman, B. P., G. S. Salvesen*, et al.* (2006). "Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family." EMBO Rep **7**(10): 988-94.

Elmore, S. (2007). "Apoptosis: A review of programmed cell death." Toxicol. Pathol.**34**(4): 495-516.

Endoh, Y., K. Sakata*, et al.* (2000). "Cellular phenotypes of differentiated-type adenocarcinomas and precancerous lesions of the stomach are dependent on the genetic pathways." J Pathol **191**(3): 257-63.

Evans, J. D., P. A. Cornford*, et al.* (2001). "Detailed tissue expression of bcl-2, bax, bak and bcl-x in the normal human pancreas and in chronic pancreatitis, ampullary and pancreatic ductal adenocarcinomas." Pancreatology **1**(3): 254-62.

Fazeli, A., R. G. Steen*, et al.* (1997). "Effects of p53 mutations on apoptosis in mouse intestinal and human colonic adenomas." Proc Natl Acad Sci U S A **94**(19): 10199-204.

Fearnhead, N. S., J. L. Wilding*, et al.* (2002). "Genetics of colorectal cancer: hereditary aspects and overview of colorectal tumorigenesis." Br Med Bull **64**: 27-43.

Fearon, E. R., S. R. Hamilton*, et al.* (1987). "Clonal analysis of human colorectal tumors." Science **238**(4824): 193-7.

Fearon, E. R. and B. Vogelstein (1990). "A genetic model for colorectal tumorigenesis." Cell **61**(5): 759-67.

Ferrara, N., H. P. Gerber*, et al.* (2003). "The biology of VEGF and its receptors." Nat Med **9**(6): 669-76.

Figueiredo, C., J. C. Machado*, et al.* (2005). "Pathogenesis of Helicobacter pylori Infection." Helicobacter **10 Suppl 1**: 14-20.

Fiocca, R., L. Villani*, et al.* (1987). "Characterization of four main cell types in gastric cancer: foveolar, mucopeptic, intestinal columnar and goblet cells. An histopathologic, histochemical and ultrastructural study of "early" and "advanced" tumours." Pathol Res Pract **182**(3): 308-25.

Folkman, J., (1990). "What is the evidence tumours are angiogenesis dependent?" J. Nat. Can. Inst **82**(1): 4-6.

Fox, J. G., A. B. Rogers, *et al.* (2003). "Helicobacter pylori-associated gastric cancer in INS-GAS mice is gender specific." Cancer Res **63**(5): 942-50.

Fox, J. G., T. C. Wang, *et al.* (2003). "Host and microbial constituents influence Helicobacter pylori-induced cancer in a murine model of hypergastrinemia." Gastroenterology **124**(7): 1879-90.

Fulda, S. and K. M. Debatin (2006). "Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy." Oncogene **25**(34): 4798-811.

Geboes, K. and P. Van Eyken (2000). "The diagnosis of dysplasia and malignancy in Barrett's oesophagus." Histopathology **37**(2): 99-107.

Gerdes, J., H. Lemke, *et al.* (1984). "Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67." J Immunol **133**(4): 1710-5.

Gerson, L. B. and G. Triadafilopoulos (2002). "Screening for esophageal adenocarcinoma: an evidence-based approach." Am J Med **113**(6): 499-505.

Ghaneh, P., E. Costello, *et al.* (2007). "Biology and management of pancreatic cancer." Gut **56**(8): 1134-52.

Gilliam, A. D. and S. A. Watson (2007). "G17DT: an antigastrin immunogen for the treatment of gastrointestinal malignancy." Expert Opin Biol Ther **7**(3): 397-404.

Gilliam, A. D., S. A. Watson, *et al.* (2004). "A phase II study of G17DT in gastric carcinoma." Eur J Surg Oncol **30**(5): 536-43.

Gleissner, B., R. Schlenk, *et al.* (2007). "Gemtuzumab ozogamicin (mylotarg) for the treatment of acute myeloid leukemia--ongoing trials." Onkologie **30**(12): 657-62.

Goetze, J. P., F. C. Nielsen, *et al.* (2000). "Closing the gastrin loop in pancreatic carcinoma: coexpression of gastrin and its receptor in solid human pancreatic adenocarcinoma." Cancer **88**(11): 2487-94.

Gonzalez, C. A., P. Jakszyn, *et al.* (2006). "Meat intake and risk of stomach and esophageal adenocarcinoma within the European Prospective Investigation Into Cancer and Nutrition (EPIC)." J Natl Cancer Inst **98**(5): 345-54.

Gonzalez, G. and A. Lage (2007). "Cancer vaccines for hormone/growth factor immune deprivation: a feasible approach for cancer treatment." Curr Cancer Drug Targets **7**(3): 229-41.

Goodwin, C. S. and J. A. Armstrong (1990). "Microbiological aspects of Helicobacter pylori (Campylobacter pylori)." Eur J Clin Microbiol Infect Dis **9**(1): 1-13.

Grabowska, A. M., J. Hughes, *et al.* (2007). "Use of interfering RNA to investigate the role of endogenous gastrin in the survival of gastrointestinal cancer cells." Br J Cancer **96**(3): 464-73.

Green, D. R. and J. C. Reed (1998). "Mitochondria and apoptosis." Science **281**(5381): 1309-12.

Griffin, M. and E. C. Sweeney (1987). "The relationship of endocrine cells, dysplasia and carcinoembryonic antigen in Barrett's mucosa to adenocarcinoma of the oesophagus." Histopathology **11**(1): 53-62.

Groden, J., A. Thliveris*, et al.* (1991). "Identification and characterization of the familial adenomatous polyposis coli gene." Cell **66**(3): 589-600.

Guo, Y. S., J. Z. Cheng*, et al.* (2002). "Gastrin stimulates cyclooxygenase-2 expression in intestinal epithelial cells through multiple signaling pathways. Evidence for involvement of ERK5 kinase and transactivation of the epidermal growth factor receptor." J Biol Chem **277**(50): 48755-63.

Hahn, W. a. W., RA. (2002). http://www.nature.com/nrc/posters/subpathways/index.html downloaded 1.5.09.

Hahn, W. C. and R. A. Weinberg (2002). "Rules for making human tumor cells." N Engl J Med **347**(20): 1593-603.

