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“Ghrelin, Motilin in Health and Disease”

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Abbreviations

5-HT	5-hydroxytryptamine
AChE	Acetylcholine esterase
ACTH	Adreno-corticotrophin
AgRP	Agouti related peptide
ANOVA	Analysis of variance
ASA	Aminosalicylates
AUC	Area under curve
BMI	Body mass index
BSA	Bovine serum albumin
CARD	C aspase recruitment domain
CD	Crohns's disease
cDNA	Complement deoxyribonucleic acid
CRP	C-Reactive protein
CSF	Cerebrospinal fluid
DAB	3,3'-diaminobenzidine
DMSO	Dimethyl sulfoxide
EBV	Epstein barr virus
ECL	Enterochromaffin-like cells
EDTA	Ethylenediaminetetra-acetic acid
EFS	Electrical field stimulation
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay

FBS	Foetal bovine serum
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GHRP	Growth hormone releasing peptide
GHS	Growth hormone secretagogue
GHSR	Growth hormone secretagogue receptor
GI	Gastrointestinal
HBAI	Harvey Bradshaw activity index
HOMA-IR	Homeostatic model assessment- insulin resistance
HRP	Horseradish-peroxidase
HRP	Horseradish peroxidase
IκB	Inhibitory Kappa B
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin growth factor
IHC	Immunohistochemistry
IL	Interleukin
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
NASH	Non-alcoholic steatohepatitis
NFκB	Nuclear factor kappa B
NOD	nucleotide-binding oligomerization domain
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
OB	Obesity gene

OD	Optical Density
PBS	Phosphate buffered saline
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PVDF	Polyvinylidene fluoride
RIPA	Radio-immuno precipitation assay
RPMI	Roswell Park Memorial Institute
SEM	Standard error of the mean
TEMED	Tetramethylethylenediamine
TNF	Tumour necrosis factor

Declaration

The content of this thesis is original and has never been a subject of submission for any degree at other universities. All experiments were conceptualised, designed and performed by myself under guidance of my supervisor, Professor Chuka Nwokolo, except for the following:

Sean James, histopathology technician at University Hospital Coventry who assisted and demonstrated the rudiments and procedures of immunohistochemistry.

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Summary

Ghrelin is a 28 amino-acid peptide produced predominantly by the stomach. Two main isoforms of ghrelin are currently known (octanoyl- and desoctanoyl ghrelin). It functions as a circulating orexigenic hormone. In addition, it has an effect on the nervous, cardiovascular and immune system. Current data suggest that ghrelin may have beneficial anti-inflammatory effects. Chapter 3 in this thesis primarily examines the relationship between ghrelin and inflammation in Crohn's disease (CD). Modulation of inflammation with infliximab, a powerful anti-TNF α antibody therapy, can increase total ghrelin concentration by 25%. In addition, a normal physiological post-prandial decrease in ghrelin following a meal is restored when infused with infliximab, suggesting a dysregulation of ghrelin in CD patients with active inflammation. At cellular level, there is evidence that ghrelin may have an immunosuppressive effect on activated T-lymphocytes. Chapter 4 of this thesis examines the effect of ghrelin, a manufactured agonist and des-octanoyl ghrelin on NF κ B activation on a human B-lymphocyte cell line. This study demonstrated that exposure to octanoyl ghrelin confers an initial increase of NF κ B activation in inactivated cells of up to 50% which suggests a pro-inflammatory effect. However, NF κ B activation appears to decrease at much higher concentrations of octanoyl ghrelin, which may indicate toxicity at supra-physiological levels. Ghrelin is also involved in the regulation of gastric motility and has structural similarities to motilin. Symptoms of delayed gastric emptying can occur long after cancer chemotherapy has ended. Chapter 5 of this thesis compares the contractility and pro-motility neurotransmitter expression in chemotherapy and non-chemotherapy exposed stomach tissues obtained from patients undergoing surgery for oesophago-gastric cancers. Chemotherapy exposed tissues have reduced contractility to carbachol and apparent destruction of the cholinergic activity. The tendency for ghrelin receptors to increase suggests an attempt to upregulate compensating systems. In conclusion, ghrelin can be altered by inflammation and may have beneficial effects on gastric motility.

Chapter 1

Introduction

1.1 “Starve the fever, feed the cold”: The benefit of hunger or starvation

Hunger is a natural self-preservation instinct incorporated in all living animals. As it drives food intake it preserves energy balance and ensures survival. However, it is generally accepted that unwell animals do not eat well. Hunger, in particular is severely diminished in many disease states. It is also true that in many disease states energy expenditure is usually increased, particularly in inflammatory conditions.

To preserve energy to deal with the illness, physical activity is decreased as a result of lethargy, hence the common medical advice of ‘bed rest’. It is indeed questionable why animals lose the hunger mechanism, if energy expenditure is increased. Clearly ongoing severe inflammatory process will lead to detrimental consequences to the body, in particular cachexia, in event of inadequate feeding. This is contrary to survival instinct in non-diseased animals.

Normal hunger mechanism is usually re-established once disease associated inflammatory process have resolved, usually by natural biological mechanisms or medical intervention. Crisis arises if the disease maintains a chronic inflammatory state, and if not adequately treated will lead to health decline. This eventually leads to malnutrition and vulnerability to secondary diseases e.g. Poor wound healing, infection etc. which may lead to the animal’s demise.

There may be a plausible explanation to why hunger is so severely diminished in the disease state. Could there be a physiological need for starvation during acute illness? Is there a possibility that not eating during the illness, at least in the short term may be of benefit to the animal? If so, could it be possible that hunger and/or starvation actually alter an animal's immune activity and help it to survive an illness? These questions are currently not adequately explained by current understanding of the hunger mechanisms in inflammatory related illnesses.

“Fasting is a great remedy for fever” a familiar anecdote first described by a 16th century lexicographer John Withals in 1574, from which the widely believed phrase “starve the fever, feed the cold” is thought to have originated. This has been widely dismissed as a myth and deemed unscientific. More recently, Dutch scientists have shown the feeding stimulated the release of IFN- γ which is a key signalling cytokine in defence against viral infection (van den Brink *et al.* 2002). Fasting, on the other hand appeared to increase IL-4 production, which is implicated in stimulation of B-cells and proliferation of T-cells which, in turn, may stimulate humoral defence mechanisms against extracellular organisms. Hence, the bona fide ancient idiom may have finally earned its legitimacy.

It is quite possible that the phrase may have originated from the observation that nutrient intake can beneficially alter immune response. Indeed, there is evidence that even trace nutrients e.g. Glutamine can have beneficial effects in mortality particularly in patients with critical illness (Avenell *et al.* 2009). Indeed, glutamine has been shown to prevent apoptosis, attenuates inflammation and decreases insulin

resistance (Singleton *et al.* 2008; Wischmeyer *et al.* 2008; Wischmeyer *et al.* 2007) thus may be clinically beneficial in critically unwell patients.

The mechanism of appetite loss in inflammation is poorly understood. In unwell animals with high inflammatory load, appetite and food intake is reduced, partly due to circulating cytokines e.g. TNF α directly inhibiting the hunger centre. This thesis describes a novel hunger mechanism that is uncovered when modulating inflammation in Crohn's disease, and a proposed mechanism for appetite loss in patients with high inflammatory load.

There is currently a growing interest in nutritional support for acutely ill patients. It is estimated that approximately one third of patients admitted to hospital for an acute illness are at least moderately malnourished, and early detection and recognition of these patients was poor (McWhirter *et al.* 1994). Early artificial nutritional intervention is now associated with better outcomes, including shorter hospital stay (Von Meyenfeldt *et al.* 1992; Delmi *et al.* 1990), reduction in complications, including infections (Heyland *et al.* 1998) and promotes recovery.

Such compelling evidence of nutrition support in acutely ill patients has precipitated much interest in delivering nutrition as part of overall treatment for these patients. In the UK, the National Institute of Clinical Excellence (NICE), an independent organisation responsible for providing national guidance on promoting good health, preventing and treating ill health has developed national guidance on nutrition support to improve outcomes of patients in hospital (NICE CG32, 2006). There is clinical

evidence that nutrition delivery during acute illnesses, where hunger mechanisms were severely diminished, may actually improve outcomes of these patients.

This effect may have evolved possibly from the improved overall well being of the patient after being fed, or perhaps nutrition itself has immune-modulating activity in regulating inflammatory process. It has been previously reported that different nutrients have an impact on the overall function of the immune system. In general, malnutrition per se is associated with a decrease in immune cell functions, hence their vulnerability to poor healing and tissue regeneration.

Deficiencies in nutrients in particular protein, iron, zinc and certain vitamins have an overall decrease in immune cell activity and even complement system (Shronts *et al.* 1993). When nutrient deficiencies are replenished, this would restore optimum immune function, and benefit patients in clinical outcomes with early nutritional intervention. This evidence would contradict the natural response of animals to stop eating, with loss of appetite during illnesses. Further studies are needed to determine if starvation during illnesses, particularly in the short term during acute illness, confers any benefit.

1.2 Ghrelin

1.2.1 The Origins, Structure and Genetics of Ghrelin

Ghrelin is a novel 28 amino acid peptide predominantly by the stomach .In humans, ghrelin is genetically encoded in chromosome 3 (Seim *et al.* 2007). It is highly conserved between species and structurally related to motilin in amino acid sequence. Soon after the discovery of ghrelin, another peptide isolated from the stomach by another group was described as “motilin related peptide” as it was noted to have similar structure to motilin (Tomasetto *et al.* 2000). The structure was in fact ghrelin prior to octanoylation. The association was elusive as it was not known that the N-terminus could undergo post translational modification. If ghrelin has such structural similarities, it would be expected to have functional characteristics of motilin. In animal and human studies, ghrelin is involved in the modulation of gastric and intestinal motility, including migrating motor complex, which are physiological peristaltic waves transporting stomach contents distally in the GI tract (Kitazawa *et al.* 2005; Tack *et al.* 2006).

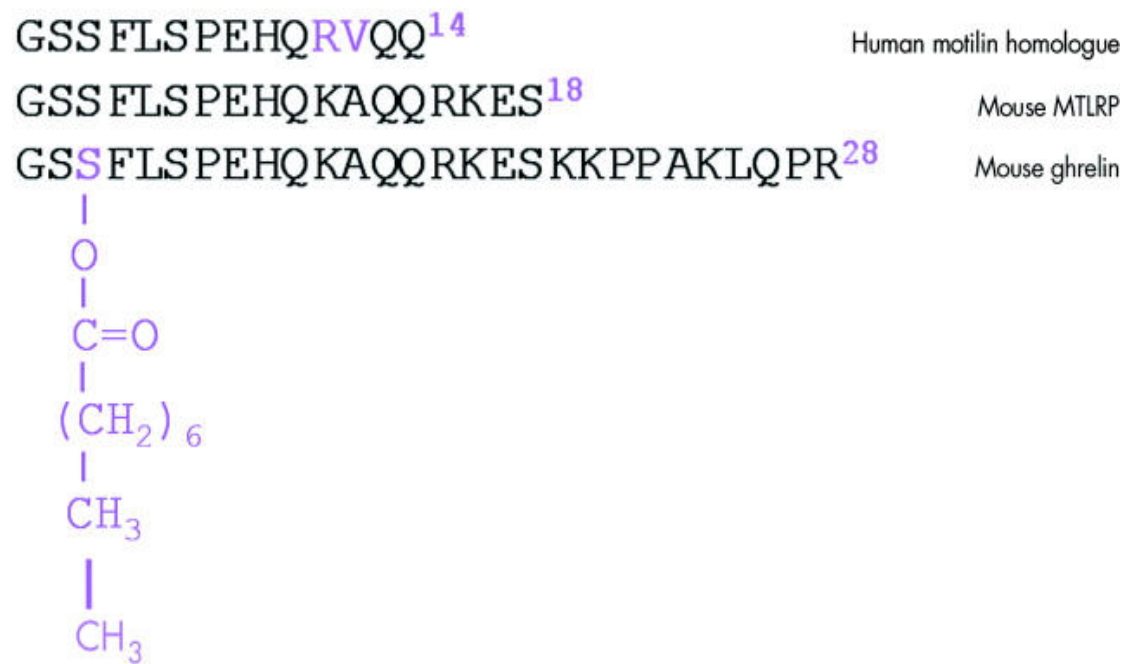


Figure 1.1 The amino acid sequence of mouse ghrelin (adapted from Peeters TL, Gut 2005;54:1638-1649). This illustrates the striking similarities in the sequence of rat and human motilin, differing in only 2 amino acid residues, which is probably due to differences between species.

Studies have shown that ghrelin is decreased following gastrectomy and the stomach produces approximately two thirds of circulating ghrelin (Ariyasu *et al.* 2001; Dornonville de la *et al.* 2005). Ghrelin is produced in abundance by the X/A neuro-endocrine cells of the stomach, mainly in the fundus (Date *et al.* 2000). The ghrelin producing cells GI tract have been characterised in animal studies and these cells were confined mainly to the mucosal layer.

Morphological differences of these ghrelin-producing cells have been described previously. The stomach and intestinal cells have been shown to differ in shape and structure. In the stomach the cells are so called “closed-type” cells and in the intestines “open-type” cells (Sakata *et al.* 2002). The “closed” type cells tend to be small and round shaped. In contrast, the “open-type” cells are elongated. “Open-type” cells are usually positioned to be in contact with the lumen, presumably regulated by luminal contents including nutrients and acidity. “closed-type” cells on the other hand where buried deep in the glandular pits are probably regulated hormonally, neuronally or even by mechanical distension (Sakata *et al.* 2010).

The density of the ghrelin producing cells decrease towards the lower GI tract. Apart from the stomach, smaller amounts of ghrelin has been produced by many other organs including pancreas (Broglia *et al.* 2003b), kidneys (Mori *et al.* 2000; Yabuki *et al.* 2006), placenta (Gualillo *et al.* 2001), testes (Ishikawa *et al.* 2007), pituitary (Korbonits *et al.* 2001) and bowel (Krsek *et al.* 2002). The diverse distribution of ghrelin production raises the possibility that every tissue may contribute to the overall energy homeostasis of an intact animal. Another possibility could be that ghrelin may be involved in regulation of an unknown function specific to the tissues involved.

The ghrelin gene is encoded in chromosome 3p25-p25 (locus tag UNQ524/PRO1066). This gene encodes the ghrelin-obestatin preproprotein. Subsequent cleavage of this preproprotein produces ghrelin and obestatin.

Figure 1.2 indicating the non-sequence coordinate location of the ghrelin sequence on chromosome 3. (Source : Gene database, Pubmed)

The ghrelin gene is conserved is highly conserved in cow, chimpanzee, mouse, dog and rat. There are many variants of mRNA transcripts identified but some do not translate into protein. There are 5 known mRNA transcript and protein variants:

5. Isoform 5 : NM_001134946.1 → NP_001128418.1. This variant is also generated from a distal promoter and includes an alternate first exon which contains an upstream in-frame AUG codon and omits 2 in frame coding exons compared to isoform 1. This isoform is shorter and has a distinct N-terminus compared to isoform 1. Also includes the obestatin ligand

There are 3 known ghrelin single nucleotide polymorphisms

1. rs34911341

CCGGGCGGAGCCAGCCTGCTAGAGCT [C/T] GGGCTGCAGCTTGGCTGGTGGCTT

2. rs4684677

CCAGGGCCTGGCTGTGCTGCTGGTAC [A/T] GAACCCCTGACAGCTTGATTCCAAC

3. rs696217

TGCAGAGGTACCGACCCGGACTTCCA [G/T] TTCATCCTCTGCCCCTTCTGCTTGA

Ghrelin SNP is associated with hypertension but also thought to have some protective effects against gestational diabetes (dos, I *et al.* 2010). A study on Amish population suggests that these polymorphisms are also associated with increased risk of metabolic syndrome (Steinle *et al.* 2005). It is also possible that ghrelin SNPs are associated with increased susceptibility to bulimia nervosa (Ando *et al.* 2006).

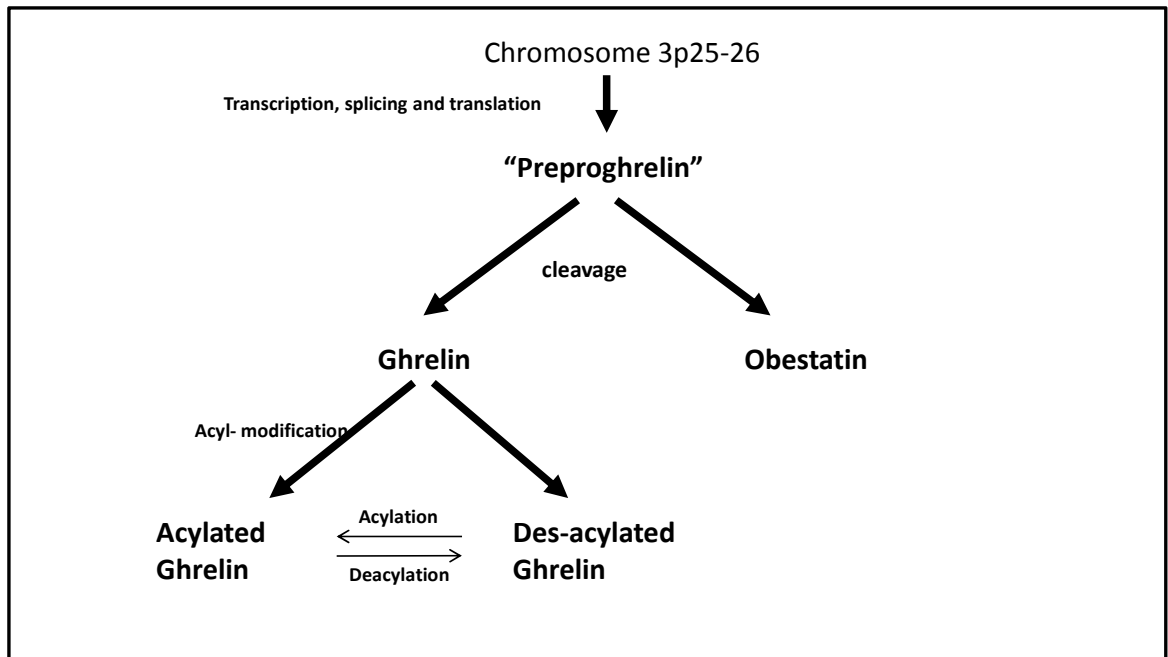


Figure 1.3 Schematic diagram of the ghrelin gene to active peptide. Ghrelin is encoded on chromosome 3. Resulting exon from transcription and splicing is translated into a 117 amino acid precursor protein preproghrelin. Subsequent cleavage of this precursor protein produces ghrelin and obestatin. Ghrelin then undergoes acyl-modification to produce a 28 amino acid ‘active’ acylated ghrelin. Des-acylated ghrelin is also thought to be either a precursor to acylated ghrelin or a product of deacylation of acylated ghrelin

Subsequent cleavage of the pre-proghrelin precursor produces 2 main forms of ghrelin (figure 1.3). The first is a 28 amino acid with post-translational n-octanoylation of serine residue at position 3, which is known as acyl-ghrelin, also thought to be the 'biologically active' isoform. The octanoylation of ghrelin is essential for binding to the GHSR-1a receptor to exert its biological activity (Kojima *et al.* 1999). The second form of ghrelin produced is the des-[Gln¹⁴] formed from alternated splicing of the ghrelin gene (Hosoda *et al.* 2000), known as des-acyl ghrelin. This isoform of ghrelin may represent the product of deacylation of the acylated ghrelin or possibly a precursor to prior to acylation (Kojima *et al.* 2005). When devoid of the octanoylation of serine³ at the N terminus, the biological activity of ghrelin is reduced by a factor of 2300 (Matsumoto *et al.* 2001). This form of ghrelin can also undergo octanoylation and become biologically active. Ghrelin has a short half life and vulnerable to degradation by circulating proteases. Hence it is recognized as a dynamic hormone in which circulating concentrations vary rapidly within minutes. The half life of total ghrelin is estimated to be 27-31 minutes and acylated ghrelin 9-13 minutes (Akamizu *et al.* 2004).

1.2.2. Ghrelin: The Receptor and Regulatory Mechanisms

The ghrelin receptor, GHSR-1a, is widely expressed. This receptor for ghrelin is found in abundance in the arcuate nucleus of the hypothalamus (Howard *et al.* 1996b). This receptor is also widely expressed other various tissues e.g. Pancreas, myocardium, adipose tissue, kidney, liver, placenta and even lymphocytes (Gnanapavan *et al.* 2002). The distribution of this receptor suggests a wide range of possible activity and function of ghrelin, which is discussed later in this chapter.

A variant of this receptor, the GHSR-1b, arise from the same genetic locus. A study using a synthetic GH secretagogue MK0677 showed low affinity of the GH secretagogue for the GHSR1-b and failed to demonstrate the intracellular release of calcium in the COS-7 cells which over expresses the GHSR 1b cDNA (Howard *et al.* 1996a). This suggests that the GHSR-1b is a redundant receptor and currently thought to have no functional properties. It is accepted that the GHSR-1a is the predominant active ghrelin receptor, particularly in regulation of appetite and GH release.