Haigh, C. R., S. E. Attwood*, et al.* (2003). "Gastrin induces proliferation in Barrett's metaplasia through activation of the CCK2 receptor." Gastroenterology **124**(3): 615-25.

Hamamoto, Y., T. Endo*, et al.* (2004). "Usefulness of narrow-band imaging endoscopy for diagnosis of Barrett's esophagus." J Gastroenterol **39**(1): 14-20.

Hanahan D. and J. Folkman (1996). "Patterns and emmerging mechanisms of the angiogenic switch during tumorigenesis." Cell **86**: 353-364.

Harris, J. C., P. A. Clarke*, et al.* (2004). "An antiapoptotic role for gastrin and the gastrin/CCK-2 receptor in Barrett's esophagus." Cancer Res **64**(6): 1915-9.

Harris, J. C., Clarke, P. & Watson, S. A. (2005). "Gastrin mediated XIAP up-regulation and associated cisplatin resistance in the pre-malignant and malignant oesophagus." Gastroenterology **128**(Suppl.3 A42).

Hashizume H., P. Bulk, *et al*. (2000). "Openings between defective endothelial cells explains tumour vessel leakiness." Am. J. Path. **156**(4): 1363-1380.

Heissmeyer, V., D. Krappmann*, et al.* (1999). "NF-kappaB p105 is a target of IkappaB kinases and controls signal induction of Bcl-3-p50 complexes." Embo J **18**(17): 4766-78.

Hellmich, M. R., X. L. Rui*, et al.* (2000). "Human colorectal cancers express a constitutively active cholecystokinin-B/gastrin receptor that stimulates cell growth." J Biol Chem **275**(41): 32122-8.

Henwood, M., P. A. Clarke*, et al.* (2001). "Expression of gastrin in developing gastric adenocarcinoma." Br J Surg **88**(4): 564-8.

Hill, D. R., N. J. Campbell*, et al.* (1987). "Autoradiographic localization and biochemical characterization of peripheral type CCK receptors in rat CNS using highly selective nonpeptide CCK antagonists." J Neurosci **7**(9): 2967-76.

Hirayama, F., S. Takagi*, et al.* (1996). "Establishment of gastric Helicobacter pylori infection in Mongolian gerbils." J Gastroenterol **31 Suppl 9**: 24-8.

Hirota, W. K., T. M. Loughney*, et al.* (1999). "Specialized intestinal metaplasia, dysplasia, and cancer of the esophagus and esophagogastric junction: prevalence and clinical data." Gastroenterology **116**(2): 277-85.

Hobbs S. K., Monsky W. L., *et al.* (1998). Regulation of transport pathways in tumour vessels; Role of tumour type and microenvironment." Proc. Nat. Acad. Sci. **95**(8): 4607-4612.

Hockenbery, D., G. Nunez*, et al.* (1990). "Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death." Nature **348**(6299): 334-6.

Holmqvist, K., M. J. Cross*, et al.* (2004). "The adaptor protein shb binds to tyrosine 1175 in vascular endothelial growth factor (VEGF) receptor-2 and regulates VEGF-dependent cellular migration." J Biol Chem **279**(21): 22267-75.

Holroyde, C. P., C. L. Skutches*, et al.* (1984). "Glucose metabolism in cachectic patients with colorectal cancer." Cancer Res **44**(12 Pt 1): 5910-3.

Houghton, J., C. Stoicov*, et al.* (2004). "Gastric cancer originating from bone marrow-derived cells." Science **306**(5701): 1568-71.

Howell, G. M., B. L. Ziober*, et al.* (1995). "Regulation of autocrine gastrin expression by the TGF alpha autocrine loop." J Cell Physiol **162**(2): 256-65.

Howes, N., M. M. Lerch*, et al.* (2004). "Clinical and genetic characteristics of hereditary pancreatitis in Europe." Clin Gastroenterol Hepatol **2**(3): 252-61.

Hsu, S. J., A. Patel*, et al.* (2008). "Development of XPA067.06, a potent high affinity human anti-gastrin monoclonal antibody." Biochem Pharmacol **76**(3): 340-52.

Huang, H., N. Ansorge*, et al.* (2007). "The CCK-2/gastrin splice variant receptor retaining intron 4 transactivates the COX-2 promoter in vitro." Regul Pept **144**(1-3): 34-42.

Inberg, M., P. Lauren*, et al.* (1965). "Factors influencing survival after radical operation for gastric cancer." J Int Coll Surg **44**(6): 682-6.

Isomoto, H., Y. Nishi*, et al.* (2007). "Immune and Inflammatory Responses in GERD and Lansoprazole." J Clin Biochem Nutr **41**(2): 84-91.

Iwase, K., B. M. Evers*, et al.* (1997). "Regulation of growth of human gastric cancer by gastrin and glycine-extended progastrin." Gastroenterology **113**(3): 782-90.

Jakubowska, A., K. Nej*, et al.* (2002). "BRCA2 gene mutations in families with aggregations of breast and stomach cancers." Br J Cancer **87**(8): 888-91.

Jankowski, J. (1993). "Gene expression in Barrett's mucosa: acute and chronic adaptive responses in the oesophagus." Gut **34**(12): 1649-50.

Jankowski, J. A., N. A. Wright*, et al.* (1999). "Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus." Am J Pathol **154**(4): 965-73.

Jemal, A., R. Siegel*, et al.* (2006). "Cancer statistics, 2006." CA Cancer J Clin **56**(2): 106-30.

Jiang, X. H. and B. C. Wong (2003). "Cyclooxygenase-2 inhibition and gastric cancer." Curr Pharm Des **9**(27): 2281-8.

Jones, D. M., A. Curry*, et al.* (1985). "An ultrastructural study of the gastric campylobacter-like organism 'Campylobacter pyloridis'." J Gen Microbiol **131**(9): 2335-41.

Josenhans, C., A. Labigne*, et al.* (1995). "Comparative ultrastructural and functional studies of Helicobacter pylori and Helicobacter mustelae flagellin mutants: both flagellin subunits, FlaA and FlaB, are necessary for full motility in Helicobacter species." J Bacteriol **177**(11): 3010-20.

Kakiuchi, H., T. Ushijima*, et al.* (1993). "Rare frequency of activation of the Ki-ras gene in rat colon tumors induced by heterocyclic amines: possible alternative mechanisms of human colon carcinogenesis." Mol Carcinog **8**(1): 44-8.

Karikari, C. A., I. Roy*, et al.* (2007). "Targeting the apoptotic machinery in pancreatic cancers using small-molecule antagonists of the X-linked inhibitor of apoptosis protein." Mol Cancer Ther **6**(3): 957-66.

Karlseder, J., D. Broccoli*, et al.* (1999). "p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2." Science **283**(5406): 1321-5.

Karpeh M., K. D., Tepper J (2001). "Cancer of the Stomach." Cancer: Principles and Practice of Oncology: 1092-1126.

Kawasaki, D., Y. Emori*, et al.* (2008). "Effect of Z-360, a novel orally active CCK-2/gastrin receptor antagonist on tumor growth in human pancreatic adenocarcinoma cell lines in vivo and mode of action determinations in vitro." Cancer Chemother Pharmacol **61**(5): 883-92.

Kerbel R. S., (2000). "Tumour angiogenesis past, present and future." Carcinogenesis **21**: 505-515.

Kluger, H. M., M. M. McCarthy*, et al.* (2007). "The X-linked inhibitor of apoptosis protein (XIAP) is up-regulated in metastatic melanoma, and XIAP cleavage by Phenoxodiol is associated with Carboplatin sensitization." J Transl Med **5**: 6.

Koh, T. J., C. J. Bulitta*, et al.* (2000). "Gastrin is a target of the beta-catenin/TCF-4 growth-signaling pathway in a model of intestinal polyposis." J Clin Invest **106**(4): 533-9.

Koh, T. J., G. J. Dockray*, et al.* (1999). "Overexpression of glycine-extended gastrin in transgenic mice results in increased colonic proliferation." J Clin Invest **103**(8): 1119-26.

Kolch, W. (2000). "Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions." Biochem J **351 Pt 2**: 289-305.

Konturek, P. C., W. Bielanski*, et al.* (2002). "Progastrin and cyclooxygenase-2 in colorectal cancer." Dig Dis Sci **47**(9): 1984-91.

Konturek, P. C., J. Kania*, et al.* (2003). "Influence of gastrin on the expression of cyclooxygenase-2, hepatocyte growth factor and apoptosis-related proteins in gastric epithelial cells." J Physiol Pharmacol **54**(1): 17-32.

Konturek, P. C., K. Rembiasz*, et al.* (2006). "Effects of cyclooxygenase-2 inhibition on serum and tumor gastrins and expression of apoptosis-related proteins in colorectal cancer." Dig Dis Sci **51**(4): 779-87.

Kovacs, T. O., J. H. Walsh*, et al.* (1989). "Gastrin is a major mediator of the gastric phase of acid secretion in dogs: proof by monoclonal antibody neutralization." Gastroenterology **97**(6): 1406-13.

Kowalski-Chauvel, A., L. Pradayrol*, et al.* (1997). "Tyrosine phosphorylation of insulin receptor substrate-1 and activation of the PI-3-kinase pathway by glycine-extended gastrin precursors." Biochem Biophys Res Commun **236**(3): 687-92.

Krasner N. A, A. W. (2005). "A. Management of non-dysplastic columnar-lined oesophagus." BSG guidelines in Gastroenterology.

Kuipers, E. J., G. I. Perez-Perez*, et al.* (1995). "Helicobacter pylori and atrophic gastritis: importance of the cagA status." J Natl Cancer Inst **87**(23): 1777-80.

Kumagai, H., K. Mukaisho*, et al.* (2003). "Cell kinetic study on histogenesis of Barrett's esophagus using rat reflux model." Scand J Gastroenterol **38**(7): 687-92.

Kune, G. A., S. Kune*, et al.* (1988). "Colorectal cancer risk, chronic illnesses, operations, and medications: case control results from the Melbourne Colorectal Cancer Study." Cancer Res **48**(15): 4399-404.

Kushima, R., W. Muller*, et al.* (1996). "Differential p53 protein expression in stomach adenomas of gastric and intestinal phenotypes: possible sequences of p53 alteration in stomach carcinogenesis." Virchows Arch **428**(4-5): 223-7.

Kusters, J. G., A. H. van Vliet*, et al.* (2006). "Pathogenesis of Helicobacter pylori infection." Clin Microbiol Rev **19**(3): 449-90.

Lagergren, J., R. Bergstrom*, et al.* (1999). "Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma." N Engl J Med **340**(11): 825-31.

Lanas, A. (2005). "Potent gastric acid inhibition in the management of Barrett's oesophagus." Drugs **65 Suppl 1**: 75-82.

Lane, D. P. (1992). "Cancer. p53, guardian of the genome." Nature **358**(6381): 15-6.

Lauren, P. (1965). "The Two Histological Main Types Of Gastric Carcinoma: Diffuse And So-Called Intestinal-Type Carcinoma. An Attempt At A Histo-Clinical Classification." Acta Pathol Microbiol Scand **64**: 31-49.

Layke, J. C. and P. P. Lopez (2004). "Gastric cancer: diagnosis and treatment options." Am Fam Physician **69**(5): 1133-40.

Lessey, B. A., I. Yeh*, et al.* (1996). "Endometrial progesterone receptors and markers of uterine receptivity in the window of implantation." Fertil Steril **65**(3): 477-83.

Leung, W. K., E. K. Ng*, et al.* (2006). "Effects of long-term rofecoxib on gastric intestinal metaplasia: results of a randomized controlled trial." Clin Cancer Res **12**(15): 4766-72.

Levi, F., F. Lucchini*, et al.* (2004). "Monitoring falls in gastric cancer mortality in Europe." Ann Oncol **15**(2): 338-45.

Levi, S., K. Beardshall*, et al.* (1989). "Campylobacter pylori and duodenal ulcers: the gastrin link." Lancet **1**(8648): 1167-8.

Li, P., D. Nijhawan*, et al.* (1997). "Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade." Cell **91**(4): 479-89.

Liang, Y., R. A. Brekken*, et al.* (2006). "Vascular endothelial growth factor induces proliferation of breast cancer cells and inhibits the anti-proliferative activity of anti-hormones." Endocr Relat Cancer **13**(3): 905-19.