Ghrelin is regulated by many factors including hormonal, neural and local controls. Ghrelin has diverse physiological functions (see below) and as expected, there is usually a putative feedback mechanism that self regulates ghrelin release in many instances. This balance may in fact be crucial in normal physiological function. In disease states, the regulation of ghrelin release can be profoundly affected. Based on current evidence, it is still unclear if ghrelin is a major player in some pathological states or a small player in a complex repertoire of hormonal regulation.

Ghrelin secretion can be influenced by dietary manipulation. Carbohydrate rich meals has been shown to decrease ghrelin secretion (Shiia *et al.* 2002) and this effect is proportional to the calorific load (Callahan *et al.* 2004). Protein ingestion appears to increase plasma ghrelin, and this effect may be augmented by gastrin stimulation (Erdmann *et al.* 2003). In contrast, high fat meal decrease ghrelin secretion, probably because of high calorie load. However, lipid when infused intravenously did not influence ghrelin concentration (Mohlig *et al.* 2002). This suggests the stomach, via humoral and neural mechanisms, may regulate ghrelin secretion by macronutrient

intake locally. It is also possible that ghrelin secreting cells are capable of detecting high calorific substances on ingestion.

Insulin has been implicated in regulating ghrelin release and it is shown to be inversely correlated to ghrelin (Blom *et al.* 2005; Purnell *et al.* 2003). The association between insulin and ghrelin regulation has not been fully explained. Intravenously administered glucose and insulin did not alter ghrelin concentration significantly in healthy human subjects (Caixas *et al.* 2002). However, a study has shown that insulin resistance, measured by HOMA-IR, is positively associated with acylated ghrelin, but negatively associated with desacylated ghrelin and total ghrelin in patients with metabolic syndrome. The investigators found that obesity with metabolic syndrome had lower total and desacylated ghrelin but comparable acylated ghrelin levels when compared to non-obese individuals.

Obesity has been shown to alter the ghrelin profile. Obese individuals were found to have higher acylated to des-acylated ratio (Pacifico *et al.* 2009) and higher acylated ghrelin is associated with insulin resistance. In addition, glucagon and somatostatin appear to have a role in negatively influencing ghrelin secretion (Norrelund *et al.* 2002; Arafat *et al.* 2006). An animal study have implicated glucagon in satiety and meal termination (Le *et al.* 1993). It was postulated that ghrelin is most likely mechanism of which glucagon exert this effect. The mechanism underlying the ghrelin regulation by glucagon is via the hypothalamic-pituitary axis. Other appetite suppressing peptides e.g. Peptide YY and oxyntomodulin have also been shown to decrease ghrelin concentrations (Batterham *et al.* 2003; Cohen *et al.* 2003).

Many factors may increase ghrelin concentrations apart from fasting. Acetylcholine has been demonstrated to increase ghrelin secretion in humans by direct stimulation of muscarinic receptors (Broglia *et al.* 2004). This relationship is also reciprocal in effect (see below). In a study of undernourished patients with anorexia nervosa, it was demonstrated that oestrogen and recombinant insulin growth factor 1(IGF-1) administration in these patients significantly increase ghrelin concentration (Grinspoon *et al.* 2004).

Ghrelin secretion also has neural regulation. One study has demonstrated an increase in plasma ghrelin after truncal vagotomy (Lee *et al.* 2002). This would be in keeping with neural control during the fasting state where vagal activity is decreased. This brain-gut neural axis influence is probably essential in initiating hunger. It has been reported that gastrin administration can synergistically increase ghrelin levels (Fukumoto *et al.* 2008) which results in increasing gastric acid secretion. However, this effect of acid secretion is abolished in vagotomised animals. This suggests that gastrin probably directly stimulates ghrelin secretion, and its effect on gastric acid secretion is regulated by a neural pathway. In addition, a study previously undertaken in this department has shown an increase in circulating ghrelin and intra-gastric acidity following *Helicobacter Pylori* eradication (Nwokolo *et al.* 2003) with an observed non-significant decrease in gastrin concentration. The increase in gastric acid secretion by ghrelin may be influenced by neural regulation.

1.3 Ghrelin: Physiological Function and Therapeutic Use

1.3.1 Appetite and Hunger

Ghrelin is a potent orexigen. When infused into humans, ghrelin stimulates appetite initiates feeding without time or food related cues (Cummings *et al.* 2004). It has been shown that ghrelin increases food intake in animals (Asakawa *et al.* 2001) and humans (Wren *et al.* 2001). It is widely regarded as the only known circulating ‘hunger hormone’ in intact mammals and is thought to play an important role in energy balance. A study on GHSR-1a knockout mice has shown that these mice loses its ability to respond to ghrelin (Chen *et al.* 2004), suggesting this receptor is crucial for the augmentation of hunger.

Ghrelin has also been shown to stimulate the release of Neuropeptide-Y(NPY) (Wren *et al.* 2002), the most powerful orexigen known, and also Agouti-related Peptide (AgRP) in the arcuate neurons of the hypothalamus (Nakazato *et al.* 2001). The ability of ghrelin to stimulate hunger is eliminated in NPY/AgRP knockout mice, which reinforces the suggestion that it is the likely mechanism for the hunger stimulating effects of ghrelin (Chen *et al.* 2004). There is evidence that ghrelin also acts peripherally to exert its orexigenic effects. In a rat study, gastric afferent vagotomy or vagal nerve block appears to abolish the hunger effects of peripheral ghrelin administration (Date *et al.* 2002). Therefore, ghrelin has central and peripheral mechanisms in inducing hunger.

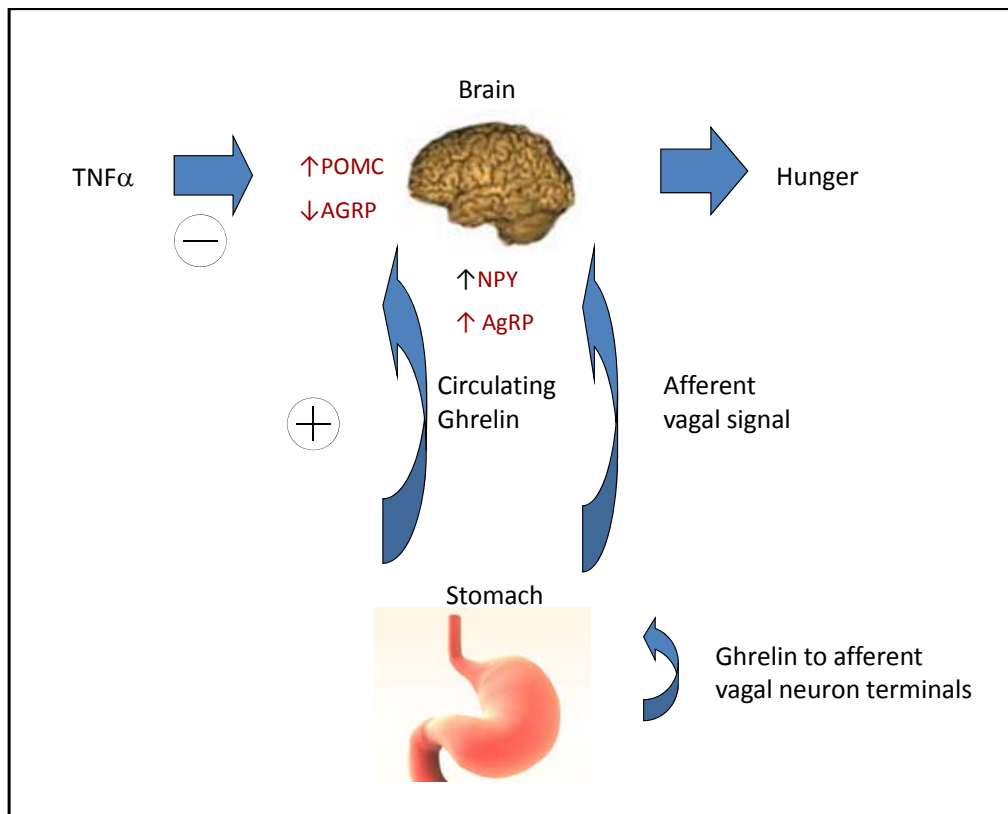


Figure 1.4 Ghrelin mechanism of hunger. Ghrelin can exert hunger effects via afferent vagus nervous system and circulating ghrelin can also act directly on the hypothalamus to induce secretion of neuropeptide-Y (NPY), a potent orexigen, and also agouti-related peptide (AgRP). In contrast, circulating $\text{TNF}\alpha$ has an anorexigenic effect by inducing pro-opiomelanocortin (POMC) and also decreases expression of AgRP, diminishing hunger in presence of inflammation. It is not clear if $\text{TNF}\alpha$ has direct influences on ghrelin secretion.

In humans, ghrelin concentrations are high pre-prandially and associated with a decrease post-prandially (Callahan *et al.* 2004; Cummings *et al.* 2001) which coincides well with the concept of ghrelin as a signal inducing hunger before meals.

The stomach is the first major organ to receive and store food; therefore, it seems appropriate that it has an important biological function as a control centre for appetite control and food intake. Hence, the stomach has earned its role as an important endocrine organ contributing to the regulation of energy homeostasis in an intact animal.

In obese individuals, the post-prandial decrease in ghrelin is blunted or abolished, suggesting a dysregulation of ghrelin in these individuals which may contribute to abnormal energy homeostasis (English *et al.* 2002). This has created much interest in the targeted signalling approach in treatment of obesity.

As a hunger-stimulating hormone, ghrelin may have a role in the treatment of cachexia. For example, in rats with heart failure ghrelin treatment is associated with an increase in body mass with an increase in gastronemius muscle weight to bone ratio (Nagaya *et al.* 2001). This increase in body weight by ghrelin administration is probably GH-independent (Tschop *et al.* 2000). Ghrelin is also known to have beneficial effects on cancer cachexia. A small randomised placebo- controlled human study on cachexia in cancer patients demonstrated an 31% increase in energy intake following ghrelin infusion (Neary *et al.* 2004). In addition, a randomised placebo controlled study demonstrated that ghrelin has therapeutic potential in improving appetite and food intake in patients with post total gastrectomy cachexia (Adachi *et al.* 2010).

1.3.1.1 Leptin, Ghrelin and obesity

Leptin is a 16kDa hormone, which is a product of the obesity gene (OB) encoded in chromosome 7 (7q31.3). First identified in 1994 (Zhang *et al.* 1994), leptin was found in abundance in the adipose tissues, although other tissues e.g. Placenta, stomach and heart secrete smaller quantities (Sobhani *et al.* 2000; Hoggard *et al.* 2001; Green *et al.* 1995). The primary secretors of leptin in animals are white adipose tissues. Leptin acts centrally, in part by crossing the blood brain barrier and the cerebrospinal fluid concentration appears to correlate with BMI (Schwartz *et al.* 1996c).

The leptin receptor is encoded on chromosome 1 and widely expressed in the hypothalamus and cerebellum (Chung *et al.* 1996). Leptin is thought to have influences in both orexigenic (e.g. NPY and AgRP) and anorexigenic peptides (Pro-opiomelanocortin, corticotrophin-releasing hormone and brain derived neurotrophic factor (Sahu *et al.* 1998; Bariohay *et al.* 2005). Leptin functions as a long term signalling peptide reflecting the energy stores, in particular body adiposity (Schwartz *et al.* 1996) and also functions as a long term mediator of energy balance. Therefore, an increase in BMI may result in an increase in circulating leptin concentration.

In animal studies, leptin has been found to be an important hormone in decreasing food intake and regulating energy homeostasis (Halaas *et al.* 1995). In addition, leptin maintains weight, increases physical activity and resting metabolic state in animals.

There have been implications on the interplay between ghrelin and leptin in obesity. It has been observed that obese individuals have high leptin and low ghrelin

concentrations, although the converse is expected (Tschop *et al.* 2001; Lonnqvist *et al.* 1995). However, leptin treatment in obese individuals did not have the desired effect of weight loss (Heymsfield *et al.* 1999; Hukshorn *et al.* 2000). Chronic treatment of leptin appears to decrease the hypothalamic sensitivity to leptin (Sahu *et al.* 2002).

Many authors conclude obese individuals are resistant to leptin. In obese individuals lower CSF concentration of leptin is observed in comparison to plasma concentration (Schwartz *et al.* 1996) which suggests another altered regulating mechanism at the leptin transporter level. There is evidence that leptin resistance is demonstrated in peripheral but not central administration of leptin (Van *et al.* 1997). This implicates that the blood brain barrier may act as a mechanism for insulin resistance.

1.3.1.2 Leptin, Ghrelin and Inflammation

The involvement of leptin in inflammation is poorly understood. The structure of leptin have a resemblance to the cytokine family (Zhang *et al.* 1997) with a four helix bundle strikingly similar to the long-chain helical cytokines. The leptin receptor bears resemblance to the class 1 cytokine receptor family, and have signalling capabilities of the IL-6 receptor which are gp-130 related (Baumann *et al.* 1996). It is possible that leptin has an immune regulatory function. Studies have shown that leptin levels are increased in presence of acute inflammation and infectious stimuli, in particular bacterial lipopolysaccharide and cytokines (Grunfeld *et al.* 1996; Sarraf *et al.* 1997). Leptin has been shown to have a role in modulating macrophage phagocytosis and cytokine production (Lee *et al.* 1999; Loffreda *et al.* 1998). In addition leptin also

modulates lymphocyte proliferation and sensitivity to pro-inflammatory stimulus (Faggioni *et al.* 1999; Howard *et al.* 1999). Leptin is classified as a cytokine because of its ability to influence the immune system and promote inflammation.

Ghrelin on the other hand was initially thought to have a reciprocal relationship to leptin in regulating the immune system. This is unsurprising since ghrelin and leptin has opposing effects in the control of food intake and energy balance. The interplay between these two hormones may contribute to the regulation of the immune response to an inflammatory stimulus.

The imbalance of the two hormones may drive or promote inflammation. A study on haemodialysis patients with protein energy wasting have demonstrated low circulating ghrelin, high leptin and CRP levels in these patients (Carrero *et al.* 2010). This would support the conclusion that inflammation may be associated with the imbalance of both these hormones. In contrary, there is evidence that modulating inflammation may not affect either ghrelin or leptin concentrations significantly (Gonzalez-Gay *et al.* 2009). The role of ghrelin and leptin in inflammation may be disease specific or perhaps related to adiposity and energy balance as a primary function. Further studies are needed to establish the relationship between ghrelin, leptin and inflammation, including trial therapy with ghrelin may shed some light into its potential as a therapeutic target

1.3.2 Endocrine function

1.3.2.1 Growth Hormone Release

Although ghrelin was initially discovered as a growth hormone secretagogue, its physiological role is still undetermined despite being potent stimulator of growth hormone release in humans (Arvat *et al.* 2000). Ghrelin is not known to be important in the physiological regulation of GH secretion, as ghrelin-null mice are not growth impaired (Sun *et al.* 2003). In addition, normal physiological release of GH is unaffected by the absence of ghrelin. Ghrelin appears to regulate the pulsatile release of GH (Sun *et al.* 2004), and it is possible that ghrelin may somewhat regulate anabolic metabolism although the physiological effects of this is still unclear. Ghrelin-induced GH secretion is shown to be unrelated to IGF-1 influences, which may differentiate it from GHRH-mediated GH secretion (Goodyear *et al.* 2010).

It has been reported that ghrelin administration in animals can possibly reverse certain characteristics of aging, particularly improving food intake, body weight and maintaining low adiposity (Ariyasu *et al.* 2008). These effects presumably related to GH secretion by ghrelin as GH increases protein anabolism and adipose utilisation. Ghrelin is also shown to decrease adipose utilisation quite the opposing effect to that of GH. Perhaps this is beneficial in the physiological sense to maintain body adiposity. In addition exogenous GH administration appear to decrease ghrelin secretion which suggest a feedback mechanism of self regulation (Eden *et al.* 2003).

1.3.2.2 Other Endocrine Effects

Apart from regulating the release of GH, ghrelin is also known to have other endocrine actions. For example, growth hormone secretagogue has been shown to have stimulatory effects on the hypothalamic-pituitary-adrenal axis. The postulated mechanism via the arginine-vasopressin release rather than the corticotrophin-releasing hormone stimulation (Korbonits *et al.* 1999).

In a human study, ghrelin induces a gender independent release of prolactin, ACTH (adreno-corticotrophin hormone) and cortisol. Ghrelin also decreases insulin and increase glucose levels, which are independent of both age and gender. Glucagon levels were unaffected by ghrelin administration (Broglia *et al.* 2003). Therefore, ghrelin may act directly on the hepatic glycogenolysis rather than the pancreas. These findings are interesting which adds to the wider activity and function of ghrelin, which the clinical significance is still yet to be explored.

1.3.3 Gastrointestinal Tract

1.3.3.1 Gastric acid secretion

The reported effects of ghrelin on gastric acid secretion on animals are conflicting (Takayama *et al.* 2007; Sibilio *et al.* 2002). Under normal physiological conditions, gastric acid secretion is initiated by the release of gastrin usually after a meal which in turn stimulates the release of histamine by enterochromaffin-like cells (ECL). This then stimulates the parietal cells in the stomach to produce acid. There are also vagal influences on the parietal and ECL cells exerting further control of acid secretion.

In a study on rats, ghrelin appear to have little effect on the activation of G-cells nor ECL cells when administered subcutaneously to an animal. However, it is believed that ghrelin, when administered intravenously can increase gastric acid secretion via vagal pathways (Masuda *et al.* 2000). This is consistent with evidence that sight and smell of food induces gastric acid secretion (Feldman *et al.* 1986) and ghrelin may be part of this mechanism. Ghrelin has been also shown be increased up to 75% following the successful eradication of *Helicobacter Pylori*, a bacterium capable of surviving in an acidic environment of the stomach. This effect appears to increase intragastric acidity by 14% (Nwokolo *et al.* 2003). It was postulated that this rise in ghrelin may increase appetite and food intake, and possibly have contributed to the rise in obesity in the developed world. This may in turn explain the rise in incidence of gastro-oesophageal reflux disease (GORD) and in turn may be associated with an observed increase in incidence of oesophageal cancers in western countries.

1.3.3.2 Gastrointestinal Motility

Ghrelin is capable of regulating gastrointestinal motility. In animal studies, ghrelin promotes gastric contractility and postulated to act via vagal innervations (Masuda *et al.* 2000). Similarly, it has been shown to stimulate small intestinal motility. This action was inhibited by atropine, which suggests it acts locally via the cholinergic innervation at the neuromuscular level (Edholm *et al.* 2004).

There has been much interest in the pro-motility therapeutic potential of ghrelin. Ghrelin has been shown to alleviate gastroparesis in animals (Trudel *et al.* 2002) and humans (Tack *et al.* 2005). The therapeutic potential of ghrelin was soon realised. A pilot randomised placebo controlled study had demonstrated that ghrelin infusion improves gastric emptying in diabetic gastroparesis (Murray *et al.* 2005).

The other most obvious therapeutic application would be related to post-operative patients where ileus is a common complication. Ghrelin has been shown to be an effective pro-motility agent in post-operative animal models with ileus (Poitras *et al.* 2005; Trudel *et al.* 2003). This warrants further human studies on the efficacy of ghrelin in post-operative ileus. Another application of ghrelin as a pro-motility agent is in cancer associated symptoms of gastroparesis. It has been demonstrated in rodents that ghrelin alleviates the cancer chemotherapy associated dyspepsia (Liu *et al.* 2006b). This will be discussed in detail in Chapter 4 of this thesis.

1.3.4 Cardiovascular Effects

Ghrelin receptors has been shown to be expressed in cardiac tissues in animals (Bodart *et al.* 1999) and humans (Muccioli *et al.* 2000). In an animal study, administration of ghrelin resulted in a significant vascular vasodilatory effects (Okumura *et al.* 2002). This is consistent with a human study where healthy volunteers showed a significant decrease in mean arterial blood pressure when administered with ghrelin (Nagaya *et al.* 2001a) .

Ghrelin is also shown to increase cardiac output and also improve cardiac cachexia associated with heart failure (Nagaya *et al.* 2003). Therefore, ghrelin may not only exerts protective effects on the cardiovascular system but be beneficial in reversing cachexia in chronic heart failure. It is possible that some of this effect may be related to GH secretion, but the side effects of fluid retention and heart failure by GH may be counteracted by effects of ghrelin on the heart. This warrants further clinical studies.

1.3.5 Immunity and inflammation

Appetite control and energy homeostasis is a well studied function of ghrelin. More recently, there is emerging evidence that ghrelin may have some immune-modulatory function. The discovery of GHSR receptors in human immune cell lines suggests that ghrelin may play a role in the function or activity of human lymphocytes (Hattori *et al.* 2001).

This has created much speculation to the exact role of ghrelin in the immune system. Subsequent study has demonstrated that ghrelin dosing of activated primary human immune cells, in particular T-cells and monocytes, decreases the production of pro-inflammatory cytokines in human monocytes and lymphocytes, namely TNF α , IL-1 β and IL-6. This appear to be reciprocal to that of leptin (Dixit *et al.* 2004).