Liang, Y. and S. M. Hyder (2005). "Proliferation of endothelial and tumor epithelial cells by progestin-induced vascular endothelial growth factor from human breast cancer cells: paracrine and autocrine effects." Endocrinology **146**(8): 3632-41.

Lin, Y. C., K. Brown*, et al.* (1995). "Activation of NF-kappa B requires proteolysis of the inhibitor I kappa B-alpha: signal-induced phosphorylation of I kappa B-alpha alone does not release active NF-kappa B." Proc Natl Acad Sci U S A **92**(2): 552-6.

Louis, H. (2007). "Reflux disease and Barrett's esophagus." Endoscopy **39**(11): 969-73.

Lowe, S. W. and A. W. Lin (2000). "Apoptosis in cancer." Carcinogenesis **21**(3): 485-95.

Lund, T., A. H. Geurts van Kessel*, et al.* (1986). "The genes for human gastrin and cholecystokinin are located on different chromosomes." Hum Genet **73**(1): 77-80.

Luo, J., B. D. Manning*, et al.* (2003). "Targeting the PI3K-Akt pathway in human cancer: rationale and promise." Cancer Cell **4**(4): 257-62.

MacFarlane, A. J. and P. J. Stover (2007). "Convergence of genetic, nutritional and inflammatory factors in gastrointestinal cancers." Nutr Rev **65**(12 Pt 2): S157-66.

Magistrelli, P., R. Coppola*, et al.* (2006). "Apoptotic index or a combination of Bax/Bcl-2 expression correlate with survival after resection of pancreatic adenocarcinoma." J Cell Biochem **97**(1): 98-108.

Makino, H., T. Ushijima*, et al.* (1994). "Absence of p53 mutations in rat colon tumors induced by 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole, 2-amino-3-methylimidazo[4,5-f]quinoline, or 2-amino-1-methyl-6-phenylimidazo]4,5-b]pyridine." Jpn J Cancer Res **85**(5): 510-4.

Malka, D., P. Hammel*, et al.* (2002). "Risk of pancreatic adenocarcinoma in chronic pancreatitis." Gut **51**(6): 849-52.

Mantovani, A., F. Bussolino*, et al.* (1992). "Cytokine regulation of endothelial cell function." Faseb J **6**(8): 2591-9.

Mantovani, A., S. Sozzani*, et al.* (2002). "Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes." Trends Immunol **23**(11): 549-55.

Mao, J. D., P. Wu*, et al.* (2005). "Correlation between expression of gastrin, somatostatin and cell apoptosis regulation gene bcl-2/bax in large intestine carcinoma." World J Gastroenterol **11**(5): 721-5.

Mao, X. Y., X. G. Wang*, et al.* (2007). "COX-2 expression in gastric cancer and its relationship with angiogenesis using tissue microarray." World J Gastroenterol **13**(25): 3466-71.

Marnett, L. J. (1992). "Aspirin and the potential role of prostaglandins in colon cancer." Cancer Res **52**(20): 5575-89.

Marshall, B. J. and J. R. Warren (1984). "Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration." Lancet **1**(8390): 1311-5.

Martin, S. J. and D. R. Green (1995). "Protease activation during apoptosis: death by a thousand cuts?" Cell **82**(3): 349-52.

Masood, R., J. Cai*, et al.* (2001). "Vascular endothelial growth factor (VEGF) is an autocrine growth factor for VEGF receptor-positive human tumors." Blood **98**(6): 1904-13.

McCurrach, M. E., T. M. Connor*, et al.* (1997). "bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis." Proc Natl Acad Sci U S A **94**(6): 2345-9.

McDonnell, T. J., N. Deane*, et al.* (1989). "bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation." Cell **57**(1): 79-88.

McWilliams, D. F., S. A. Watson*, et al.* (1998). "Coexpression of gastrin and gastrin receptors (CCK-B and delta CCK-B) in gastrointestinal tumour cell lines." Gut **42**(6): 795-8.

Mehta, V. B. and G. E. Besner (2007). "HB-EGF promotes angiogenesis in endothelial cells via PI3-kinase and MAPK signaling pathways." Growth Factors **25**(4): 253-63.

Merchant, J. L., B. Demediuk*, et al.* (1991). "A GC-rich element confers epidermal growth factor responsiveness to transcription from the gastrin promoter." Mol Cell Biol **11**(5): 2686-96.

Merwin, R. M. and G. H. Algire (1956). "The role of graft and host vessels in the vascularization of grafts of normal and neoplastic tissue." J Natl Cancer Inst **17**(1): 23-33.

Mitka, M. (2008). "Colorectal cancer screening rates still fall far short of recommended levels." Jama **299**(6): 622.

Miyake, A. (1995). "A truncated isoform of human CCK-B/gastrin receptor generated by alternative usage of a novel exon." Biochem Biophys Res Commun **208**(1): 230-7.

Molina, M. A., M. Sitja-Arnau, *et al.* (1999). "Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs." Cancer Res **59**(17): 4356-62.

Moore, T. C., L. I. Jepeal, *et al.* (2004). "Gastrin stimulates receptor-mediated proliferation of human esophageal adenocarcinoma cells." Regul Pept **120**(1-3): 195-203.

Mori, T., R. Doi, *et al.* (2007). "Effect of the XIAP inhibitor Embelin on TRAIL-induced apoptosis of pancreatic cancer cells." J Surg Res **142**(2): 281-6.

Moser, A. R., H. C. Pitot, *et al.* (1990). "A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse." Science **247**(4940): 322-4.

Muller, P., H. G. Dammann, *et al.* (1989). "Human gastric acid secretion following repeated doses of AG-1749." Aliment Pharmacol Ther **3**(2): 193-8.

Murata, H., S. Kawano, *et al.* (1999). "Cyclooxygenase-2 overexpression enhances lymphatic invasion and metastasis in human gastric carcinoma." Am J Gastroenterol **94**(2): 451-5.

Nishida, A., K. Miyata, *et al.* (1994). "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." J Pharmacol Exp Ther **269**(2): 725-31.

Noble, F. and B. P. Roques (1999). "CCK-B receptor: chemistry, molecular biology, biochemistry and pharmacology." Prog Neurobiol **58**(4): 349-79.