In an animal study, ghrelin has been demonstrated to attenuate pancreatic damage induced by caerulein in rats possibly by suppressing the production of pro-inflammatory IL-1 β cytokine (Dembinski *et al.* 2003). In endotoxaemia induced by bacterial LPS (lipopolysaccharide), ghrelin proved to be beneficial in reducing mortality and alleviates hypotension related to sepsis (Chang *et al.* 2003). Ghrelin has also been demonstrated to be protective in ethanol induced gastric ulceration (Sibilia *et al.* 2003; Brzozowski *et al.* 2004; Konturek *et al.* 2004).

In addition, ghrelin agonists have been shown to improve experimentally induced inflammatory arthritis presumably by suppressing the production of IL-6 (Granado *et al.* 2005). Further in vitro studies on human endothelial cells suggest that ghrelin may suppress TNF α -induced pro-inflammatory cytokine production. This may, in part, play an important role in the development of atherosclerotic disease in obese patients, where ghrelin levels are reduced (Li *et al.* 2004).

In patients with metabolic syndrome, which encompasses a spectrum of clinical conditions including hyperlipidaemia, hypertension, obesity and type 2 diabetes, the development of insulin resistance is thought to be related to a pro-inflammatory process. A previous study has demonstrated that patients with NASH have lower

acylated ghrelin concentration compared to controls, due to dysregulation of ghrelin, which may provide a pro-inflammatory environment for progressive liver damage. In addition, the prevalence of small intestinal bacterial overgrowth in NASH patients may facilitate the production of significant endogenous ethanol causing further damage to the liver (Sajjad *et al.* 2005).

There is a recent suggestion that caloric restriction can prolong lifespan by delaying age-onset disease, improving stress resistance and reduce functional decline over time (Ingram *et al.* 2004). Intermittent fasting in an intact animal is shown to have beneficial in glucose regulation and also protects against neuronal injury (Anson *et al.* 2003). As fasting is associated with an increase in circulating ghrelin and a decrease in leptin, it is possible that in a fasting state these changes may have anti-inflammatory properties that may, in turn, be life-prolonging (Dixit *et al.* 2005). This certainly warrants further investigation.

As previously described, ghrelin administration has a beneficial effect on the cardiac function in patients with chronic heart failure. A recent animal study in rats post myocardial infarction has suggested that the beneficial effects of ghrelin may be mediated by the anti-inflammatory effects of ghrelin. This includes attenuation of TNF α and IL-1 β , with a reduction of matrix metalloproteinase expression, such as MMP-2 and MMP-9, which leads to improved cardiac remodelling, decreased ventricular dilatation with decreased cardiac cell apoptosis and limits cardiac scar thickness (Huang *et al.* 2009).

If ghrelin has an immune-modulatory role, it is possible that circulating ghrelin concentration may change when cytokine profile is altered in chronic inflammatory conditions since there is now evidence that ghrelin may in some way influence immune activity and inflammation. It would be desirable to observe the consequence to ghrelin once inflammation is subdued. A hypothesis that modulating an inflammatory condition may alter circulating plasma ghrelin was developed and investigated as part of this thesis.

1.4 Aims of this Thesis

This department has previous experience in studying ghrelin and its dynamics (Goodyear *et al.* 2010; Goodyear *et al.* 2010; Mottershead *et al.* 2007; Nwokolo *et al.* 2003; Sajjad *et al.* 2005) . This has been invaluable in the conceptualisation of study design in this thesis, particularly in profiling of ghrelin and data analysis. This thesis primarily investigates the effects of ghrelin on immune system and gastrointestinal motility.

This thesis has the following aims:

- **To investigate the consequence of modulating inflammation on the ghrelin profile in patients with Crohn's disease.**

Crohn's disease a chronic inflammatory bowel disease that can affect the entire luminal gastrointestinal tract. This study investigates the profile of ghrelin in patients with Crohn's disease before and after administration of anti TNF α therapy.

- **To investigate the effects of ghrelin dosing on NFκB activation and cytokine production in human lymphocyte cells lines.**

Recent studies on primary human lymphocytes suggest that ghrelin attenuates the release of pro-inflammatory cytokines and exerts its anti-inflammatory properties at cellular level. In B-cells, NFκB acts as a gene enhancer for κ light chain and also crucial in the development and maturation of B-cell (Sha *et al.* 1995b) . It also has a pivotal role in driving and coordinating the innate and adaptive immune response to an antigen (Bonizzi *et al.* 2004a). This experiment will explore the effects of ghrelin and a ghrelin agonist on NFκB in a lymphocyte cell line, which will provide some insight into the function of ghrelin and the immune system, upstream from cytokine production.

- **To investigate the effects of cancer chemotherapy on motility gastric neurotransmitters and gastric smooth muscle contractility.**

It is evident that a cancer chemotherapy regime can induce gastric dysmotility in animal studies. This may lead to symptoms that resemble dyspepsia, frequently experienced by these patients following treatment. This experiment compares the gastric contractility and neurotransmitter expression in chemotherapy exposed and non-chemotherapy exposed gastric tissues.

Chapter 2

Research Methodology

This chapter will outline and describe the general experimental methods and materials used in this thesis. Information on specific methods for each experiment will be discussed in the appropriate chapters.

2.1 Patient recruitment: Ghrelin in Crohn's disease

Patients with Crohn's disease were recruited from the hospital database on patients scheduled to receive infliximab therapy. Patients were contacted prior to their scheduled infliximab treatment and outline of the study was conveyed. All patients receiving infliximab therapy were included in this study. Patients with previous gastric surgery were excluded, as the stomach is the predominant ghrelin producer and can affect the physiological profile. In addition, patients with ulcerative colitis or indeterminate colitis receiving infliximab therapy were also excluded.

2.1.2 Experimental protocol

Each patient was studied on two occasions, immediately before and 1 week after an infusion of infliximab (5 mg/kg). All studies were started between 08:00 and 10:00 h. All patients were fasted overnight and on arrival consent was obtained. An 18 G cannula port-free was inserted into a vein in the forearm. A needle free port (SmartSite needle free injection port, Alaris Medical Cat: 2000E) was attached to cannula to enable blood sampling without the need for repeated venopuncture. Blood

samples were collected in a lithium heparin tube (BD Vacutainer Cat: 367880, BD, NJ, USA) and also in a spray coated silica tube with polymer gel separation (BD Vacutaner SST Cat: 367986, BD, NJ USA) to collect serum equivalent samples. All blood tubes were added with 20 U/mL of aprotinin (Trasyol, Bayer Healthcare, NJ, USA). Aprotinin is a 58 amino acid bovine lung derived protease inhibitor. It binds to active sites of proteases and forms stable complexes. In this study, it provides protection against the breakdown of ghrelin by inhibiting protease enzymes.

Firstly, an initial fasting blood sample was taken. This is followed by consumption of a 450 cal meal consisting of a cereal bar, a slice of buttered toast, a glass of orange juice (250 mL) and a cup of tea with milk (180 mL). Minor variations to this diet was allowed e.g. for dairy intolerance, omission of butter or food dislikes, coffee rather than tea. However, an identical meal was consumed in the second study occasion.

After the meal is consumed, further blood samples were taken every 20 minutes for 2 hours. During this time, data was collected on the patients' weight, height, site of disease and concurrent drug treatment. Once this was completed, the patients proceeded to receive their infliximab therapy. In exactly 1 week, an identical study was conducted without the infliximab infusion. In five patients, the study conducted in reverse order i.e. A study '1 week after' infliximab was performed first followed by the 'immediately before' study performed just before the next infusion, usually 7 weeks later. This excludes any effects that could be attributed to the order in which the studies were conducted.

Blood samples collected were centrifuged immediately (2000 rpm for 15 minutes for plasma, 3000 rpm for 10 minutes for serum), plasma extracted and stored at -80°C for later analysis. This was later used for assays were performed for plasma total ghrelin, acylated ghrelin, desacyl ghrelin, C-reactive protein and a panel of cytokines. Total, acylated and desacylated ghrelin were assayed using commercially available ELISA based kits. In this experiment, plasma samples were compatible with the commercial assays available. Initial experiments with serum samples did not reveal significant differences in the results when either plasma or serum samples were used. All commercially available assay kits were compatible with use of plasma samples.

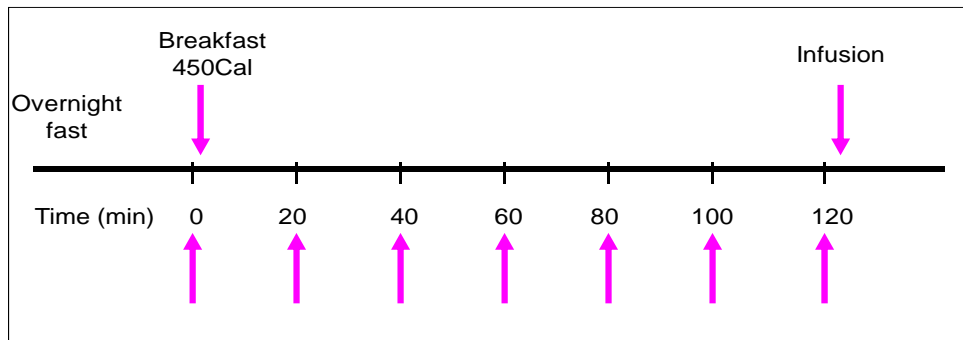


Figure2.1 Study blood sampling scheme

Figure 2.1. Schematic diagram of the blood sampling protocol. After an overnight fast, an initial blood sample of taken at $t=0$ followed by ingestion of a 450Cal meal. Blood is then sampled every 20 minutes followed by infusion of infliximab. In the next visit, this is repeated without the infusion. Some patients are studied in reverse order.

2.1.3 Principles of Enzyme Immunoassays

The enzyme immunoassays are based on a double-antibody sandwich system. The commercially available EIA kits comprise usually of antibody coated wells on a plate specifically to the antigen in question e.g. Ghrelin. The antibody binds to the antigen when samples were introduced to the wells. A secondary antibody with an attached enzyme (usually a peroxidase or alkaline phosphatase) is then introduced to these wells, which then binds to the primary antibody to the antigen.

Following an incubation period, excess secondary antibody is removed by another series of washes. Finally, an addition of an enzyme substrate will then colour the wells. When the reaction is complete, the intensity of the colour is analysed using a plate reader. The optical density, which reflects the colour intensity, is proportional to the amount of primary antibody-antigen complex in each well. The EIA kits also contain controls and samples of known varying quantities of the antigen. Using the optical densities of the 'known' samples, a standard curve is then generated. Using this curve, specific quantification of the antigen in unknown samples can be determined. All enzyme immunoassays performed in this thesis were assayed in duplicates.

2.1.4 Principles of Multiplex Assay

Multiplex assays techniques allow the measurement of multiple analytes efficiently from a small sample. This utilizes the colour-coded beads which are coated for the analyte specific primary antibody. Detection of the analyte is then made possible using the antibody-antigen sandwich, similar to the enzyme immunoassays that

measure a single analyte. Expression levels are determined following incubation with a biotinylated detection antibody and streptavidin-conjugated phycoerythrin (PE). Using a laser analyser, the colour of each bead is detected and the magnitude of the phycoerythrin signal is determined. This is proportional to the quantity of analyte in the sample.

2.2 Lymphocyte Cell Line Experiment

2.2.1 Lymphocyte cell-line selection

Four lymphocyte cell lines were selected for experimental use: 2 B-lymphocyte (WILCL, ECACC Cat: 89120565; DAUDI, ECACC: Cat: 85011437) and 2 T-lymphocyte (JM, ECACC Cat: 86010201; HUT-78, ECACC Cat: 88041901).

All lymphocyte cell lines were cultured in accordance to previously described methods unless specified otherwise. Specific requirements for individual cell lines described were in accordance to methods recommended by the commercial supplier.

2.2.1.1 WILCL (Normal B-Lymphocyte)

WILCL is a normal caucasian human B cell line of a patient with a lung tumour. Morphologically it is a lymphoblast, and commercial karyotype analysis of this particular cell line was normal. WILCL is cultured in media containing RPMI 1640 + 2mM Glutamine + 10% Foetal Bovine Serum (FBS).

2.2.1.2 JM cell line

JM is a human T-cell lymphoblast cell line. Cells were cultured in RPMI 1640 enriched with 2mM Glutamine + 10% Foetal Bovine Serum.

2.2.1.3 Hut 78

Hut 78 is a T-cell lymphoma cell line derived from a 50 year old male with Sezary syndrome. This cell line has characteristics of a mature T-cell line with an inducer and helper phenotype. An established product of this cell line is Interleukin-2 (Gootenberg *et al.* 1981)

2.2.1.4 DAUDI

DAUDI is a human Burkitt's B-cell lymphoma cell line. It is derived from a 16 year old black male. Positive for EBNA, carries EBV markers, complement receptors, surface bound immunoglobulin and surface markers for the Fc fragment of IgG (Klein *et al.* 1968; Klein *et al.* 1967; Nilsson *et al.* 1977). Media requirement are RPMI 1640 + 2mM Glutamine + 10% FBS.

2.2.2 Cell culture methods

2.2.2.1 General lymphocyte cell line resuscitation

Cell line resuscitation and culture procedures and protocols provided by the commercial distributor ECACC (European Collection of Cell Cultures) were

followed, including specific media requirements for each cell line described further in the chapter.

All cell line work was performed in level 2 microbiology safety cabinets. On every occasion the cabinets were sterilised using 70% alcohol to prevent contamination of cell culture. Protective face masks and sterile gloves were used for protection. Prepared media was pre-warmed in the water bath prior to every cell line procedure for 30 minutes. On receipt of the cell line ampoule, it is then transferred immediately and slowly swirled a water bath but not immersed at 37°C.

Once thawed, the ampoule was cleaned using 70% alcohol. The ampoule was then centrifuged and the cryoprotectant was removed by pipette. 1 ml of freshly prepared warm media was introduced and mixed well with the collected cells. The contents of the ampoule are then transferred to fresh media in a 25cm² Greiner® culture flask containing 8mls of media. The cells were incubated in an environment of 5% CO₂ at 37°C. Once cell proliferation is established, the cells are then sub-cultured in larger 50cm² Greiner® culture flasks maintaining cell culture density of 3×10^6 . Penicillin/streptomycin was added to all cell culture medium for antimicrobial protection.

2.2.2.2 Assessment of cell viability

Cell viability was assessed using trypan blue by dye exclusion. A 0.5mls of homogenised sample of cells is then collected and 0.1mls of 0.4% trypan blue (Sigma Aldrich Cat. No. T8154, Dorset, UK) added. The sample was then vortexed

and a small sample transferred to a haemocytometer. Observed under a microscope, viable cells do not take up staining, hence excluded from staining whereas non-viable cells take up dye and stained blue.

2.2.2.3 Cell count and quantification

Cell count is useful to ensure optimum cell density is achieved to keep cells healthy. For all cells, cell density was maintained at 3×10^6 in culture. It is also useful in assessment of cell viability. Firstly, cells were collected from the flasks and centrifuged in a 50ml falcon flask. 10mls of fresh media was then added and cells were mixed well using a pipette. A small aliquot of the homogenised sample was taken and used for cell counting. A 10 μ l sample was extracted and used to fill a chamber in the bright line haemocytometer (Sigma-Aldrich, cat, no. Z359629, Dorset, UK) with cover slips.

The haemocytometer was viewed under a light microscope and the number of cells in four of the 1mm X 1mm squares were counted. The following formula was then used to estimate cell concentration: $[\text{Total number of cells}(y) \times \text{dilution (10mls)}] \div 4 = y \times 10^4$, hence concentration would be $0.0y \times 10^6$. The amount of media that needs to be added to the original 10ml cell suspension to obtain optimum cell density can be estimated.

Similarly, for estimating percentage of viable cells using trypan blue, percentage viable cells are calculated using the following formula:

$$(\text{Total no. of viable cells} \div \text{Total no. of cells}) \times 100.$$

2.2.2.4 Cell subculture

Initially cells were extracted, counted and resuspended in appropriate media volume to a cell density of 1×10^6 (as described in 2.7). The cell suspension was then transferred to a 50cm² Greiner® culture flasks and incubated in an environment of 5% CO₂ at 37°C with the flask lying flat. A small sample from each flask is usually inspected under a microscope every 1-2 days to ensure the morphology appearances of cells are healthy. Every third day, the cells were again collected, counted, and resuspended in a calculated volume of media to cell density of 1×10^6 and allowed to proliferate. Cells were inspected regularly using light microscopy to ensure intact morphology and healthy proliferation. On rare occasions, bacterial contamination can be seen as well. If detected the flask of cells were discarded.

2.2.2.5 Cell cryopreservation

Cryopreservation of cells was required to maintain a supply of cells in event of culture failure that are unforeseen. This is achieved using a freeze medium which contains 70% cell medium, 20% foetal bovine serum and 10% DMSO (dimethyl sulfoxide, Sigma-Aldrich, cat. No. C6164, Dorset, UK). Following successful cell proliferation and subcultures, cells were collected by centrifuge (150g for 5 minutes) and resuspended in pre-prepared freeze medium with cell density of 3×10^6 . 1ml aliquots of cells were transferred to ampoules transferred to a -20°C freezer for 4 hours then transferred to a -80°C freezer overnight. On the following day, the ampoules were then transferred to liquid nitrogen for storage. Approximately 3-5days later, an

ampoule of cells were retrieved, resuscitated and resuspended in media to assess viability.

2.2.3 Compounds used in cell culture treatment

2.2.3.1 Ghrelin and Analogues

3 different compounds were tested on the cell lines : Octanoyl ghrelin (Phoenix, CA, USA; Cat. 031-39), des-octanoyl Ghrelin (Phoenix, CA,USA; Cat. 031-32) and a non-peptide ghrelin agonist (Pfizer CP-464709). The ghrelin agonist was provided by our collaborators at GlaxoSmithKline as part of our shared experimental interest in motility work. The compound CP-464709 has previously been studied in animals and demonstrated to have prokinetic properties in fed animals with moderate enhanced motility stimulation in the gastric antrum (Komada T *et al.* 2008). This compound has also been shown to induce colonic propulsion and defecation in intact rats when administered centrally into the spinal cord (Shimizu *et al.* 2006). In contrast, previous animal studies have shown that ghrelin administered peripherally did not produce significant colonic peristalsis (Ohno *et al.* 2010). This suggests that ghrelin may not cross the blood brain barrier significantly to induce such an effect.

2.2.3.2 Phytohemagglutinin (PHA)

Phytohemagglutinin (Cat. No. L1668, Sigma, UK) is a lectin derived from mainly from plants. It is found in high concentrations in red kidney beans, which are toxic to humans in its raw form if ingested. It is potent mitogen in lymphocytes, inducing cell

division and proliferation. In this study, it is used as a lymphocyte activator, in particular to artificially induce the NFκB machinery. This allows the study of the effects of NFκB activation by different compounds on activated and non-activated cells. In powder form, it is diluted in 5 mls of PBS for cell treatment use. Pokeweed (*phytolacca americana*, another source of lectin) was used in isolation as control for NFκB activation did not show significant difference in NFκB activation compared to PHA in these cell lines.

2.2.4 Cell treatment methods

Cells cultured in flasks were centrifuged (150g for 10 minutes) and resuspended in fresh media. Following this, cells were resuspended in 10mls of fresh media and homogenised. Cell count was performed and a calculated amount of fresh media was added to dilute to the required cell density of $1 \times 10^6/\text{ml}$. Subsequently, 1ml containing 1×10^6 cells was added to each well of a 6-well culture plate. Initial experiments were conducted to determine the optimal concentration of PHA for these cells. Cell viability was determined at 6 and 24 hour following dosing with PHA at 5μg/ml, 0.5μg/ml and 0.05μg/ml concentrations and compared to controls. 0.5 μg/ml appears optimal without excessive toxicity.

The cells were then re-cultured, collected, diluted and added to the 6-well culture plates as described above. 0.5 μg/ml of PHA was added to half the wells as ‘activated’ cells. Phytohemagglutinin (PHA) was used for lymphocyte activation. The optimal concentration of PHA was estimated using trypan blue staining for cell viability.

PHA concentration of 0.5µg/ml was optimal for WILCL cell activation. We assessed the effect of the compounds on activated and resting cells.

To assess the effect of ghrelin isoforms and a non-peptide ghrelin agonist on the cell line, each treated well was compared to control wells with no additions. A screening experiment (n=3) using time points of 6, 24 and 48 hours were performed using octanoyl ghrelin on resting and activated cells. A response was detected at 6 hours to PHA activation and octanoyl ghrelin. In all subsequent experiments 6 hours incubation duration was applied.

Octanoyl-ghrelin, des-octanoyl ghrelin and the ghrelin agonist was added to the wells to make up concentrations of 1nM, 10nM and 100nM. . These compounds were added to the wells with resting and PHA activated cells to make up concentrations of control cells (no addition), 1nM, 10nM and 100nM. For the ghrelin receptor agonist and desoctanoyl ghrelin that are not natural ligands of the ghrelin receptor, we widened the concentration range to include 0.1nM and 1000nM. The culture plates were then incubated in an environment of 5% CO₂ at temperatures of 37°C. The cells were then washed, collected and frozen to -80°C. The cell pellets were later lysed and protein content were extracted and quantified.

2.2.5 Cell preparation and protein extraction

2.2.5.1 Cell lysis and protein extraction

Cell lysis and protein solubilisation was achieved with RIPA (Radio-Immuno precipitation Assay) buffer (Cat. No. R0278, Sigma-Aldrich, UK) containing 50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulphate. Cells were collected from each well, centrifuged, washed 3 times with PBS and collected. Subsequently 200µL of RIPA buffer was added to the cell pellet. With gentle pipetting, the cells were mixed well with the RIPA buffer which promoted mechanical and chemical lysis. The mixture is then left to incubate on ice for 5 minutes. Finally, the mixture is centrifuged and the supernatant RIPA buffer containing the solubilised cellular protein is extracted and frozen to -80°C for later analysis. Remaining cell debris is discarded.

2.2.5.2 Protein quantification

To determine the protein quantification extracted from cells the Bio Rad DC protein assay kit (Bio Rad Laboratories, Ca, USA) was used. This kit comprises of 3 reagents: REAGENT A, an alkaline copper tartrate solution; REAGENT B, a dilute Folin Reagent 5 and REAGENT S, a surfactant containing solution. Initially, 20µL of Reagent S was mixed well with 980µL of Reagent in a curvette, a glass test tube (Startstedt, Numbrecht, Germany). 5µL of protein containing RIPA buffer was added to this mixture. In addition, 1ml of Reagent B was added to each curvette gently swirled to mix well. The mixture is then incubated at room temperature for 15 minutes.

Bovine serum albumin (First Link LTD, UK) was used to make up known concentrations of protein which is then used create a standard curve in which concentrations of protein in unknown can be determined. 0.01g of BSA was dissolved in 1ml of PBS and serial dilutions were then performed and the same protein quantification kit described above is used.

Using a spectrophotometer (6505 UV, Jenway, UK), the absorbance of the BSA samples were determined and a standard curve was generated. Following this, absorbance of each sample was measured, plotted on the standard curve and the protein quantity determined.

2.2.6 Detection of cellular ghrelin receptors

2.2.6.1 General principles of western blot

Western blot is an immunoassay-based technique that allows identification and quantification of specific proteins. In this thesis, it is used to detect the presence of both ghrelin receptors, GHSR-1A and 1B in all four cell lines. Proteins contained in cellular extracts undergo electrophoresis through a gel under electrical stimulation. Proteins was separated according to the molecular weight in this manner. The product of the electrophoresis was then transferred to a nitrocellulose membrane. Specific antibodies were used to bind to the protein of interest and visualised using an chemiluminescent system. Detailed methodology are described below

2.2.6.2 Preparation of samples.

Cell line protein extracts were thawed and vortexed. Volume of samples containing 20µg of protein was calculated. These samples were then transferred to appendorfs and 10 µL loading buffer was added. Finally, dH₂O was added up to standardised volume for all the samples. To identify the protein of interest, a rainbow molecular weight marker (Amersham Cat: RPN800 (10,000-250,000 kDa), Amersham Biosciences, UK), was used. Following electrophoresis, the rainbow marker will allow identification of a range of proteins with different molecular weights using a colour coded molecular weight spectrum following electrophoresis. The sample mixture was heated at 95°C to denaturise the protein.

2.2.6.3 Western blot set up and electrophoresis

Gels were prepared using an apparatus according to manufacturers' instructions. This consists of 2 sheets of glass, a glass holder with clamp and a comb which created the wells for sample introduction. The two glass plates were slotted in to the apparatus on to a foam bed and clamped. Water tightness is tested using dH₂O. Resolving gel (see table 2.2.6.3) was added between the glass plates until it is $\frac{3}{4}$ height of the smaller plate. dH₂O was added on top to prevent formation of bubbles within the gel. This was then left to set for 30 minutes at room temperature.

Following this, the dH₂O on the top layer was expelled leaving the set resolving gel. Stacking gel (see table 2.2.6.3) is then added above the resolving gel. Wells were formed by inserting a 1.5mm thickness comb with 10 teeth. Air bubble formation was prevented by inserting the comb at an angle. During the time taken to set, any shrinkage of gel leaving air spaces was replaced by added gel using a hypodermic needle and syringe. Once set, the comb was removed vertically with care.

The gel formed between the glass sheets are immersed in a tank containing electrode buffer. In the tank, it was separated into 2 different compartments, a central compartment containing the gel and a peripheral compartment containing electrode buffer, which was added. Compartmenting in this manner will then allow completion of an electrical circuit through the buffer for electrophoresis. Samples were then added to the wells carefully and all air bubbles expelled. Electrophoresis was performed at 150v until the blue protein front has run of the bottom of the gel.

10%Resolving Gel (Reagents)	Quantity added
Protogel	6.6mls
Protogel resolving buffer	5.2mls
dH ₂ O	7.8mls
Ammonium persulphate	200μL
TEMED	20μL
Stacking Gel (Reagents)	
Protogel	1.3mls
Protogel stacking buffer	2.5mls
dH ₂ O	6.1mls
Ammonium persulphate	50μL
TEMED	10μL

Table 2.2.6.3 Composition of 10% resolving and stacking gel used for Western Blot experiments

2.2.6.4 Eletrophoretic Transfer to Membrane (Blotting)

For electrophoretic transfer, Immobilon-P PVDF membranes (Millipore, Bedford, USA) was transiently immersed in 100% methanol. The membranes were then briefly washed with distilled H₂O and soaked in transfer buffer for 5 minutes. The gel containing resolved proteins were carefully separated from the glass sheets and the stacking gel was discarded. Gels were then immersed in transfer buffer for 10 minutes at room temperature. 2 sets of fibre and filter papers were immersed in transfer buffer in preparation for use.

Following this, a fibre pad was layered with filter paper and doused with transfer buffer on a transfer casing. The gel was then placed with the protein side facing up. The Immobilon-P membrane was then layered over the gel. Any bubbles formed between the membrane and gel was removed by adding transfer buffer. A further

layer of filter and fibre pad was added. The sandwich is then doused with further transfer buffer. The casing was then sealed and transferred to a tank, filled to the top with transfer buffer. An ice pack was added to keep the tank cool and a magnetic flea placed at the bottom of the tank to circulate the transfer buffer. The apparatus was connected to an electrical circuit at 100v and placed on top of a magnetic stirrer in a cold room for 1 hour.

Upon completion of transfer, the casing and the layers of filter and fibre pads were removed, carefully extracting the membrane from the gel. The membrane was then washed in a solution of 10% milk solution (10g milk powder, 50mls PBS and 50mls PBS-Tween) on an orbital shaker for 1 hour to minimise non-specific background protein binding. Following this, the membranes were washed 3 times with PBS and once with PBS-tween. This wash process was repeated twice.

2.2.6.4 Primary Antibody Application

Primary antibody was added to a solution of 50% PBS and 50% PBS-tween in a 50mls centrifuge tube, ensuring sufficient volume to cover the membrane. The membrane is then carefully rolled-up and inserted into the centrifuge tube. This was then placed in an orbital shaker overnight at 4°C. The next day, the membrane was removed and washed 3 times with PBS and once in PBS-tween. The membranes were then washed 3 times with PBS-tween on an orbital shaker for 10 minutes.

2.2.6.5 Secondary Antibody Application

Secondary antibody was added to a solution of 50% PBS and 50% PBS-tween. Similar to the preparation of the primary antibody application, the membranes were incubated at room temperature with the secondary antibody on an orbital shaker for 1 hour. Again, the membrane was washed 3 times with PBS and once in PBS-tween. The membranes were then washed 3 times with PBS-tween on an orbital shaker for 10 minutes.

2.2.6.6 Detection of Antibody Labelled Proteins

Detection of specific protein with bound primary and secondary antibody was detected using the ECL plus western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). In this method, the secondary antibody conjugated with horseradish-peroxidase (HRP) was bound to the primary antibody attached to the protein of interest. Using this detection agent, the combined HRP and peroxidase elicits a peroxidase catalysed oxidation of luminol and subsequently enhanced chemiluminescence. The resulting light emitted was captured in a photographic X-ray film (Hyperfilm ECL Western, Amersham, UK) within seconds and minutes.

Following PBS and PBS-tween washes after secondary antibody incubation, ECL plus (Cat. No. RPN2132, Amersham, UK) reagent (a mix 2mls reagent A and 50 μ L reagent B) was applied onto the membrane and incubated at room temperature for 5 minutes. This was then placed in a plastic wallet cleaned thoroughly with 70% ethanol and excess ECL+ was removed. The membrane in the cassette was exposed to the X-

ray hyperfilms in a dark room for various times and processed using a developer until visible protein band of interest is clearly visible.

2.2.7. NFκB Assay

TransAM NFκB (Cat. No. 40096, Active Motif, Rixensart, Belgium) is a commercially available ELISA based kit to detect and quantify NFκB activation.

This kit utilises a 96 well plate coated with oligonucleotide containing NFκB consensus site (5'-GGGACTTCC-3'). The activated NFκB P65 subunit binds specifically to this oligonucleotide.

Using an antibody that binds to this the NFκB-oligonucleotide complex allows detection of activated NFκB. The primary antibodies detect NFκB by recognising the P65 epitope which is accessible only when NFκB is activated and bound to the target DNA. 0.5µg of cellular extract is recommended by manufacturer for optimum results. The procedural principles are otherwise similar to ELISA techniques as described in 2.1.3. This assay provides detection and quantitative measures of activated NFκB within 5 hours. This assay kit has a detection limit of <0.5µg nuclear or whole cell extract/well. This provides a detection range of 0.2-0.5µg of nuclear or whole cell extracts.

2.3 Ghrelin receptor and gastric motility study

2.3.1 Immunohistochemistry of archival samples

Immunohistochemistry is the localization of antigens or proteins in tissue sections by the use of labelled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, or colloidal gold. This method was used to detect neuromuscular agents implicated in gastric motility.

2.3.2 Immunohistochemistry methods

Gastric tissues taken from patients who did not have chemotherapy and in those receiving oesophageal-chemotherapy (cisplatin + 5-fluorouracil) or gastric chemotherapy (Cisplatin, 5-fluorouracil and capecitabine); n=6 each. Histological paraffin embedded tissue blocks were used for IHC. 5µm thick sections were baked onto 3-aminopropyltriethoxy-silane coated slides. Specific antibodies were used to look for the expression of specific antigens.

After comparison against enzymatic digestion, (Sigma α -chymotrypsin, Cat. C-4129) and pH 6 buffer (Vector Antigen Unmasking Solution Cat. H-3300), optimum retrieval was performed by pressure cooking. Antigen retrieval was carried out by pressure cooking in Tris EDTA buffer pH 7.8 for 80 seconds. A commercially available tween-based buffer was used to prepare all solutions used in this technique (excepting DAB) for all washes, detection kit solutions and antibody dilutions

(Menarini Optimax Wash Buffer Cat. HK 583-5K). Endogenous peroxidase was blocked by incubating sections in a 1.5% Hydrogen peroxide solution for 20 minutes.

Vector Universal Elite ABC kit (Cat. No. PK-6200; Burlingame, CA, USA) with a diaminobenzidine tetrahydrachloride visualisation agent (Vector ImPACT DAB substrate, Cat. No. SK-4105, Burlingame, CA, USA) was used for detection of bound antibody. Following a serum block of 20minutes at room temperature, primary antibody was applied to the sections for 1 hour, also at room temperature. Optimised antibody dilutions were determined for motilin receptor (MBL International Corp, Woburn, MA, USA, Cat. No. LS-A134), ghrelin receptor (Chemicon, Billerica, MA, USA, Cat. No. AB9543), nNOS (Abcam, Cambridge, UK, Cat. No. ab40662) and AChE (Abcam, Cambridge, UK, Cat. No. ab2803) (see Table 2.3.2)

Sections were then washed twice, 5 minutes each time. The avidin-biotin complex solution was applied to the sections, and incubated for 30 minutes at room temperature (Vector Elite ABC reagent). Sections were then washed for 5 minutes in buffer. DAB solution was prepared according to the manufacturer's instructions using de-ionised water, and applied to the sections for 5 minutes (Menarini Concentrated Substrate Cat. HK 153-5K.). Sections were rinsed with de-ionised water, and counterstained for 1 minute in Mayers Haematoxylin. Sections were blued in Scott's tap water, dehydrated, cleared and mounted.

Antibody	Dilution
5HT4 Receptor (Novus Biologicals Inc, Littleton, CO, Cat No: NLS656)	1/300
Motilin Receptor (MBL International Corp, Woburn, MA. Cat no; LS-A134)	1/1500
Growth Hormone Secretagogue Receptor (Chemicon, Billerica, MA. Cat No; AB9543)	1/1000
nNos (Abcam, Cambridge, UK. Cat No; ab40662)	1/10,000
Acetylcholinesterase (Abcam, Cambridge, UK. Cat No; ab2803)	1/350

Table 2.3.2 shows optimum dilutions of the commercially available primary antibodies against motility agents of interest. All antibodies have been tested previously for immunohistochemistry based on product specification by the manufacturer.

2.4. Gastric Contractility Methods

Gastric specimens were obtained at the time of surgery from the tumour free margins of the stomach. The specimens were washed with saline to remove any debris or blood, and 2 sutures were placed to identify the longitudinal orientation. It was then immediately immersed in cold carbogenated (bubbled with 95% O₂/ 5% CO₂ for at least 2 hours) Gey's salt solution overnight at 4°C to preserve tissue viability. In some cases, the following morning the tissues were placed in fresh carbogenated Gey's solution and then transported to the laboratory.

On arrival the specimens were placed in fresh, oxygenated Krebs solution (containing in mM NaCl 121.5, CaCl₂ 2.5, KH₂PO₄ 1.2, KCl 4.7, MgSO₄ 1.2, NaHCO₃ 25, glucose 5.6) which had been equilibrated with 5% CO₂ and 95% O₂. The mucosa was removed and strips (2-4 x 10-15 mm) were cut parallel to the longitudinal or the circular muscle. The strips (2-4 from each patient) were mounted in tissue baths (10 ml) containing Krebs solution at 37°C and gassed with 5% CO₂ in O₂. Tension was measured using Dynamometer UF1 force-displacement transducers (Pioden Control Ltd., UK). Data acquisition and analysis were performed using MP100 hardware and AcqKnowledge software (Biopac Systems Inc., USA). Tissues were initially suspended under 2-3g tension and allowed to equilibrate for at least 45 min during which time bath solutions were changed every 15 min. During this time, muscle tension stabilised at ~1 g.

At the end of the equilibration period, longitudinal muscle strips were stimulated via two parallel platinum ring electrodes connected to a stimulator (STG2008, Scientifica

Ltd, UK). The stimulation parameters were 50 V (~200 mA), 0.5 ms bipolar pulse duration, applied for 10 s, every 1 min. The frequency was changed every 3 min to produce a frequency-response curve, using frequencies of 1, 2, 5, 10, 15 and 20 Hz.

After washing, 10 μ M erythromycin was added for 15 min. The tissues were washed and left for 30 min. For both longitudinal and circular muscle strips, a carbachol concentration-response curve (1nM – 10 μ M) was then constructed, with each additional concentration being added after a maximum response was observed to the previous, usually between 2-8 min). Changes in muscle tension (g) evoked by each carbachol concentration were expressed in grams.

2.5 Statistical Analysis

All statistical analysis were performed using the GraphPad Prism software version 4.0. Threshold for significance is $p < 0.05$.

2.5.1 Chapter 3 “Ghrelin in Crohn’s Disease”

Data was described in non-parametric terms and comparisons were made using Wilcoxon’s Matched pair non-parametric tests. Values were expressed in median (range). Interquartiles ranges is incorporated in the ghrelin graphs.

2.5.2 Chapter 4 “Ghrelin, NFκB and Human Lymphocytes”

Data in this chapter is reported as mean±SEM. Statistical analysis is undertaken using the paired student's t-test to compare mean values.

2.5.3 Chapter 5 “Cancer Chemotherapy and Gastric Contractility”

Data in this chapter is expressed as mean±SEM or mean with ranges. In the gastric contractility study, differences in response to carbachol between the chemotherapy exposed and non-chemotherapy exposed tissues were analysed using the 2-way ANOVA with Bonferroni post-hoc test. Data from immunohistochemistry study was expressed in mean±SEM and analysed using the Student's paired t-test.

Chapter 3

Ghrelin in Crohn's Disease

3.1.1 Overview

Crohn's disease is a form of inflammatory bowel disease characterised by patchy, transmural inflammation that can affect any areas of the gastrointestinal tract. The aetiology of Crohn's disease is unknown, it is understood to have several contributing factors including genetic, environmental, infective and even psychological. The disease pattern varies, although the terminal ileum remains the most commonly affected site. It can also be differentiated into fistulating, inflammatory or structuring disease. Mainstay of treatment is immune-suppression for mucosal healing and alleviation of symptoms.

3.1.2 Prevalence and incidence of Crohn's disease

Crohn's disease has an incidence of 5-10 per 100,000 per year with a prevalence of 50-100 per 100,000 (Loftus, Jr. et al. 2004). There has been high incidence of CD in certain ethnic groups eg. Ashkenazi Jews, suggesting a genetic predisposition in an endogamous population. Peak incidence of CD is between ages 10 to 40 years.

3.1.3 Pathogenesis of Crohn's disease

Crohn's disease was thought to arise from an environmental precipitant in a genetically susceptible individual. Among environmental agents implicated in Crohn's disease are infection, drugs, diet, vaccination, water supply and cigarette smoking (Calkins et al. 1989). Smoking is not only implicated as a precipitant but also associated with a more phenotypically aggressive course of disease. Women with CD who smoke are more likely to have recurrent surgery (Sutherland et al. 1990) and likely to require immunosuppressant because of recurrent disease (Cosnes et al. 1999). Dietary association of CD is inconsistent although low residue and high refined sugar diet were implicated (Riordan et al. 1998).

There has been considerable interest in genetic mutations that are implicated in CD. The first Crohn's susceptibility gene (IBD1) was identified in 2001 on Chromosome 16 (NOD2/CARD15) (Hugot *et al.* 2001). The relative risk of developing Crohn's disease following mutation of this gene locus is increased by 2-4 fold if heterozygote and 28-40 fold if homozygous. Mutation in this gene locus is associated with a decrease in NF κ B activity in response to bacterial LPS and peptiglycans. This impairs the ability to clear bacterium and fails to offer protection to the intestinal barrier to bacterial invasion. This mutation is associated with small bowel CD in Caucasian population. Other lesser known genetic associations include OCTN1 and 2 on chromosome 5 and DLG5 on Chromosome 10 (Gazouli et al. 2005).

3.1.4 Clinical features of Crohn's disease

The cardinal symptoms of CD include chronic diarrhoea, weight loss and abdominal pain. Colonic involvement of disease can also include rectal bleeding and urgency. Fever, malaise and anorexia can be manifestations of CD. In more severe phenotypes of CD, fistulae (see below) and abscess formation is a common feature. In addition, chronic luminal inflammation may lead to structuring disease and can result in bowel obstruction, which occurs in 20-30% of patients. Clinical features of CD can vary depending on the site of disease. Small intestinal involvement presents with diarrhoea, weight loss, abdominal pain and malabsorption. Colonic involvement frequently presents with diarrhoea, rectal bleeding and pain. Anorectal disease may involve perianal abscess formation, fistulation and fissures. CD is also known to involve the stomach and oesophagus and usually presents with odynophagia, abdominal pain, dyspepsia, bleeding and dysphagia.

Fistula formation is a frequent complication of luminal enteric CD. Entero-enteric, entero-vesical and entero-peritoneal fistulations are typical in CD. Patients can present with pyrexia, abdominal pains, pneumaturia, recurrent urinary infections and also cutaneous discharge. Initial symptoms of obstruction are typically due to intestinal inflammation and oedema. Persistent active disease will lead to progressive fibrosis, causing a fixed stenosis of the bowel. Mucosal inflammation in CD is transmural, hence it is possible that perforation of the bowel can occur. It is a rare complication of CD but can be serious and life threatening. Very occasionally, perforation can be the initial presentation which leads to the discovery of CD.

Other clinical features of CD are aphthous ulceration, fatigue, iron deficiency anaemia, osteoporosis and bile acid malabsorption. There is an increased incidence of bowel malignancy in long-standing CD, particularly in the diseased colon. Surveillance colonoscopy is therefore recommended 10 years after disease development. There are other systemic complications that are associated with CD (Table 1). These extra-manifestations of CD occur in about 24% of patients (Rankin et al. 1979).