Ochiai, A., W. Yasui*, et al.* (1985). "Growth-promoting effect of gastrin on human gastric carcinoma cell line TMK-1." Jpn J Cancer Res **76**(11): 1064-71.

Okami, J., H. Yamamoto*, et al.* (1999). "Overexpression of cyclooxygenase-2 in carcinoma of the pancreas." Clin Cancer Res **5**(8): 2018-24.

Oliveira, C., P. Ferreira*, et al.* (2004). "E-Cadherin (CDH1) and p53 rather than SMAD4 and Caspase-10 germline mutations contribute to genetic predisposition in Portuguese gastric cancer patients." Eur J Cancer **40**(12): 1897-903.

Oliveira, C., R. Seruca*, et al.* (2003). "Genetic screening for hereditary diffuse gastric cancer." Expert Rev Mol Diagn **3**(2): 201-15.

Omura, K. (2008). "Advances in chemotherapy against advanced or metastatic colorectal cancer." Digestion **77 Suppl 1**: 13-22.

Orsini, B., B. Ottanelli*, et al.* (2003). "Helicobacter pylori cag pathogenicity island is associated with reduced expression of interleukin-4 (IL-4) mRNA and modulation of the IL-4delta2 mRNA isoform in human gastric mucosa." Infect Immun **71**(11): 6664-7.

Oshima, M., J. E. Dinchuk*, et al.* (1996). "Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2)." Cell **87**(5): 803-9.

Pajares, J. M. and J. P. Gisbert (2006). "Helicobacter pylori: its discovery and relevance for medicine." Rev Esp Enferm Dig **98**(10): 770-85.

Parkin, D. M., F. I. Bray*, et al.* (2001). "Cancer burden in the year 2000. The global picture." Eur J Cancer **37 Suppl 8**: S4-66.

Parkin, D. M. and C. S. Muir (1992). "Cancer Incidence in Five Continents. Comparability and quality of data." IARC Sci Publ(120): 45-173.

Patan S., L. L. Munn, *et al* (1996). "Intussusceptive microvascular growth in a human colon adenocarcinoma xenograft: A novel mechanism of tumour angiogenesis." Microvasc. Res. **51**: 260-272.

Pelengaris, S., M. Khan*, et al.* (2002). "c-MYC: more than just a matter of life and death." Nat Rev Cancer **2**(10): 764-76.

Pikarsky, E., R. M. Porat*, et al.* (2004). "NF-kappaB functions as a tumour promoter in inflammation-associated cancer." Nature **431**(7007): 461-6.

Potter, J. D. (1997). "food, nutrition and the prevention of cancer: A global perspective."

Powell, S. M., N. Zilz*, et al.* (1992). "APC mutations occur early during colorectal tumorigenesis." Nature **359**(6392): 235-7.

Pritchard, D. M. and S. M. Przemeck (2004). "Review article: How useful are the rodent animal models of gastric adenocarcinoma?" Aliment Pharmacol Ther **19**(8): 841-59.

Ptak-Belowska, A., M. W. Pawlik*, et al.* (2007). "Transcriptional upregulation of gastrin in response to peroxisome proliferator-activated receptor gamma agonist triggers cell survival pathways." J Physiol Pharmacol **58**(4): 793-801.

Reubi, J. C. (2003). "Peptide receptors as molecular targets for cancer diagnosis and therapy." Endocr Rev **24**(4): 389-427.

Reubi, J. C., B. Waser*, et al.* (1999). "Receptor autoradiographic evaluation of cholecystokinin, neurotensin, somatostatin and vasoactive intestinal peptide receptors in gastro-intestinal adenocarcinoma samples: where are they really located?" Int J Cancer **81**(3): 376-86.

Rmali, K. A., M. C. Puntis*, et al.* (2007). "Tumour-associated angiogenesis in human colorectal cancer." Colorectal Dis **9**(1): 3-14.

Rudi, J., C. Kolb*, et al.* (1998). "Diversity of Helicobacter pylori vacA and cagA genes and relationship to VacA and CagA protein expression, cytotoxin production, and associated diseases." J Clin Microbiol **36**(4): 944-8.

Satin, B., N. Norais*, et al.* (1997). "Effect of helicobacter pylori vacuolating toxin on maturation and extracellular release of procathepsin D and on epidermal growth factor degradation." J Biol Chem **272**(40): 25022-8.

Sawaoka, H., S. Tsuji*, et al.* (1997). "Expression of the cyclooxygenase-2 gene in gastric epithelium." J Clin Gastroenterol **25 Suppl 1**: S105-10.

Sawaoka, H., S. Tsuji*, et al.* (1999). "Cyclooxygenase inhibitors suppress angiogenesis and reduce tumor growth in vivo." Lab Invest **79**(12): 1469-77.

Schlansky, B., A. J. Dimarino, Jr.*, et al.* (2006). "A survey of oesophageal cancer: pathology, stage and clinical presentation." Aliment Pharmacol Ther **23**(5): 587-93.

Schmitz, F., M. N. Goke*, et al.* (2001). "Cellular expression of CCK-A and CCK-B/gastrin receptors in human gastric mucosa
CCK-B/gastrin receptors in human colorectal cancer." Regul Pept **102**(2-3): 101-10.

Schmitz, F., J. M. Otte*, et al.* (2001). "CCK-B/gastrin receptors in human colorectal cancer." Eur J Clin Invest **31**(9): 812-20.

Scott, D. R., E. A. Marcus*, et al.* (2002). "Mechanisms of acid resistance due to the urease system of Helicobacter pylori." Gastroenterology **123**(1): 187-95.

Segal, E. D., J. Cha*, et al.* (1999). "Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori." Proc Natl Acad Sci U S A **96**(25): 14559-64.

Seva, C., C. J. Dickinson*, et al.* (1994). "Growth-promoting effects of glycine-extended progastrin." Science **265**(5170): 410-2.

Shimizu, D., D. Vallbohmer*, et al.* (2006). "Increasing cyclooxygenase-2 (cox-2) gene expression in the progression of Barrett's esophagus to adenocarcinoma correlates with that of Bcl-2." Int J Cancer **119**(4): 765-70.

Shimpi, R. A., J. George*, et al.* (2007). "Staging of esophageal cancer by EUS: staging accuracy revisited." Gastrointest Endosc **66**(3): 475-82.