Related to disease activity	Erythema nodosum Aphthous ulceration Episcleritis Uveitis Episcleritis Erythema nodosum Arthritis Osteoporosis Pyoderma gangrenosum
Not related to disease activity	Gallstones Sacroileitis Ankylosing spondylitis Osteomalacia Primary sclerosing cholangitis

Table 3.1 Illustrates the extraintestinal manifestations in Crohn's disease, both related and unrelated to disease activity.

3.1.5 Diagnosis of Crohn's Disease

The diagnosis of Crohn's disease should be sought in patients with history of chronic persistent bloody diarrhoea, recurrent perianal fistulation and abscess, abdominal pain, fever of unknown origin and intestinal malabsorption. Laboratory tests are usually non-specific. If there is small bowel involvement, there may be deficiencies of vitamin B12, iron and vitamin D. Significant diarrhoea can cause electrolyte disturbances, namely hyponatraemia, hypokalaemia and hypomagnesaemia.

Low albumin can reflect luminal disease with protein losing enteropathy, malabsorption or significant inflammatory activity. Anaemia is a common finding, which may result from gastrointestinal blood loss or chronic inflammation affecting the bone marrow. Finally, abnormalities of the liver function can represent an associated liver disease eg. Primary sclerosing cholangitis, which is a condition, related to CD. A combination of endoscopic, radiological and pathological examination is usually required to establish not only the diagnosis but also the extent of the disease.

Endoscopic examinations e.g. Colonoscopy can firstly establish any macroscopic inflammation in the colon and terminal ileum, with the benefit of biopsies to confirm. Moreover, colonoscopy can also be used to assess disease extent and severity. In addition, endoscopic examination of the bowel can be used for cancer surveillance in patients with duration of disease exceeding 10 years. Typical endoscopic findings are ulceration of aphthous and deep fissuring, granular mucosa with friability,

‘cobblestone’ appearances reflecting the irregular mucosa due to submucosal inflammation, pseudopolyps, patchy inflammation with ‘skip’ lesions and also stenosing disease. MRI visualisation of the small bowel has become an important modality in assessing the disease in extent, activity and also assessing for complications of CD. Static and dynamic images obtained are particularly helpful in assessing the disease. More recently, capsule endoscopy is shown to be a useful tool in diagnosing more subtle CD particularly in the small bowel, which is currently difficult to reach with push endoscopy. Barium studies are helpful in characterising and assessing the luminal disease extent. Depending on the expertise of the radiologist, small bowel barium enema is an alternative method of examining the extent of disease in the small bowel. Typical finding of CD in barium studies are ulceration, loss of mucosa detail, mucosal oedema and structuring disease.

Histological diagnosis of CD are made with finding of cryptitis, crypt abscesses, inflammatory cell infiltrate affecting all layers of the bowel wall, ulceration and also the presence of non-caseating granulomata, a collection of macrophages with or without giant cells, are more suggestive of CD.

3.1.6 Treatment of CD

The treatment of CD is complex, as it is dependent on the site, severity and type of disease. Treatment of CD involves mainly immunomodulation which is why other causes of similar symptoms have been considered including infection. Ileocolonic disease can be treated initially with mesalazine, a 5-aminosalicylates (5-ASA) in mild

disease (Hanauer et al. 2004). Failure to respond to mesalazine warrants steroid treatment which is tapered gradually over 2 months. Budesonide is a suitable alternative to systemic corticosteroid compared to placebo which can be used in ileocaecal disease (Seow et al. 2008).

In minor distal colonic disease topical treatments with 5-ASA and steroids are effective treatments in delivering the drug to diseased areas. In more severe cases, intravenous steroids e.g. hydrocortisone 100mg four times daily is suitable for severe in-patient management of an acute flare. Concomitant use of metronidazole with corticosteroids are recommended especially in cases where septic complication is suspected. For maintenance therapy, 6-thiopurines (Azathioprine and 6-Mercaptopurine) are considered for rapid escalation in mesalazine treatment failure. Thiopurines are recognised as effective steroid sparing agents.

In patients who failed thiopurine treatment, anti-TNF therapy has been demonstrated to be an effective treatment for CD. Infliximab is a chimeric antibody active against TNF α is an effective treatment for moderately severe Crohn's disease (Hanauer *et al.* 2002). It is effective in both induction of remission and maintenance therapy. Further discussions regarding the modes of action, side effects and efficacy of these treatments are beyond the scope of this thesis. Finally, nutritional support should always be integral part of CD treatment in every patient. Consideration of elemental or polymeric feeds and total parenteral nutrition can be considered as part of treatment of CD.

In perianal disease, metronidazole and ciprofloxacin are recommended first line treatment. In complex fistulating disease, azathioprine and infliximab are effective medical management. Surgery, including seton drainage and fistulectomy are appropriate alternative to failed medical therapy. In all complex disease, treatment is based on assessment of anatomy using imaging, availability of nutritional support and assessment for surgery. Surgery should always be considered particularly when medical therapy has failed, or in event of transmural disease complications eg. abscess formation, perforation or fistulating disease.

3.2 Study design : Ghrelin profiling in patients with Crohn's disease

As discussed in chapter 1 of this thesis, ghrelin may have immune-modulating properties. In general, the evidence from intact animal studies suggests that ghrelin may be anti-inflammatory. Currently there are no human studies in support of this finding. The suggested ability of ghrelin to reduce inflammation raises an exciting prospect that may prove to be useful in Crohn's disease where anti-inflammatory properties are desirable. The other possible clinical use of ghrelin would be alleviation of cachexia which is a common presentation in presence of active inflammation.

The relationship between ghrelin and inflammation is poorly understood. It is quite possible that inflammation will reciprocally regulate ghrelin, which could be a plausible mechanism of appetite loss and cachexia in patients with active disease.

In this study, we hypothesize that circulating ghrelin concentration may change when TNF α activity is altered by infliximab in patients with Crohn's disease. We investigated the effect of infliximab infusion on the total, acylated and desacyl isoforms of ghrelin in patients with Crohn's disease and related this to changes in biochemical and clinical measures of inflammation in Crohn's disease. It is quite possible that if inflammatory process were altered, this will in turn affect circulating ghrelin concentration in a dynamic fashion.

3.3 Methods

3.3.1 Patient characteristics

Fifteen patients with Crohn's disease who qualified for infliximab treatment were recruited. These include patients with moderately severe disease with fistulation, who are refractory to other immunomodulators or in which surgery is avoided. Exclusion criteria include patients with gastrectomy or gastric by-pass surgery as this may affect the ghrelin excretion and regulation. Patients with ulcerative colitis and indeterminate diagnosis were also excluded.

Mean age for all patients was 31 (range 21-50) and 9 were male. Mean body mass index was 23 (range 21-34). Table 1 shows demographic and clinical characteristics of each patient. There was no change in drug treatment during the study.

3.3.2 Protocol

Detailed experimental protocols are discussed in Chapter 2. Briefly, each patient was studied on two occasions, immediately before and 1 week after an infusion of infliximab (5mg/kg). In 5 patients, the study done “one week after” infliximab was performed first followed by the “immediately before” study performed just before the next infusion usually 7 weeks later. On each occasion, an 18G cannula was inserted into a forearm vein.

An initial fasting blood sample was taken followed by consumption of a 450 Cal meal by the patient. Following this, further samples were taken every 20 minutes for 2 hours. Blood samples were centrifuged immediately, plasma extracted and stored at -80⁰ Celsius for later analysis. Assays were performed for plasma total ghrelin, acylated ghrelin, desacyl ghrelin, C-reactive protein and a panel of cytokines. The Harvey Bradshaw Activity Index (HBAI) were calculated for each patient on each study occasion.

3.3.3 Assays

All samples were assayed in duplicate. Only plasma samples were used. Product specific protocols for commercially available assays were followed. General principals and assay techniques is described in Chapter 2.

3.3.3.1 Total ghrelin and desacyl ghrelin assays

Total ghrelin, was measured by ELISA (Diagnostics Systems Laboratory, Texas, USA; Catalogue DSL-10-33700). Desacyl ghrelin was assayed using an EIA kit (SPI-BIO, Montigny le Bretonneux, France; Catalogue A05119). The Tecan GENios plate reader with Magellan ver.4 software was used to analyse the results.

3.3.3.2 Acyl ghrelin

Acylated ghrelin was assayed using Linco Gut Panel Multiplex (Linco Research, St. Charles, MO, USA; Catalogue HGT-68K). A multiplex analyser, the Bio-Plex system (Bio-Rad Laboratories Inc.) were used to analyse the samples. Results were generated using Bio-Plex Manager software ver.4.

3.3.3.4 C-Reactive Protein (CRP)

CRP was measured by ELISA using the High Sensitivity C-Reactive Protein EIA (Life diagnostics Inc., West Chester, PA, USA; Catalogue 2210).

3.3.3.5 Cytokines

TNF α , IL-1 β , IL-6 and IFN- γ was measured using the High Sensitivity Human Cytokine Lincoplex Kit (Linco Research, St. Charles, MO, USA; Catalogue: HSCYTO-60SK) with the Bio-Plex system (Bio-Rad Laboratories Inc.). Results were generated using Bio-Plex Manager software ver.4.

3.4 Data processing

The principles of data processing was initially agreed with Professor Chuka Nwokolo and adhered to strictly. Data loss was defined as failure of an assay to record a value for a time point. Since assays were done in duplicate when data was unavailable for one or both wells this was regarded as data loss for that time point. If a single time point is lost a value is calculated which is the average of the values of the 2 adjacent time points. If the lost time point is either the first or last time point in the profile then the single adjacent value was assigned to that time point. If data is lost for 2 adjacent time points in a 2 hour profile such a profile was declared invalid and the patient excluded from the final analyses. The reason for data losses is unclear but may be related to post venopuncture processing, including acute haemolysis that may interfere with the assays, a faulty batch of blood collection tubes or degradation of the protein prior to analysis.

3.5 Statistics

For each patient, a 2 hour profile of ghrelin concentration before and after infliximab is available. From this data a 2 hour integrated ghrelin concentration or area under the curve (AUC) for the study period was calculated using the trapezoid rule.

CRP, cytokines, and glucose and insulin were measured in fasting samples taken before infliximab in the first study occasion and after infliximab on the second study occasion or vice-versa

Data is described in non-parametric terms and since all comparisons were paired the Wilcoxon's matched pair non-parametric test was used throughout. Graphpad Prism software was used for the statistical analysis.

3.6 Ethical Considerations

The study was approved by Coventry Research Ethics Committee and the R & D Department of the University Hospital of Coventry. Written informed consent was obtained for all participating patients.

3.7 Results

Following adjustment for data loss, data was available for 11, 9 and 15 patients in the total ghrelin, acylated ghrelin and des-acyl ghrelin groups respectively. Patient demographics are illustrated in table 3.2. The assay for acylated ghrelin was subject to most data loss partly because acylated ghrelin is a small fraction of all circulating ghrelin isoforms. Moreover, this form of ghrelin is very unstable and vulnerable to breakdown by protease activity, hence a half-life estimated to be 9-13 minutes (Akamizu et al. 2004).

Patient	Age	Gender	BMI	Disease site	Immunomodulation	Previous Surgery
1	27	F	22	Perianal, colonic	Azathioprine	No surgery
2	22	M	34	Ileal	Azathioprine	No surgery
3	21	M	21	Ileal, colonic	Azathioprine	No surgery
4	50	M	24	Perianal	Azathioprine	No surgery
5	27	M	22	Colonic	Azathioprine	No surgery
6	23	M	23	Ileal, colonic	Azathioprine	No surgery
7	30	M	22	Ileal, colonic	Azathioprine	Panproctocolectomy
8	39	F	21	Ileal, colonic, perianal	None	Panproctocolectomy
9	29	F	24	Ileal, perianal	None	Right hemicolectomy
10	45	M	21	Colonic, perianal	Azathioprine	No surgery
11	30	M	21	Colonic, perianal	Azathioprine	No surgery
12	23	M	19	Ileal, colonic	Azathioprine	Right hemicolectomy
13	49	F	25	Ileal, colonic	Azathioprine	No surgery
14	34	F	24	Ileal	Azathioprine	Right hemicolectomy
15	24	M	21	Colonic	Azathioprine	No surgery

Table 3.2. Patient demographics. Total of 15 patients participated in the study. 10 patients were male. Mean age was 31 and average BMI was 23. 13 patients were on Azathioprine and 5 patients underwent gastrointestinal surgery for Crohn's disease.

3.7.1 Ghrelin

3.7.1.1 Total ghrelin (n=11)

Figure 3.2 shows that following infliximab infusion there was a higher median concentration of total ghrelin during the 2 hour study period. It also shows a typical post-prandial decrease in plasma ghrelin in both profiles. Figure 3.3 shows plasma integrated total ghrelin for the 2 hour study period increased in 9 of the 11 patients following infliximab. Figure 3.4 and table 3.3 show that median plasma integrated total ghrelin for the 11 subjects increased by 25% from 162 (82-738) pg/ml.hr before infliximab to 200 (101-792) pg/ml.hr. ($p=0.02$) one week after infliximab.

4 patients were excluded from analysis due to data losses as defined in section 3.4. Three patients had data losses of more than 2 adjacent time points, in which 2 patients were involving the last time point and 1 patient involving the first time point. In one other patient, complete data loss was seen in the post-infliximab analysis apart from time point 0. All 4 patient samples were repeated with same findings which rendered them unsuitable for analysis.

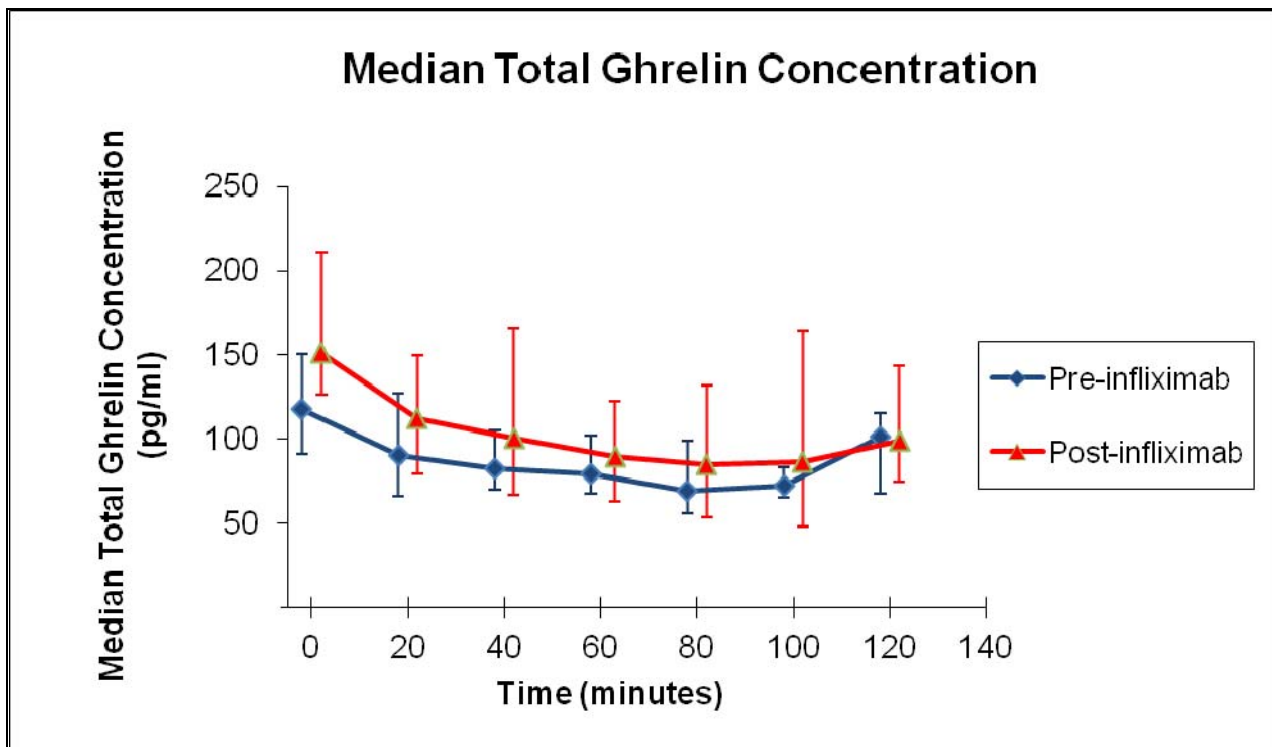


Figure 3.2. Median (inter-quartile range) plasma total ghrelin (pg/ml) concentration profiles (N=11) pre and post-infliximab infusion with interquartile range. A 450 Cal meal was eaten immediately after the fasting blood sampling at $t=0$. Blood was sampled every 20 minutes. Interquartile ranges were calculated for each 20 minute time point. Each data point has been separated at every 20 minute interval to demonstrate the interquartile range. All samples obtained from each time-point were assayed in duplicates. Both curves show the typical post-prandial decrease in total ghrelin. Median values were compared using the non-parametric Wilcoxon's matched pair test. GraphPad prism software was used to analyse results

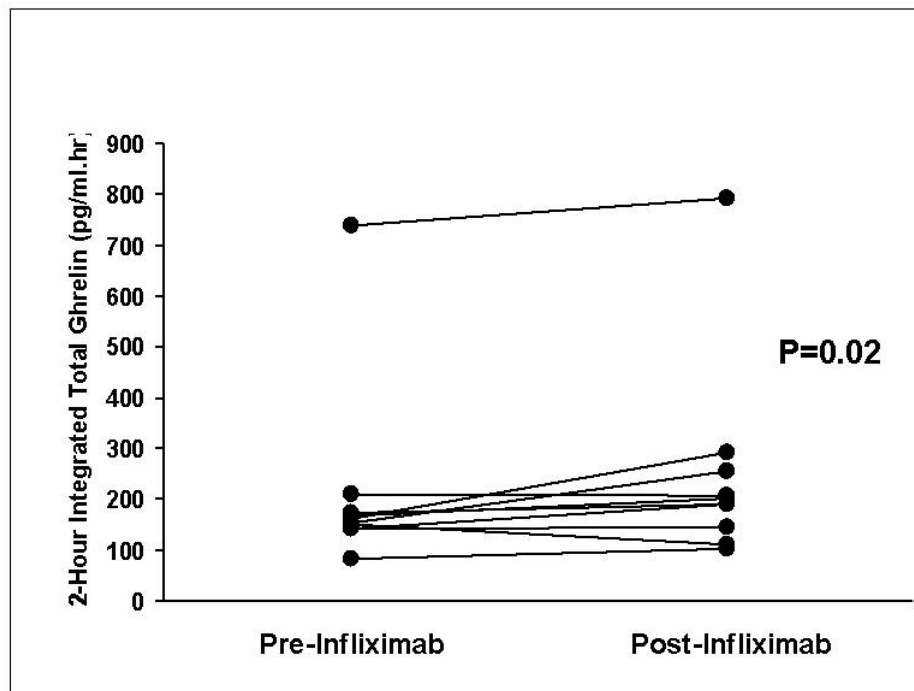


Figure 3.3. Two-hour integrated plasma total ghrelin (pg/ml.hr) (N=11) pre and post infliximab infusion for each individual patient. Integrated ghrelin is increased in 9 of 11 patients after infliximab (p=0.02). Median values were used for comparison using the non-parametric Wilcoxon's matched pair test performed using GraphPad prism software.

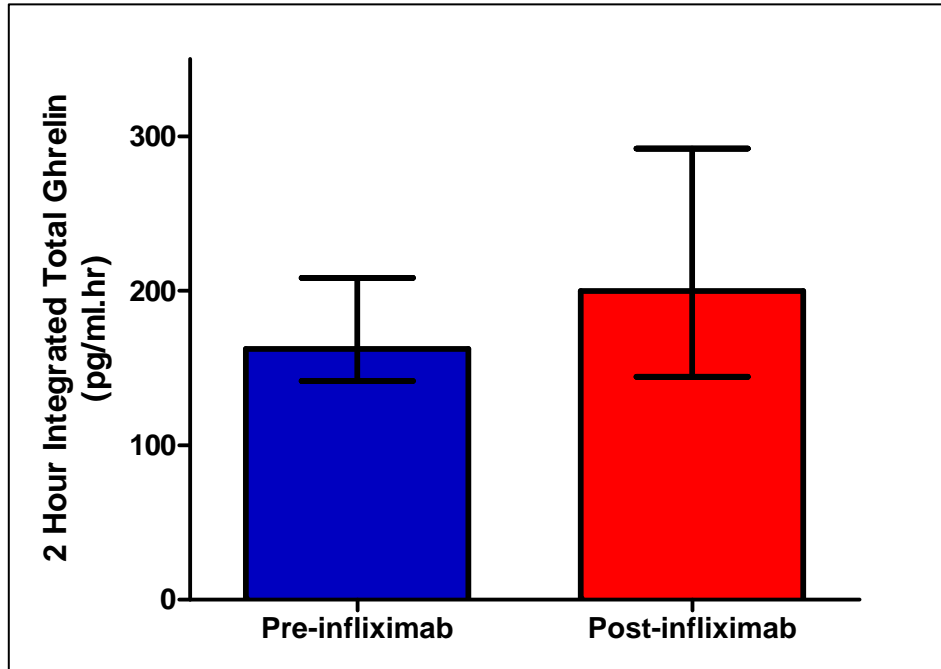


Figure 3.4. Median integrated plasma total ghrelin AUC (pg/ml.hr) (N=11) pre- and post-infliximab infusion. Wilcoxon's non-parametric matched pair test was used to compare pre- and post-infliximab median values. Statistical analysis was performed using GraphPad Prism software. Median (inter-quartile range) integrated total ghrelin increased by 25% from 162 (141-208) pg/ml.hr before to 200 (144-292) pg/ml.hr one week after infliximab infusion (p=0.02).

3.7.1.2 Acylated ghrelin (n=9)

Figure 2.5 shows the profiles of median plasma acylated ghrelin concentration before and after infliximab. A normal post-prandial decrease in ghrelin is absent in the before infliximab profile. However in the after infliximab profile, at 20 min post-meal, median acylated ghrelin concentration decreased below the fasting concentration (Table 2.2) and was also significantly lower than the same time point in the before infliximab profile (38.6 (21.9-92.0) pg/ml vs 50.3 (20.1-105.6) pg/ml) ($p=0.04$). Thus in the after infliximab profile the traditional meal-related ghrelin curve with its post-prandial dip is restored. Over the 2 hour study period integrated acylated ghrelin increased non-significantly from 91 (39-196) pg/ml.hr before infliximab to 102 (40-194) pg/ml.hr after infliximab (Table 2.2), a trend similar to that observed for total ghrelin.

Significant data loss was observed in acylated ghrelin analysis despite utilising a high sensitivity bioplex assay. Acylated ghrelin has a very short half-life and sensitive to protease degradation. Six patient samples were excluded from analysis due to data losses. In 5 patients, acylated ghrelin was undetectable by the bioplex assays in both pre- and post-infliximab samples, presumably related to post venopuncture processing. With the benefit of hindsight, it is possible that a different protease inhibitor may have limited such data losses. In one patient, sampling errors were reported by the bioplex analyser and results were unobtainable, possibly due to an unknown contaminant or significant haemolysis of the blood sample interfering with the assay.

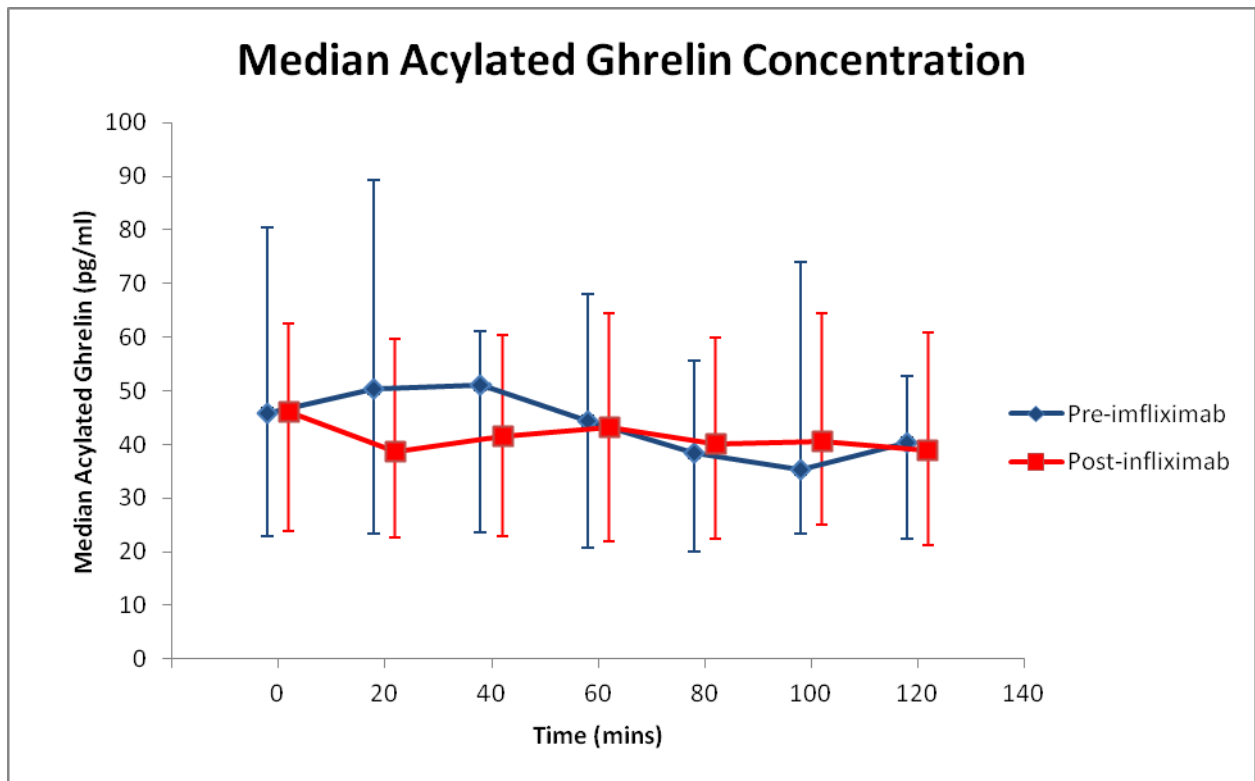


Figure 3.5. Median (interquartile range) plasma acylated ghrelin concentration (N=9) pre and post infliximab infusion. Each data point has been separated at every 20 minute interval to demonstrate the interquartile range. Unlike the pre-infliximab profile, the post-infusion shows a typical post-prandial decrease of acylated ghrelin with plasma acylated ghrelin concentration at t=20mins being significantly lower than the same time point in the pre-infliximab curve (38.6 (22.8-59.63) pg/ml vs 50.3(23.3-89.23) pg/ml) (P=0.04). Total AUC pre- and post-infliximab was not significantly different. Wilcoxon's matched pair non-parametric test was used to compare median results using GraphPad prism software.

3.7.1.3 Des-acylated ghrelin (n=15)

There was no statistical difference in integrated des-acylated ghrelin which was 397 (153-1106) pg/ml.hr before infliximab and 423 (251-888) pg/ml.hr after (Table 2.2).

	<u>Pre-Infliximab</u> (95% CI)	<u>Post-Infliximab</u> (95%CI)	<u>P value</u>
Total Ghrelin AUC (pg/ml.hr)	162 (99-341)	200 (128-387)	0.02
Acylated Ghrelin (pg/ml) at t=20 minutes	50.3 (24-64)	38.6 (26-82)	0.04
Acylated Ghrelin AUC (pg/ml.hr)	91 (49-142)	102 (47-139)	0.38
Des-acylated Ghrelin AUC (pg/ml.hr)	397 (356-648)	423 (331-651)	0.63

Table 3.3. Summary of ghrelin results. Median integrated total ghrelin (N=11) increased significantly from 162 to 200 following infliximab. Median acylated ghrelin (N=9) at t=20minutes decreased significantly from 50.3 to 38.6 following infliximab, suggesting restoration of the traditional post-prandial decrease of ghrelin following a meal. However, median integrated acylated ghrelin and desacyl ghrelin did not change significantly.

3.7.2 Cytokines (Table 2.3)

3.7.2.1 TNF α

Median (range) TNF α concentration decreased significantly from 2.8 (0.4-5.85) pg/ml before infliximab to 1.31 (0-2.08) pg/ml (p=0.002) after infliximab.

3.7.2.2 IL-1 β , IL-6, and IFN- γ

Before and after infliximab median (range) IL-1 β was 0.7 (0.27-2.82) and 0.89 (0.37-3.37) respectively, median (range) IL-6 was 20.6 (4.79-52.49) and 20.7 (4.21-48.22) respectively and median (range) IFN- γ was 10.8 (1.23-45.84) and 7.3 (0-46.71) respectively. There was no significant difference.

	<u>Pre Infliximab</u> <u>(95% CI)</u>	<u>Post Infliximab</u> <u>(95% CI)</u>	<u>P value</u>
TNFα (pg/ml)	2.8 (1.89-4.48)	1.31 (0.73-2.06)	0.002
IL-1β (pg/ml)	0.7 (0.55-1.76)	0.89 (0.69-1.96)	0.16
IL-6 (pg/ml)	20.6 (13.0-29.9)	20.7 (12.5-27.9)	0.10
IFNγ(pg/ml)	10.8 (4.7-22.3)	7.3 (4.6-23.9)	0.70

Table 3.3 Summary of cytokine results (N=15). TNF α was the only cytokine which decreased significantly from 2.8 to 1.31 following infliximab. There were no significant changes in IL-1 β , IL-6 and IFN- γ . Median values are expressed and comparisons were made using the non-parametric Wilcoxon's matched pair test performed with GraphPad prsim software.

3.7.3 C-Reactive Protein

CRP concentration decreased non-significantly from 2.1 (0-17) mg/ml before infliximab to 0.7 (0-7.8) mg/ml after infliximab.

3.7.4 Harvey Bradshaw Activity Index

Median (range) HBAI decreased by from 5 (2-28) before infliximab to 2 (0-22) after infliximab, ($p=0.001$).

3.8 Discussion

This study design extending over a 2 hour period took account of the pulsatile nature of ghrelin secretion in humans. The eating of a meal was also included in the study period to test ghrelin regulatory mechanisms. As expected, a post-prandial decrease in ghrelin followed by recovery over 2 hour period was observed presumably when food leaves the stomach. We were able to report and compare data as “area under the curve” and 2 hour concentration profiles immediately before and one week after the infusion of infliximab to improve our detection of changes in circulating ghrelin concentration.

This study provides novel evidence that systemically altering an important inflammatory mediator like TNF α can lead to changes in circulating ghrelin. The infusion of infliximab led in Crohn's disease patients to a 25% increase in total ghrelin concentration assessed during a 2 hour period after a meal. As expected there was a significant decrease in TNF α and Crohn's disease activity following infliximab treatment. A similar trend was observed for CRP. The relatively low concentrations of TNF α and CRP reflect that our study patients, the majority in maintenance infliximab programmes were in clinical remission. It would be of interest to study patients that start with a higher inflammatory burden who we believe would show an exaggeration of the changes in ghrelin observed in our study. It is possible that ghrelin-producing cells can sense and respond to changes in circulating TNF α .

This mechanism may operate at the cellular level with circulating TNF α exerting a direct effect on gastric ghrelin producing cells. Another hypothesis is that TNF α might act centrally and modulate circulating ghrelin through the efferent vagal nerves to the stomach. In a recent study, rats that were rendered hypophagic by TNF α administration were found to have decreased gastric and circulating ghrelin leading to the conclusion that some of the anorexic effects of TNF α are mediated via ghrelin. In that study the effects of hypophagia induced by TNF α could be reversed by administration of ghrelin (Endo *et al.* 2007b).

Circulating acylated ghrelin was often referred to as "active" ghrelin. This isoform stimulates hunger and growth hormone release by acting on hypothalamic nuclei. In our Crohn's patients, at a time of maximum inflammatory activity, the acylated ghrelin concentration at the 20 min time point is higher than the preceding fasting

value. There was no post-prandial decrease in acylated ghrelin concentration which is an accepted feature of a normal ghrelin curve. Similar abnormalities in post prandial ghrelin profiles have been reported in obesity and non-alcoholic steato-hepatitis, 2 diseases that have a significant inflammatory contribution to their pathophysiology (English *et al.* 2002; Sajjad *et al.* 2005). Following infliximab infusion in our patients the acylated ghrelin curve was normalised and the 20 min ghrelin concentration is lower than the preceding fasting value. Inflammation in general and perhaps TNF α specifically affects not only total ghrelin production but modifies the mechanisms that regulate ghrelin production in the stomach.

The study also showed that des-acyl ghrelin an isoform which still has an uncertain physiological role was not affected either in terms of its profile or area under the curve by infliximab. In conclusion, this study provides novel evidence that altering TNF α with a biological agent can affect circulating ghrelin in humans. It adds further evidence that ghrelin and inflammatory mediators interact in inflammatory diseases like Crohn's disease pointing to a possible immuno-modulatory role. Our study suggests that neither des-acyl nor acylated ghrelin mediate this role raising the possibility that a yet unrecognised biological isoform could be involved.

Inter-assay variability between the 3 assays used could explain the difference in results seen with the ghrelin isoforms. An infliximab-induced increase in circulating plasma ghrelin if accompanied by increased food intake suggests another mechanism by which this drug might improve well-being in Crohn's disease patients.

Chapter 4

Ghrelin, NFκB and Human Lymphocyte

4.1. Introduction

In Chapter 1, ghrelin has many physiological functions, of which hunger and energy homeostasis is most established. However, recent studies suggest that it may have an immune-modulatory role. In animal studies ghrelin seems to protect against inflammatory damage (Dembinski *et al.* 2003; Chang *et al.* 2003; Granado *et al.* 2005). The presence of ghrelin receptors on human immune cells (Hattori *et al.* 2001) and the suppression of inflammatory cytokine (IL-1 β , IL-6 and TNF α) secretion by these cells when exposed to ghrelin in-vitro suggests a possible immuno-modulatory role. This effect appears to be opposite to that of leptin (Dixit *et al.* 2004). Another study with similar findings demonstrated that ghrelin not only inhibits the proliferation of splenic T-lymphocytes but also decreases Th₁ (IL-2 and IFN- γ) and Th₂(IL-4 and IL-10) cytokines mRNA expression (Xia *et al.* 2004b). In Chapter 3 of this thesis, the study has demonstrated that modulation of inflammation can have a reciprocal effect on plasma total ghrelin. This chapter investigates the effect of ghrelin at the cellular immune level, in particular human B-lymphocyte, on NF κ B activation.

A nuclear factor kappa B (NF κ B) a nuclear transcriptional factor discovered in 1986 (Sen *et al.* 1986), is involved in the cellular responses to an environmental stress, by inducing the production of protective signals. NF κ B is held inactive in cellular cytoplasm by inhibitory kappa B (I κ B). NF κ B activation involves dephosphorylation of I κ B and releasing NF κ B (Baldwin, Jr. *et al.* 1996).

The conserved N-terminus of NF κ B then binds to the DNA and promotes the transcription of a variety of pro-inflammatory signals. In B-lymphocytes, NF κ B plays

a significant role in immunoglobulin production. The gene enhancer for κ light chain is activated by NF κ B and in animal studies found to be crucial for B-cell maturation and function against pathogens (Sha *et al.* 1995). It is recently shown that NF κ B plays an important role in driving and coordinating the innate and adaptive immune response to an antigen (Bonizzi *et al.* 2004). NF κ B has various implications on inflammatory diseases, particularly in uncontrolled activation, and has been a subject of interest as a therapeutic target.

The objective of this study is to investigate the effect of ghrelin on NF κ B activation and inflammatory cytokine production in human B-lymphocyte cell line. Immunoglobulin production is a measure of primary B-lymphocyte cell function but it is uncertain if this characteristic has been established in the cell line used. Therefore the study focused the enquiry on cell activation and NF κ B expression both upstream of immunoglobulin production with established characteristics of the cell line studied.

4.2 Materials and method

4.2.1 Cell Line Selection And Preliminary Experiments

Two cell lines, B-lymphocyte (WILCL, ECACC Cat : 89120565; DAUDI, ECACC : Cat: 85011437) and 2 T-lymphocyte (JM, ECACC Cat : 86010201; HUT-78, ECACC Cat : 88041901) were tested for expression of the ghrelin receptor. Cells were cultured in appropriate medium, collected and lysed. Total protein was extracted and quantified. Using western blotting techniques, the GHSR-1a (now known as the ghrelin receptor) and the dormant GHSR 1b receptor was detected using commercially available antibodies from Phoenix Pharmaceuticals Inc, CA, USA

(GHSR 1a, Cat G-001-62; GHSR 1b, Cat G-001-61) (Figure 4.2.1). Detailed methodology is discussed in Chapter 2.

The WILCL cell line, a normal Caucasian human B-cell lymphoblast immortalized by the Epstein-Barr virus expressed ghrelin receptors and was used for the rest of the study (figure 4.2.2).

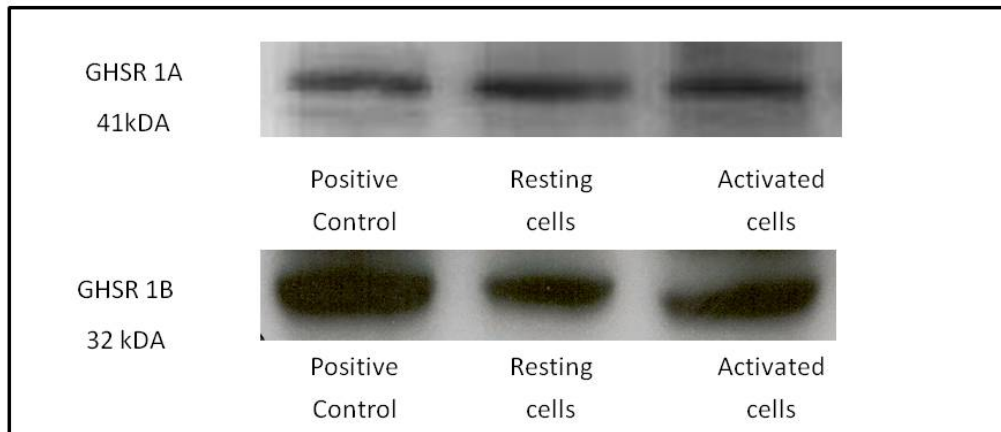


Figure 4.2.1. Western blots of the ghrelin receptor protein in WILCIL human B-lymphocyte cell line (N=1). Human adipose tissue protein extracts which has been confirmed to express both ghrelin and GHSR-1b receptors are used for positive controls. The ghrelin receptors (42 kDa) and GHSR 1b (32 kDa) are demonstrated in both resting and activated lymphocyte cells.

Figure 4.2.2 (a)

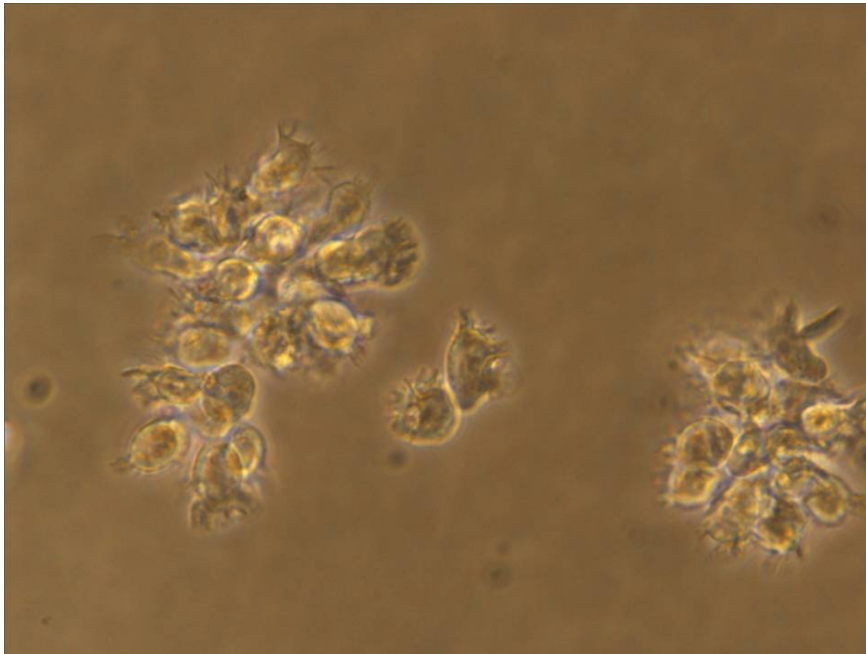


Figure 4.2.2 (b)

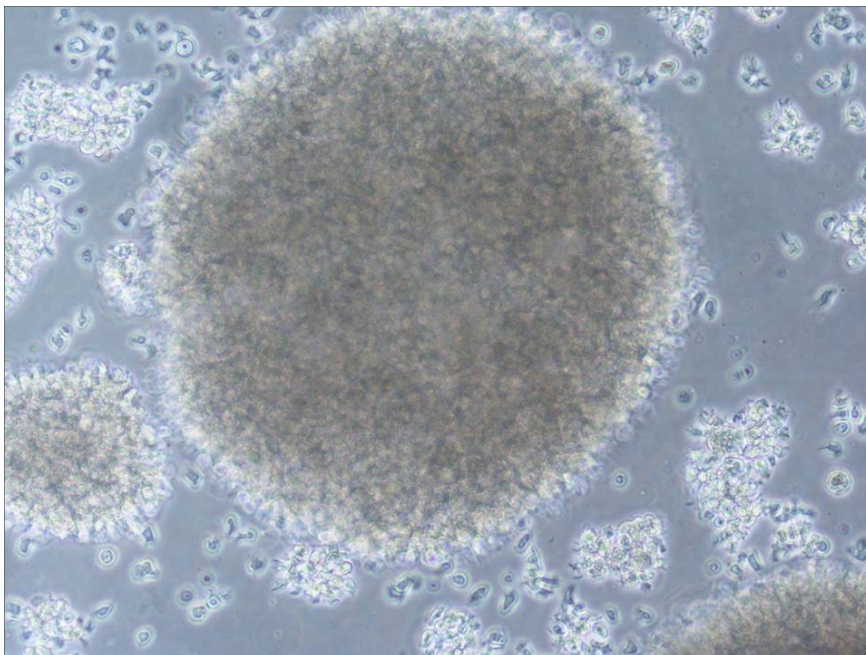


Figure 4.2.2 Light microscopy of the WILCL normal B-lymphocyte cell line at high power (a) and low power (b). Cytoplasmic extensions were clearly visible at high power which presumably allows the cells to proliferate in large aggregates [4.2.2 (b)].