Shirvani, V. N., R. Ouatu-Lascar*, et al.* (2000). "Cyclooxygenase 2 expression in Barrett's esophagus and adenocarcinoma: Ex vivo induction by bile salts and acid exposure." Gastroenterology **118**(3): 487-96.

Shoemaker, A. R., C. Luongo*, et al.* (1997). "Somatic mutational mechanisms involved in intestinal tumor formation in Min mice." Cancer Res **57**(10): 1999-2006.

Sica, A., T. Schioppa*, et al.* (2006). "Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy." Eur J Cancer **42**(6): 717-27.

Siddheshwar, R. K., J. C. Gray*, et al.* (2001). "Plasma levels of progastrin but not amidated gastrin or glycine extended gastrin are elevated in patients with colorectal carcinoma." Gut **48**(1): 47-52.

Sieber, O., K. Heinimeinn *et al* (2005). "Genomic stability and tumorigenesis." Seminars in Cancer Biology **15**(1): 61-66.

Silverstein, F. E., G. Faich*, et al.* (2000). "Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study." Jama **284**(10): 1247-55.

Singh, P., B. Rae-Venter*, et al.* (1985). "Gastrin receptors in normal and malignant gastrointestinal mucosa: age-associated changes." Am J Physiol **249**(6 Pt 1): G761-9.

Sinicrope, F. A., D. B. Evans*, et al.* (1996). "bcl-2 and p53 expression in resectable pancreatic adenocarcinomas: association with clinical outcome." Clin Cancer Res **2**(12): 2015-22.

Sinicrope, F. A. and S. Gill (2004). "Role of cyclooxygenase-2 in colorectal cancer." Cancer Metastasis Rev **23**(1-2): 63-75.

Sipponen, P., M. Kekki*, et al.* (1985). "Gastric cancer risk in chronic atrophic gastritis: statistical calculations of cross-sectional data." Int J Cancer **35**(2): 173-7.

Smith, A. M., T. Justin*, et al.* (2000). "Phase I/II study of G17-DT, an anti-gastrin immunogen, in advanced colorectal cancer." Clin Cancer Res **6**(12): 4719-24.

Smith, A. M. and S. A. Watson (2000). "Review article: gastrin and colorectal cancer." Aliment Pharmacol Ther **14**(10): 1231-47.

Smith, J. P., G. Liu, *et al.* (1994). "Identification and characterization of CCK-B/gastrin receptors in human pancreatic cancer cell lines." Am J Physiol **266**(1 Pt 2): R277-83.

Smith, J. P., C. A. Rickabaugh, *et al.* (1993). "Cholecystokinin receptors and PANC-1 human pancreatic cancer cells." Am J Physiol **265**(1 Pt 1): G149-55.

Smith, J. P., A. Shih, *et al.* (1996). "Gastrin regulates growth of human pancreatic cancer in a tonic and autocrine fashion." Am J Physiol **270**(5 Pt 2): R1078-84.

Smith, M. L., G. Hawcroft, *et al.* (2000). "The effect of non-steroidal anti-inflammatory drugs on human colorectal cancer cells: evidence of different mechanisms of action." Eur J Cancer **36**(5): 664-74.

Smoot, D. T., J. H. Resau, *et al.* (1996). "Effects of Helicobacter pylori vacuolating cytotoxin on primary cultures of human gastric epithelial cells." Gut **39**(96): 795-9.

Solcia, E., R. Fiocca, *et al.* (1996). "Intestinal and diffuse gastric cancers arise in a different background of Helicobacter pylori gastritis through different gene involvement." Am J Surg Pathol **20 Suppl 1**: S8-22.

Solomon, S. D., J. J. McMurray, *et al.* (2005). "Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention." N Engl J Med **352**(11): 1071-80.

Song, G., G. Ouyang*, et al.* (2005). "The activation of Akt/PKB signaling pathway and cell survival." J Cell Mol Med **9**(1): 59-71.

Song, I., D. R. Brown*, et al.* (1993). "The human gastrin/cholecystokinin type B receptor gene: alternative splice donor site in exon 4 generates two variant mRNAs." Proc Natl Acad Sci U S A **90**(19): 9085-9.

Souza, R. F., K. Shewmake*, et al.* (2002). "Acid exposure activates the mitogen-activated protein kinase pathways in Barrett's esophagus." Gastroenterology **122**(2): 299-307.

Spechler, S. J. and R. K. Goyal (1986). "Barrett's esophagus." N Engl J Med **315**(6): 362-71.

Spitzer, M. (2007). "Screening and management of women and girls with human papillomavirus infection." Gynecol Oncol **107**(2 Suppl): S14-8.

Stephens, M. R., A. N. Hopper*, et al.* (2007). "Prognostic significance of gastrin expression in patients undergoing R0 gastrectomy for adenocarcinoma." Gastric Cancer **10**(3): 159-66.

Strand, V. (2007). "Are COX-2 inhibitors preferable to non-selective non-steroidal anti-inflammatory drugs in patients with risk of cardiovascular events taking low-dose aspirin?" Lancet **370**(9605): 2138-51.

Strasser, A., A. W. Harris*, et al.* (1990). "Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2." Nature **348**(6299): 331-3.

Strobel, S., S. Bereswill*, et al.* (1998). "Identification and analysis of a new vacA genotype variant of Helicobacter pylori in different patient groups in Germany." J Clin Microbiol **36**(5): 1285-9.

Su, L. K., K. W. Kinzler*, et al.* (1992). "Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene." Science **256**(5057): 668-70.

Sun, W. H., S. Tsuji*, et al.* (2000). "Cyclo-oxygenase-2 inhibitors suppress epithelial cell kinetics and delay gastric wound healing in rats." J Gastroenterol Hepatol **15**(7): 752-61.

Sun, W. H., F. Zhu*, et al.* (2008). "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.

Suzuki, H., M. Mori*, et al.* (1997). "Extensive DNA damage induced by monochloramine in gastric cells." Cancer Lett **115**(2): 243-8.

Suzuki, H., K. Seto*, et al.* (1998). "Monochloramine induced DNA fragmentation in gastric cell line MKN45." Am J Physiol **275**(4 Pt 1): G712-6.