Detailed cell culture and treatment methods are discussed in Chapter 2 (Section 2.2.4). Phytohemagglutinin (PHA) was used for lymphocyte activation. PHA concentration of 0.5mg/ml was optimal for WILCL cell activation. We assessed the effect of the compounds on activated and resting cells. A screening experiment (performed in triplicate) with octanoyl ghrelin treatment using time points of 6, 24 and 48 hours indicated a response at 6 hours (Table 4.2.1). As a result 6 hours incubation was used in all experiments.

NFκB activation in WILCL at 6 Hours

	Mean and SEM	P Value		Mean and SEM	P Value
Control	0.30±0.04		Control and PHA activation	0.31±0.08	
1nM	0.29±0.05	0.42	1nM and PHA activation	0.35±0.10	0.10
10nM	0.37±0.12	0.26	10nM and PHA activation	0.34±0.13	0.09
100nM	0.32±0.06	0.34	100nM and PHA activation	0.32±0.06	0.39

NFκB activation in WILCL at 24 Hours

	Mean and SEM	P Value		Mean and SEM	P Value
Control	0.31±0.04		Control and PHA activation	0.29±0.05	
1nM	0.30±0.04	0.28	1nM and PHA activation	0.29±0.05	0.15
10nM	0.31±0.04	0.32	10nM and PHA activation	0.31±0.04	0.18
100nM	0.28±0.04	0.03	100nM and PHA activation	0.32±0.04	0.10

NFκB activation in WILCL at 48 Hours

	Mean and SEM	P Value		Mean and SEM	P Value
Control	0.43±0.10		Control and PHA activation	0.41±0.06	
1nM	0.37±0.05	0.17	1nM and PHA activation	0.37±0.04	0.26
10nM	0.42±0.11	0.43	10nM and PHA activation	0.43±0.09	0.30
100nM	0.38±0.07	0.17	100nM and PHA activation	0.45±0.08	0.14

Table 4.2.1 Results of the screening experiments (N=3) in cells dosed with octanoyl ghrelin. There was a demonstrable change in NFκB activation in the resting group at T=6 hours although this did not reach significance. Values were expressed as mean±SEM. Comparisons were made using Student's t-test analysed by GraphPad prism software.

4.2.2 Cell Resuscitation And Culture

The principles and techniques of cell resuscitation and culture are discussed in detail in section 2.2.2. Cells were resuscitated from liquid nitrogen storage and cultured in RPMI 1640 media (Invitrogen, UK) enriched with L-glutamine (200mM), penicillin (100U/ml), streptomycin (100µg/ml) and 10% fetal bovine serum (Invitrogen, UK) in an incubator. Cell density was maintained at $3-5 \times 10^6$ /ml. Media was changed every 2-3 days and cell subculture was performed to maintain cell density.

PHA (Sigma, UK) was used to activate lymphocytes as described earlier and exposed to 3 compounds which were octanoyl ghrelin (Phoenix, CA, USA; Cat. 031-39), des-octanoyl Ghrelin (Phoenix, CA,USA; Cat. 031-32) and a non-peptide ghrelin agonist (Pfizer CP-464709). These compounds were added to the wells with resting and PHA activated cells to make up concentrations of control cells (no addition), 1nM, 10nM and 100nM. For des-octanoyl ghrelin and ghrelin agonist, we increased the range of concentrations to include 0.1nM and 1000nM in attempt to detect an effect as these compounds are not natural ligands of the ghrelin receptor.

4.2.3 NFκB Assay

NFκB expression was analysed using Active Motif TransAM NFκB p65 (Cat: 31102,Ca, USA) ELISA-based assay. This assay consists of a 96 well plate each coated with an oligonucleotide containing the NFκB consensus region (5'-GGGACTTTCC-3') which the active form of NFκB from cell lysates bind. The primary antibody only binds to the epitope on p65 only when NFκB is activated and

bound to its target. A secondary HRP-conjugated antibody binds to the primary complex which then produces a signal quantifiable by spectrophotometry (measurable by optical density at 450nm wavelength).

The cell culture medium was analysed for IL-6, IL-8, IL-10 and TNF α using a high sensitivity human cytokine Lincoplex assay (Linco Research, Cat. HSCYTO-60SK, St. Charles, MO, USA) using the Bio-Plex system (Bio-Rad Laboratory Inc., Hercules, CA, USA). The principles of multiplex assay is discussed in further detail in section 2.1.4. Inter-assay variability ranged between 2 to 15% for all cytokines measured. Cells exposed to ghrelin and its agonists were compared with resting and activated controls.

4.3 Statistical Analysis

Data is reported as mean \pm SEM . A student t-test was used to compare data from cell groups using GraphPad Prism software.

4.4 Results

4.4.1 Octanoyl-Ghrelin (N=6)

In the resting state, NF κ B activity in the control cells was 0.42 ± 0.10 and increased to 0.61 ± 0.20 , 0.54 ± 0.10 , 0.52 ± 0.08 when exposed to 1nM, 10nM and 100nM octanoyl ghrelin respectively. The increase in NF κ B activity in 1nM and 10nM was statistically significant ($P=0.044$ and $P=0.043$ respectively). NF κ B activity increased by 25-50% in the octanoyl ghrelin treated cells when compared to control cells.

In the activated state, NFκB activity was 0.56 ± 0.10 in control cells and remained unchanged at 0.57 ± 0.08 , 0.60 ± 0.09 , 0.48 ± 0.07 when exposed to 1nM, 10nM and 100nM octanoyl ghrelin respectively (Figure 4.4.1).

4.4.2 Desoctanoyl Ghrelin (N=6)

In the resting state, the NFκB activity in the control cells was 0.35 ± 0.03 and remained unchanged at 0.36 ± 0.04 , 0.39 ± 0.04 , 0.36 ± 0.04 , 0.36 ± 0.03 when exposed to 0.1nM, 1nM, 10nM and 100nM des-octanoyl ghrelin respectively.

In the activated state, NFκB activity in the control cells was 0.38 ± 0.04 and remained unchanged at 0.38 ± 0.03 , 0.41 ± 0.04 , 0.39 ± 0.04 , 0.39 ± 0.04 when exposed to 0.1nM, 1nM, 10nM and 100nM des-octanoyl ghrelin respectively.

4.4.3 Ghrelin Receptor Agonist (N=6)

In the resting state, NFκB activity in control cells was 0.30 ± 0.04 and remained unchanged at 0.29 ± 0.03 , 0.31 ± 0.04 , 0.31 ± 0.05 , and 0.29 ± 0.04 when exposed to 1nM, 10nM, 100nM and 1000nM ghrelin receptor agonist respectively.

In the activated state, the NFκB activity in control cells was 0.31 ± 0.04 and remained unchanged at 0.31 ± 0.04 , 0.33 ± 0.05 , 0.32 ± 0.05 and 0.31 ± 0.04 when exposed to 1nM, 10nM, 100nM and 1000nM ghrelin receptor agonist respectively.

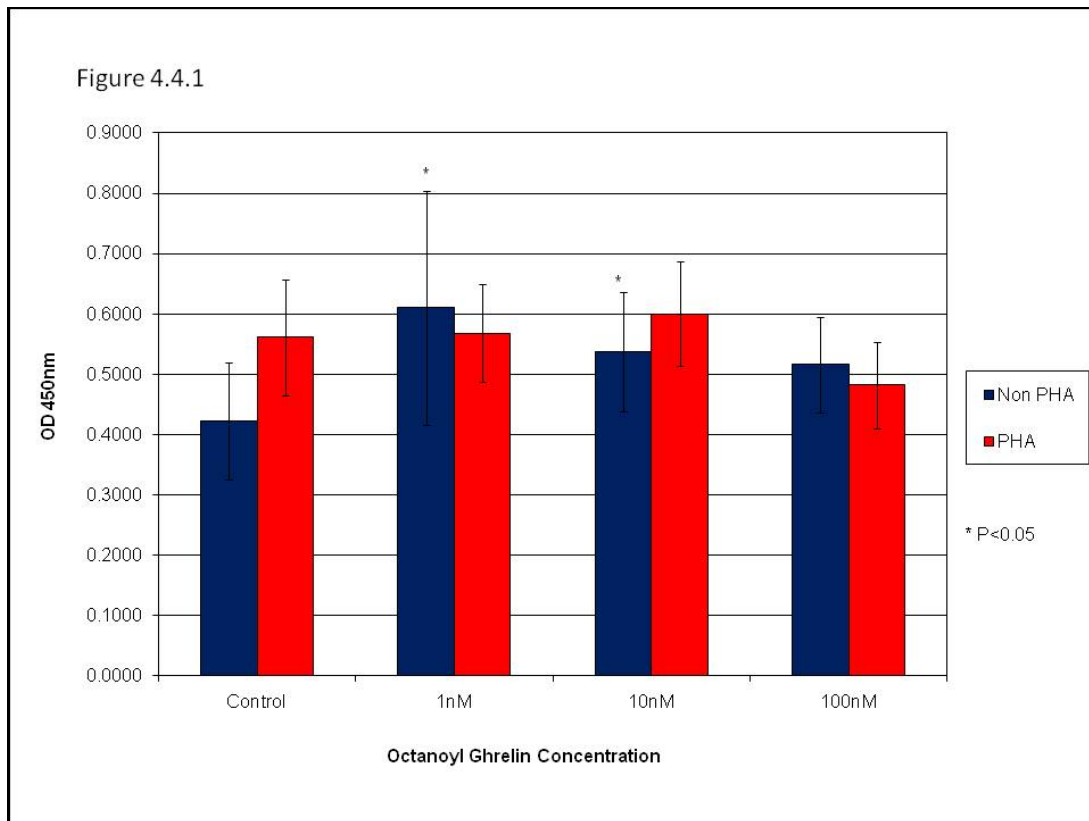


Figure 4.4.1. NFκB activation in resting and activated WILCL human B-lymphocyte cell line with increasing doses of octanoyl ghrelin (N=6). The student t-test was used to compare NFκB activation in increasing concentration of octanoyl ghrelin and controls. This illustrates the NFκB profile in resting cells and demonstrates a 25-50% increase in NFκB activation at 1nM and 10nM concentrations of octanoyl ghrelin (P<0.05). The NFκB activation in PHA activated cells and showed no significant change with increasing concentrations of octanoyl ghrelin. Values are expressed as mean±SEM. Comparisons were made using the Student's t-test analysed by GraphPad prism software.

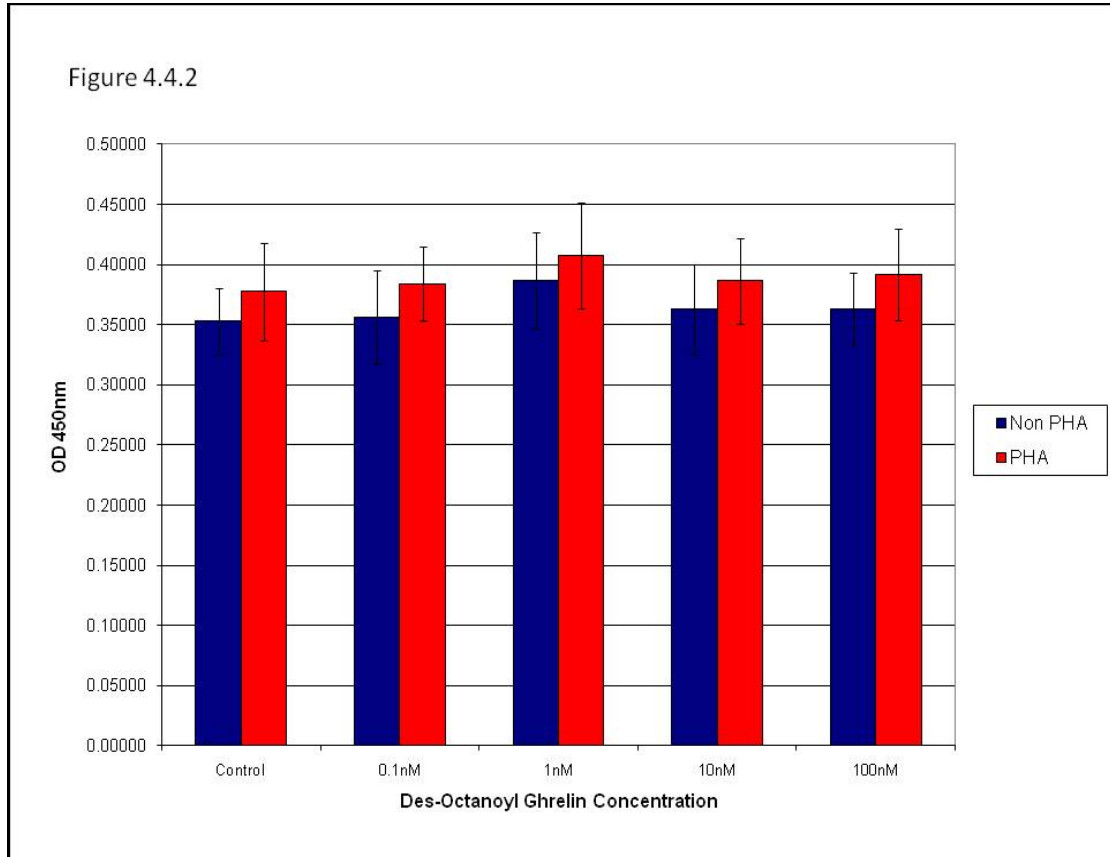


Figure 4.4.2. NFκB activation with increasing concentration of des-octanoyl ghrelin. There was no significant change in NFκB activation in both resting and PHA activated cells (N=6). . Values are expressed as mean±SEM. Comparisons were made using the Student's t-test analysed by GraphPad prism software.

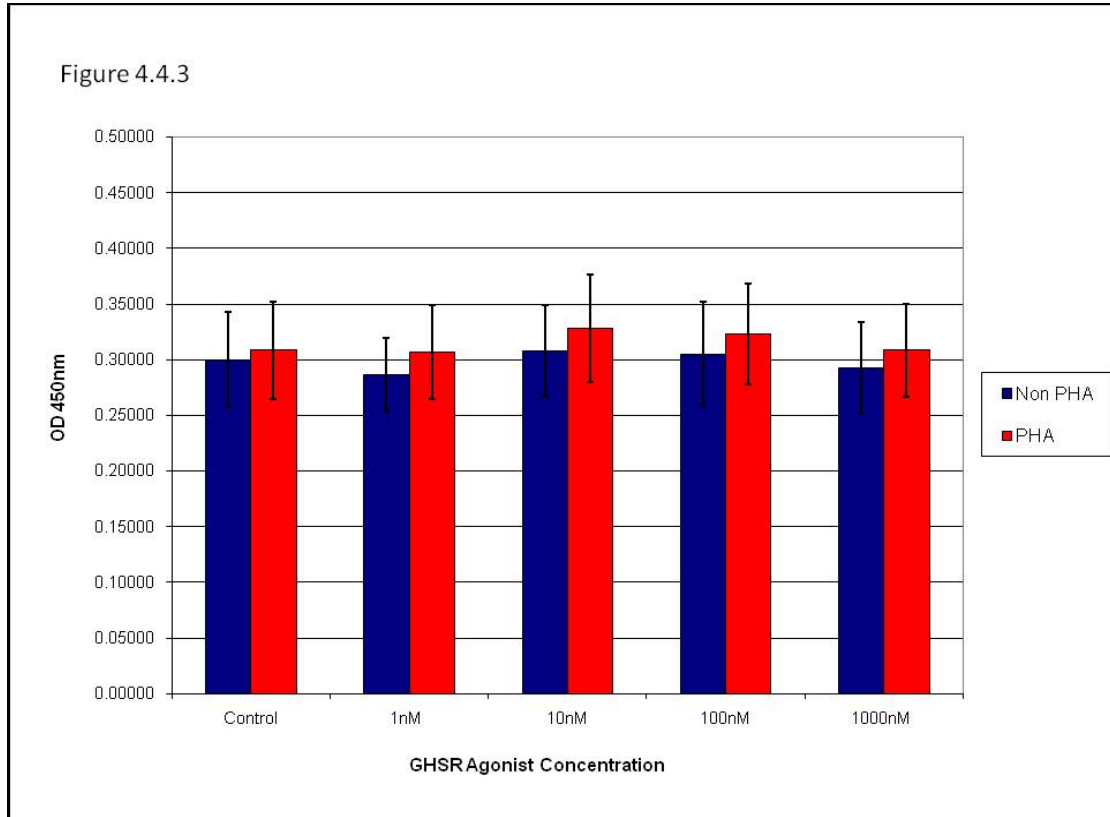


Figure 4.4.3. NF κ B activation in WILCL with increasing concentration of GHSR agonist (N=6). There was no significant change in NF κ B in both resting and PHA activated cells, suggesting a possible mechanism which may circumvent the ghrelin receptor. Values are expressed as mean \pm SEM. Comparisons were made using the Student's t-test analysed by GraphPad prism software.

4.4.4 Cytokines (N=6)

In resting and activated cells exposed to all concentrations of octanoyl ghrelin, no significant change in TNF α IL-6, IL-8, IL-10 concentration in the media was demonstrated Table 4.4.4.(a). Table 4.4.4(b) shows that the non-peptide ghrelin agonist significantly suppressed lymphocyte IL-13 production in a dose dependent manner particularly in resting lymphocytes. This effect was not associated with any changes in NF κ B. IL-13 is an anti-inflammatory cytokine and its suppression suggests that the non-peptide ghrelin agonist could be pro-inflammatory acting via a pathway independent of NF κ B. This effect was not seen with the biological ligand octanoyl ghrelin suggesting that it may be a non-specific effect of the synthetic agonist.