Syrigos, K. N., A. Zalonis*, et al.* (2008). "Targeted therapy for oesophageal cancer: an overview." Cancer Metastasis Rev.

Szabo, I., G. Rumi*, et al.* (2000). "Gastrin and pentagastrin enhance the tumour proliferation of human stable cultured gastric adenocarcinoma cells." J Physiol Paris **94**(1): 71-4.

Takahashi, M., F. Furukawa*, et al.* (1990). "Effects of various prostaglandin synthesis inhibitors on pancreatic carcinogenesis in hamsters after initiation with N-nitrosobis(2-oxopropyl)amine." Carcinogenesis **11**(3): 393-5.

Tamm, I., S. M. Kornblau*, et al.* (2000). "Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias." Clin Cancer Res **6**(5): 1796-803.

Tang, C., I. Biemond*, et al.* (1997). "Expression of receptors for gut peptides in human pancreatic adenocarcinoma and tumour-free pancreas." Br J Cancer **75**(10): 1467-73.

Tatematsu, M., M. Ichinose*, et al.* (1990). "Gastric and intestinal phenotypic expression of human stomach cancers as revealed by pepsinogen immunohistochemistry and mucin histochemistry." Acta Pathol Jpn **40**(7): 494-504.

Tatematsu, M., T. Tsukamoto*, et al.* (2003). "Stem cells and gastric cancer: role of gastric and intestinal mixed intestinal metaplasia." Cancer Sci **94**(2): 135-41.

Thomas Boenisch, F. A. J., Stead R. H, Key M, Welcher R, Harvey R, Atwood K. N. (2001). Handbook Immunochemical staining methods 3rd Edition, DakoCytomation, California.

Thompson, L. J., S. J. Danon*, et al.* (2004). "Chronic Helicobacter pylori infection with Sydney strain 1 and a newly identified mouse-adapted strain (Sydney strain 2000) in C57BL/6 and BALB/c mice." Infect Immun **72**(8): 4668-79.

Thornberry, N. A. and Y. Lazebnik (1998). "Caspases: enemies within." Science **281**(5381): 1312-6.

Todd, R. and D. T. Wong (1999). "Oncogenes." Anticancer Res **19**(6A): 4729-46.

Todisco, A., S. Ramamoorthy*, et al.* (2001). "Molecular mechanisms for the antiapoptotic action of gastrin." Am J Physiol Gastrointest Liver Physiol **280**(2): G298-307.

Todisco, A., Y. Takeuchi*, et al.* (1995). "Gastrin and glycine-extended progastrin processing intermediates induce different programs of early gene activation." J Biol Chem **270**(47): 28337-41.

Tomb, J. F., O. White*, et al.* (1997). "The complete genome sequence of the gastric pathogen Helicobacter pylori." Nature **388**(6642): 539-47.

Tran, J., J. Rak*, et al.* (1999). "Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells." Biochem Biophys Res Commun **264**(3): 781-8.

Triadafilopoulos, G. (2001). "Acid and bile reflux in Barrett's esophagus: a tale of two evils." Gastroenterology **121**(6): 1502-6.

Tsuji, S., S. Kawano*, et al.* (1996). "Evidences for involvement of cyclooxygenase-2 in proliferation of two gastrointestinal cancer cell lines." Prostaglandins Leukot Essent Fatty Acids **55**(3): 179-83.

Tsujimoto, Y., L. R. Finger*, et al.* (1984). "Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation." Science **226**(4678): 1097-9.

Tsung, K., J. P. Dolan*, et al.* (2002). "Macrophages as effector cells in interleukin 12-induced T cell-dependent tumor rejection." Cancer Res **62**(17): 5069-75.

Uchino, S., M. Noguchi*, et al.* (1993). "p53 mutation in gastric cancer: a genetic model for carcinogenesis is common to gastric and colorectal cancer." Int J Cancer **54**(5): 759-64.

Ulaner, G. A., P. M. Colletti*, et al.* (2008). "B-Cell Non-Hodgkin Lymphoma: PET/CT Evaluation after 90Y-Ibritumomab Tiuxetan Radioimmunotherapy--Initial Experience." Radiology.

van Doorn, L. J., C. Figueiredo*, et al.* (1998). "Clinical relevance of the cagA, vacA, and iceA status of Helicobacter pylori." Gastroenterology **115**(1): 58-66.

Van Nieuwenhove, Y., D. Chen*, et al.* (2001). "Postprandial cell proliferation in the esophageal epithelium of rats." Regul Pept **97**(2-3): 131-7.

Van Nieuwenhove, Y., T. De Backer*, et al.* (1998). "Gastrin stimulates epithelial cell proliferation in the oesophagus of rats." Virchows Arch **432**(4): 371-5.

Vane, J. R. and R. M. Botting (1998). "Mechanism of action of nonsteroidal anti-inflammatory drugs." Am J Med **104**(3A): 2S-8S; discussion 21S-22S.

Viala, J., C. Chaput*, et al.* (2004). "Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island." Nat Immunol **5**(11): 1166-74.

Wahl, L. M. and H. K. Kleinman (1998). "Tumor-associated macrophages as targets for cancer therapy." J Natl Cancer Inst **90**(21): 1583-4.

Walczak, H. and P. H. Krammer (2000). "The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems." Exp Cell Res **256**(1): 58-66.

Waldum, H. L., A. K. Sandvik*, et al.* (1991). "Gastrin-histamine sequence in the regulation of gastric acid secretion." Gut **32**(6): 698-701.

Walter, P. and V. R. Lingappa (1986). "Mechanism of protein translocation across the endoplasmic reticulum membrane." Annu Rev Cell Biol **2**: 499-516.

Wang, P. H., J. L. Ko*, et al.* (2008). "Clinical significance of matrix metalloproteinase-2 in cancer of uterine cervix: A semiquantitative study of immunoreactivities using tissue array." Gynecol Oncol.

Wang, T. C., S. Bonner-Weir, *et al.* (1993). "Pancreatic gastrin stimulates islet differentiation of transforming growth factor alpha-induced ductular precursor cells." J Clin Invest **92**(3): 1349-56.

Wang, T. C., C. A. Dangler, *et al.* (2000). "Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer." Gastroenterology **118**(1): 36-47.

Wang, T. C., T. J. Koh, *et al.* (1996). "Processing and proliferative effects of human progastrin in transgenic mice." J Clin Invest **98**(8): 1918-29.

Wang, W., J. L. Abbruzzese, *et al.* (1999). "Overexpression of urokinase-type plasminogen activator in pancreatic adenocarcinoma is regulated by constitutively activated RelA." Oncogene **18**(32): 4554-63.

Watanabe, H., M. Enjoji, *et al.* (1978). "Gastric lesions in familial adenomatosis coli: their incidence and histologic analysis." Hum Pathol **9**(3): 269-83.

Watanabe, T., M. Tada, *et al.* (1998). "Helicobacter pylori infection induces gastric cancer in mongolian gerbils." Gastroenterology **115**(3): 642-8.

Watkins, S. K., N. K. Egilmez, *et al.* (2007). "IL-12 rapidly alters the functional profile of tumor-associated and tumor-infiltrating macrophages in vitro and in vivo." J Immunol **178**(3): 1357-62.

Watson S.A, A. M. S., C. Parasceva, D. Michaeli, J.D. Hardcastle (1998). "Effect of gastrin neutralization on the progression of the adenoma: Carcinoma sequence in the *Min* mouse model of familial adenomatous polyposis." Gastroenterology **114**.

Watson, S. A., L. G. Durrant*, et al.* (1989). "The in vitro growth response of primary human colorectal and gastric cancer cells to gastrin." Int J Cancer **43**(4): 692-6.

Watson, S. A., L. G. Durrant*, et al.* (1991). "Intracellular gastrin in human gastrointestinal tumor cells." J Natl Cancer Inst **83**(12): 866-71.

Watson, S. A. and A. D. Gilliam (2001). "G17DT--a new weapon in the therapeutic armoury for gastrointestinal malignancy." Expert Opin Biol Ther **1**(2): 309-17.

Watson, S. A., D. Michaeli*, et al.* (1996). "Gastrimmune raises antibodies that neutralize amidated and glycine-extended gastrin-17 and inhibit the growth of colon cancer." Cancer Res **56**(4): 880-5.

Watson, S. A., D. Michaeli*, et al.* (1999). "A comparison of an anti-gastrin antibody and cytotoxic drugs in the therapy of human gastric ascites in SCID mice." Int J Cancer **81**(2): 248-54.

Watson, S. A., T. M. Morris*, et al.* (2002). "Potential role of endocrine gastrin in the colonic adenoma carcinoma sequence." Br J Cancer **87**(5): 567-73.

Watson, S. A. and A. M. Smith (2001). "Hypergastrinemia promotes adenoma progression in the APC(Min-/+) mouse model of familial adenomatous polyposis." Cancer Res **61**(2): 625-31.

Watson, S. A. and R. J. Steele (1993). "Gastrin antagonists in the treatment of gastric cancer." Anticancer Drugs **4**(6): 599-604.

Weeks, D. L., S. Eskandari*, et al.* (2000). "A H+-gated urea channel: the link between Helicobacter pylori urease and gastric colonization." Science **287**(5452): 482-5.

Weigert, N., K. Schaffer*, et al.* (1996). "Gastrin secretion from primary cultures of rabbit antral G cells: stimulation by inflammatory cytokines." Gastroenterology **110**(1): 147-54.

Weston, A. P., P. T. Krmpotich*, et al.* (1997). "Prospective long-term endoscopic and histological follow-up of short segment Barrett's esophagus: comparison with traditional long segment Barrett's esophagus." Am J Gastroenterol **92**(3): 407-13.

Wiborg, O., L. Berglund*, et al.* (1984). "Structure of a human gastrin gene." Proc Natl Acad Sci U S A **81**(4): 1067-9.

Wijnhoven, B. P., H. W. Tilanus*, et al.* (2001). "Molecular biology of Barrett's adenocarcinoma." Ann Surg **233**(3): 322-37.

Williams, C., R. L. Shattuck-Brandt*, et al.* (1999). "The role of COX-2 in intestinal cancer." Ann N Y Acad Sci **889**: 72-83.

Williams, C. S., W. Smalley*, et al.* (1997). "Aspirin use and potential mechanisms for colorectal cancer prevention." J Clin Invest **100**(6): 1325-9.

Winawer, S. J., R. H. Fletcher*, et al.* (1997). "Colorectal cancer screening: clinical guidelines and rationale." Gastroenterology **112**(2): 594-642.

Winters, C., Jr., T. J. Spurling*, et al.* (1987). "Barrett's esophagus. A prevalent, occult complication of gastroesophageal reflux disease." Gastroenterology **92**(1): 118-24.

Wu, L. B., R. Kushima*, et al.* (1998). "Intramucosal carcinomas of the stomach: phenotypic expression and loss of heterozygosity at microsatellites linked to the APC gene." Pathol Res Pract **194**(6): 405-11.

Yancopoulos. G. D., S. Davis, *et al.* (2000). "Vascular-specific growth factors and blood vessel formation." Nature **407**(6801): 242-8.

Yamamoto, Y. and R. B. Gaynor (2001). "Role of the NF-kappaB pathway in the pathogenesis of human disease states." Curr Mol Med **1**(3): 287-96.

Yang, L., Z. Cao*, et al.* (2003). "Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy." Cancer Res **63**(20): 6815-24.

Yao, M., D. H. Song*, et al.* (2002). "COX-2 selective inhibition reverses the trophic properties of gastrin in colorectal cancer." Br J Cancer **87**(5): 574-9.

Yip-Schneider, M. T., D. S. Barnard*, et al.* (2000). "Cyclooxygenase-2 expression in human pancreatic adenocarcinomas." Carcinogenesis **21**(2): 139-46.

Yokozaki, H., Y. Shitara*, et al.* (1999). "Alterations of p73 preferentially occur in gastric adenocarcinomas with foveolar epithelial phenotype." Int J Cancer **83**(2): 192-6.

Yonish-Rouach, E., D. Resnitzky*, et al.* (1991). "Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6." Nature **352**(6333): 345-7.

Zhou, J. J., M. L. Chen*, et al.* (2004). "Coexpression of cholecystokinin-B/gastrin receptor and gastrin gene in human gastric tissues and gastric cancer cell line." World J Gastroenterol **10**(6): 791-4.