Figure 4.4.4 (a)

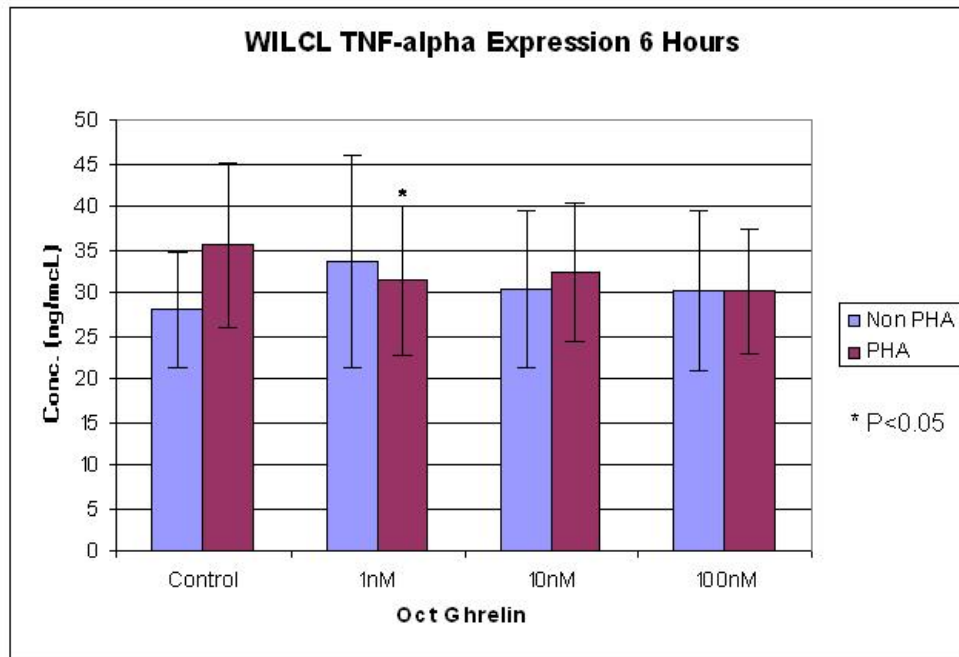


Figure 4.4.4 (b)

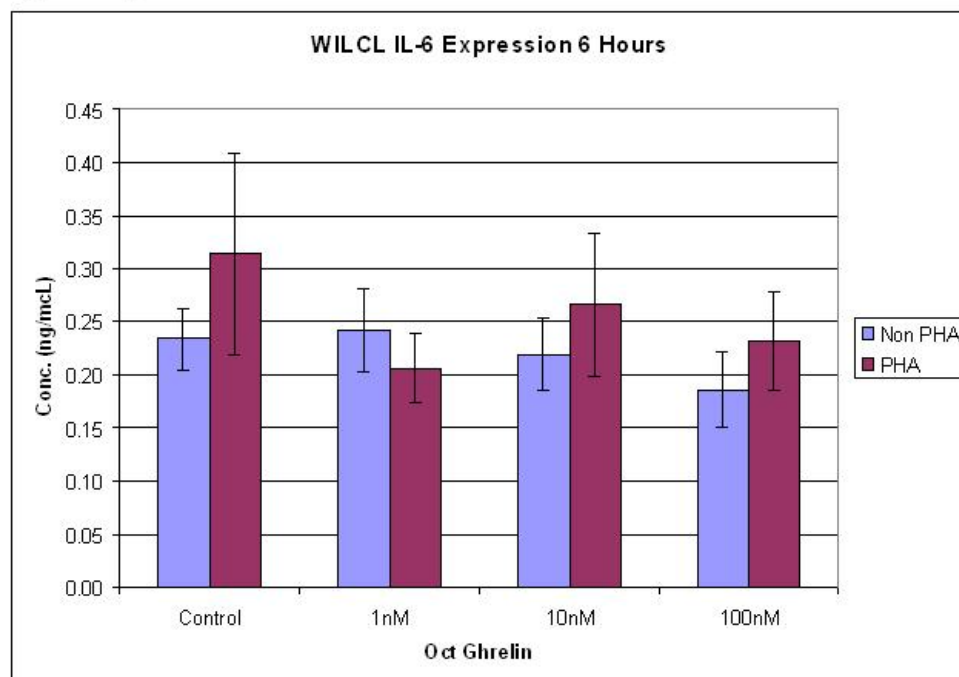


Figure 4.4.4 (d)

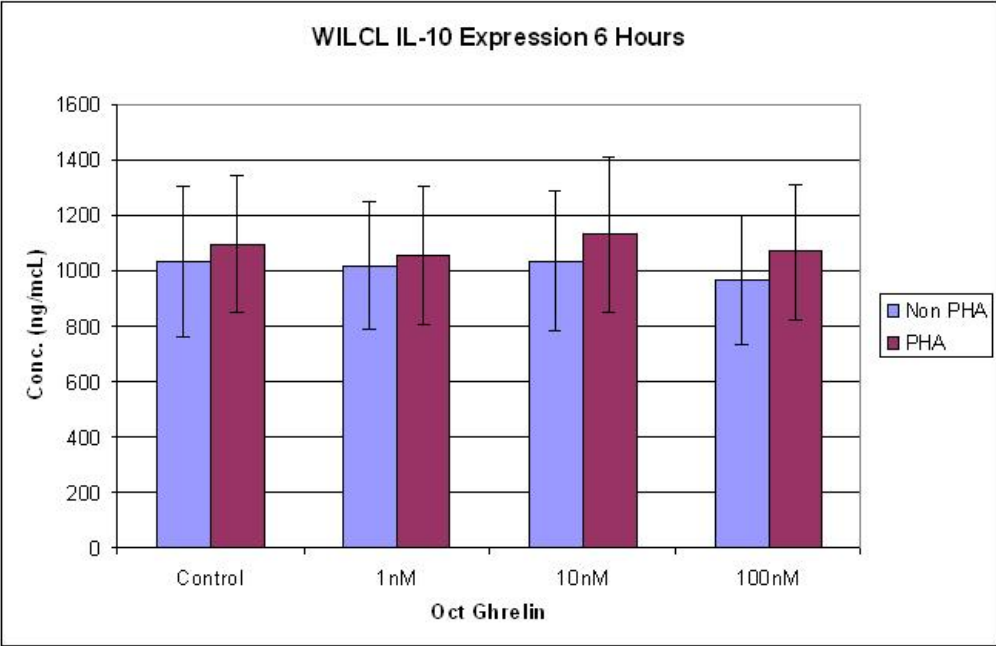


Figure 4.4.4 (c)

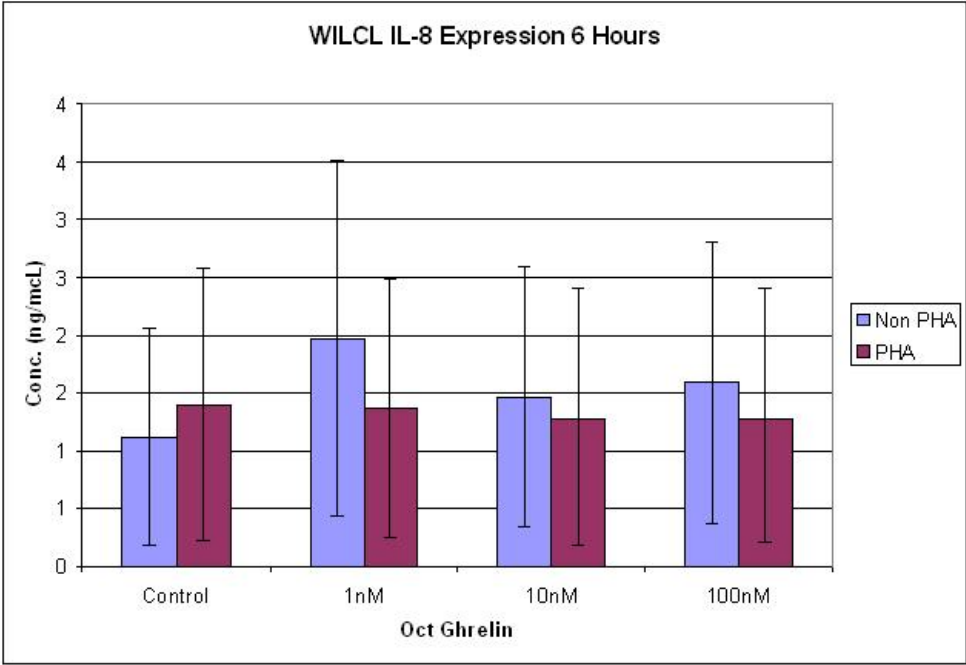


Figure 4.4.4 (e)

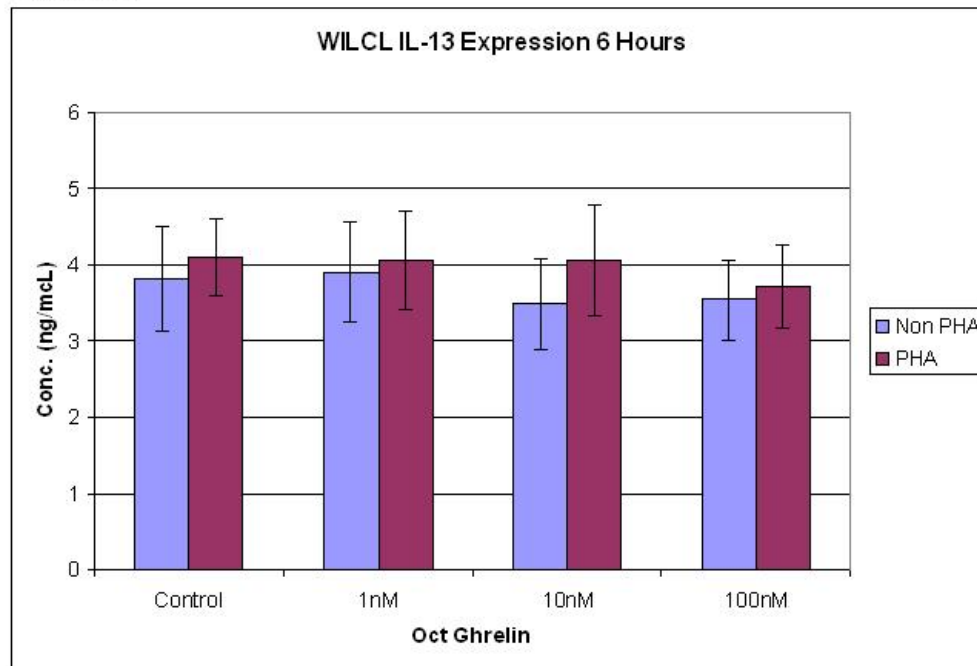


Figure 4.4.4 (a,b,c,d,e) Cytokine expression by WILCL with increasing concentration of octanoyl-ghrelin (N=6 each). Although there is significant decrease in TNF α at 1nM octanoyl-ghrelin concentration (P=0.03), this did not show a convincing trend [Figure 4.4.4 (a)]. Other cytokines measured did not show any significant change [figure 4.4.4 (b,c,d,e). Values are expressed as mean \pm SEM. Comparisons were made using the Student's t-test analysed by GraphPad prism software.

IL-6

	Non-PHA	P Value	PHA	P Value
Control	0.23 ± 0.03		0.31 ± 0.10	
1nM	0.24 ± 0.04	0.729	0.21 ± 0.03	0.2673
10nM	0.22 ± 0.03	0.252	0.27 ± 0.07	0.190
100nM	0.19 ± 0.04	0.101	0.23 ± 0.05	0.188

IL-8

	Non-PHA	P Value	PHA	P Value
Control	1.12 ± 0.93		1.40 ± 1.17	
1nM	1.97 ± 1.54	0.33	1.36 ± 1.12	0.68
10nM	1.47 ± 1.12	0.16	1.28 ± 1.09	0.11
100nM	1.58 ± 1.22	0.16	1.28 ± 1.08	0.15

IL-10

	Non-PHA	P Value	PHA	P Value
Control	1033.15 ± 270.57		1095.37 ± 246.01	
1nM	1018.35 ± 232.24	0.88	1056.51 ± 249.85	0.58
10nM	1031.57 ± 251.83	0.48	1130.65 ± 283.10	0.36
100nM	966.62 ± 232.18	0.17	1067.75 ± 241.99	0.31

IL-13

	Non-PHA	P Value	PHA	P Value
Control	3.83 ± 0.68		4.10 ± 0.50	
1nM	3.91 ± 0.65	0.80	4.06 ± 0.64	0.86
10nM	3.48 ± 0.60	0.13	4.07 ± 0.73	0.47
100nM	3.55 ± 0.52	0.22	3.72 ± 0.54	0.06

TNF α

	Non-PHA	P Value	PHA	P Value
Control	27.93 ± 6.78		35.54 ± 9.63	
1nM	33.58 ± 12.31	0.44	31.37 ± 8.63	0.03
10nM	30.40 ± 9.13	0.24	32.46 ± 7.98	0.12
100nM	30.25 ± 9.22	0.27	30.19 ± 7.24	0.10

Table 4.4.4(a) Results of the cytokine analysis of the media in the octanoyl ghrelin dosed cells (N=6). Values are expressed as mean \pm SEM. Comparisons were made using the Student's t-test analysed by GraphPad prism software.

IL-6

	Non-PHA	P Value	PHA	P Value
Control	0.25±0.06		0.26±0.05	
1nM	0.24±0.05	0.04	0.25±0.04	0.30
10nM	0.27±0.03	0.16	0.27±0.05	0.45
100nM	0.25±0.04	0.11	0.25±0.06	0.05
1000nM	0.28±0.06	0.36	0.26±0.06	0.47

IL-8

	Non-PHA	P Value	PHA	P Value
Control	12.18±3.86		11.86±3.71	
1nM	12.45±4.19	0.34	11.85±3.77	0.49
10nM	11.81±3.38	0.28	11.84±3.14	0.49
100nM	11.03±3.35	0.05	13.35±5.07	0.17
1000nM	12.40±4.46	0.38	11.74±3.93	0.38

IL-10

	Non=PHA	P Value	PHA	P Value
Control	790.84±126.17		835.59±134.23	
1nM	764.81±122.30	0.26	775.24±138.14	0.04
10nM	844.94±195.24	0.27	770.74±134.49	0.06
100nM	723.14±121.23	0.10	812.52±131.26	0.20
1000nM	759.59±127.19	0.21	753.79±127.14	0.05

IL-13

	Non-PHA	P Value	PHA	P Value
Control	5.88±0.67		5.96±0.68	
1nM	5.29±0.65	0.01	5.60±0.73	0.14
10nM	5.08±0.82	0.00	5.40±0.70	0.06
100nM	4.88±0.56	0.01	5.35±0.55	0.05
1000nM	4.97±0.63	0.00	4.55±0.42	0.00

TNF α

	Non-PHA	P Value	PHA	P Value
Control	99.68±5.29		113.49±7.43	
1nM	95.33±7.33	0.24	116.70±7.31	0.25
10nM	99.24±8.86	0.46	108.70±7.21	0.18
100nM	88.17±5.19	0.02	110.74±8.15	0.30
1000nM	92.64±8.18	0.13	107.25±8.36	0.15

Table 4.4.4(b) Results of the cytokine analysis of the media in the ghrelin agonist dosed cells (N=6). Values are expressed as mean±SEM. Comparisons were made using the Student's t-test analysed by GraphPad prism software.

4.5. Discussion

After a series of screening experiments, an immortalized B lymphocyte cell line expressing ghrelin receptors was selected as the target of investigation. Previous studies had investigated the role of ghrelin in activated T lymphocytes and monocytes but not B-lymphocytes. The physiological role of the ghrelin receptors on B-lymphocytes are currently not known. The effect of ghrelin and its agonists on NF κ B has not been investigated on B-lymphocytes. This is a novel study to investigate if NF κ B may be regulated in this particular B lymphocyte cell line by ghrelin and ghrelin receptor agonist treatment. The study shows that physiological concentrations of octanoyl ghrelin increased NF κ B activation by up to 50% when applied to this particular cell line. This effect was not dose dependent but peaked at 1 and 10nM and seemed to decline at 100nM concentration. This can be explained by the possible toxicity of ghrelin on these cell line at supra physiological levels. Cellular apoptosis is a plausible result at high concentrations of ghrelin.

As expected this increase in NF κ B activation by octanoyl ghrelin was not seen with the ghrelin receptor agonist and des octanoyl ghrelin; neither are the natural ligands for the ghrelin receptor. Another possible explanation is that octanoyl ghrelin may act on these lymphocytes via another mechanism rather than the ghrelin receptor, which may explain lack of effect seen in the ghrelin receptor agonist. The effect of octanoyl ghrelin was detected only when the cells were in a resting state. This effect was not seen when these cells were activated with PHA. It is likely that in the active state

lymphocytes are probably at a state of maximal NF κ B activation and additional stimulation via the ghrelin receptor is unable to increase this measurably.

Previous studies have demonstrated ghrelin receptors on human immune cells and also a decrease in cytokine production in T-lymphocytes and monocytes treated with ghrelin but not in B-lymphocytes. This in vitro model did not detect any consistent change in the panel of cytokines occurring in parallel with the changes in NF κ B. This however may be characteristic of this cell line. In this study, we recognize that cytokine production is not a primary function of B lymphocyte. Our experiment explored the interaction between B-lymphocyte cell activation and NF κ B expression both known characteristics of this cell line.

If this was a primary B lymphocyte cell line function would have been best assessed by immunoglobulin production but this was not a demonstrated characteristic of this particular cell line. In this research laboratory, there is more extensive experience with PHA. Although pokeweed was also tested on this cell line, it did not demonstrate greater potency in NF κ B activation in comparison to PHA.

NF κ B is known to regulate the development and maturation of lymphocytes (Siebenlist *et al.* 2005). The uncontrolled activation of NF κ B has been implicated in many inflammatory diseases and cancer. This has driven much interest in NF κ B as a therapeutic target in many diseases. There are studies that suggest an anti-inflammatory effect of ghrelin on human and animal lymphocytes (Dixit *et al.* 2004b; Xia *et al.* 2004a) and although our data points to a contrary conclusion it provides further evidence that ghrelin may be part of a complex human immune regulatory

repertoire and raises the possibility that ghrelin could be a target for immune-modulatory therapy.

Indeed there has been contrary evidence that ghrelin increases NF κ B activation and promote inflammation in the human colon (Zhao *et al.* 2006) which suggest that ghrelin may also have pro-inflammatory properties. This is the first study to demonstrate the effects of ghrelin on a human B-lymphocytes in vitro model. Further studies are warranted to explore how NF κ B regulates the immune system and this will be important in understanding a possible role of ghrelin. In addition, further studies of ghrelin dosing on other cellular types e.g. T-lymphocytes would be desirable in establishing the relationship between NF κ B and ghrelin.

Chapter 5

Cancer Chemotherapy and Gastric Contractility

5.1 Introduction

Neoadjuvant chemotherapy for oesophageo-gastric cancers prior to surgery are now common practice following clinical trials that showed survival improvement (MRC oesophageal cancer working group. 2002; Cunningham *et al.* 2006). However following completion of chemotherapy, many patients frequently continue to experience distressing symptoms resembling dyspepsia and attributed to a disorder in gastric emptying (Nelson *et al.* 2002). In a study by Riezzo *et al.* (Riezzo *et al.* 2005), for example, gastric dysmotility-like symptoms (susceptibility to nausea, early satiety, post-prandial fullness) and tachygastria were common in patients seven days after completion of chemotherapy, during which emesis was well controlled.

Such studies raise the possibility that the anti-cancer chemotherapy may somehow induce a gastric dysmotility which outlasts the treatment and which may lead to prolonged symptoms of dyspepsia. For this to occur, it is possible that chemotherapy may directly affect the integrity of the neuromuscular functions of the stomach. In rat gastro-duodenal muscle, for example, a reduction in the presence of the Ca^{2+} -calmodulin complex has been observed three days after a single treatment with cisplatin (Jarve *et al.* 1997). This thesis chapter investigates the hypothesis that cancer chemotherapy may cause long term damage to gastric motility.

5.2 Study Aims

To study the possibility that certain anti-cancer chemotherapy treatments may cause long-term damage to gastric function, we have looked at the effects of cisplatin-based treatments on the function and integrity of human stomach. For this purpose we examined the ability of human isolated stomach to contract in response to carbachol and other stimuli, following removal at surgery from patients who had undergone chemotherapy up to six weeks previously, compared to patients who had not undergone chemotherapy.

The integrity of the motor nerve system was also examined by immunohistochemical staining for acetylcholinesterase (AChE) and for the neuronal isoform of nitric oxide synthase (nNOS). In addition, we looked for changes in expression of the gastric motility stimulant motilin (Sanger *et al.* 2008) and the receptors for ghrelin. The latter was included partly because of an association between ghrelin and changes in appetite and gastric motility (Inui *et al.* 2004) and partly because of a previous study in rats, which showed that the ghrelin receptor may be up-regulated following administration of cisplatin (Malik *et al.* 2008c). The results indicate, for the first time, that marked changes in the contractility and integrity of the human stomach are caused by cisplatin-based chemotherapy. This may have impact on the clinical aspects of cisplatin-based chemotherapy which warrants further investigation.

5.3 Materials and Methods

5.3.1 Ethics

All patients enrolled were diagnosed with resectable oesophageo-gastric cancers. The patients were staged by computed tomography (CT) scan, endoscopy including endoscopic ultrasound and laparoscopy. All patients were staged as TxN0(or x)M0 by the TMN staging system and were assessed to be suitable for surgery. The study was approved by the local Coventry Research Ethics Committee and written informed consent was obtained from all patients. Particular care was taken so deceased patients were not contacted cross-referencing patient computer data and also bereavement records.

5.3.2 Patient Selection

Patients with gastric adenocarcinoma received 3 courses of epirubicin (50mgm^{-2}), cisplatin (60mgm^{-2}) and capecitabine (625mgm^{-2}). For patients with oesophageal cancers they received 2 courses of cisplatin (80mgm^{-2}) and 5-fluorouracil (1Gm^{-2}). Patients who did not undertake chemotherapy were physically frail, considered at risk of severe side effects of chemotherapy or undergoing Whipple's procedure for pancreato-biliary tumours. All patients underwent surgical resection within a 2 week window of 4 to 6 weeks following completion of chemotherapy regime, based on local cancer pathway.

Immunohistochemistry study			
	Non-chemotherapy	Oesophageal-chemotherapy	Gastric-chemotherapy
Female: Male ratio	1:5	1:5	2:4
Mean age	76	71	63
Contractility study			
	Non Chemotherapy	Chemotherapy	
Mean Age	74	63	
Female: Male ratio	2:1	0:4	

Table 5.4.1. Patient demographics in the immunohistochemistry and the contractility study.

5.3.3 Histological study (Immunohistochemistry)

Detailed immunohistochemistry methods are described in section 2.3.2. Histological paraffin embedded tissue blocks showing normal which were distant from the tumour site were selected. Specific antibodies were used to look for the expression of motilin, the ghrelin receptor, AChE and nNOS, in sections of gastric tissues taken from patients who did not have chemotherapy and in those receiving oesophageal-chemotherapy (cisplatin + 5-fluorouracil) or gastric chemotherapy (capecitabine); n=6 each. Following antigen retrieval by pressure cooking, optimised antibody dilutions were determined for motilin receptor (MBL International Corp, Woburn, MA, USA, Cat. No. LS-A134), ghrelin receptor (Chemicon, Billerica, MA, USA, Cat. No. AB9543), nNOS (Abcam, Cambridge, UK, Cat. No. ab40662) and AChE (Abcam, Cambridge, UK, Cat. No. ab2803).

The intensity and distribution of peroxidase staining was examined and scored by a histopathologist who was blinded to the group the patients were assigned to. For all procedures, a semi-quantitative scoring system for staining intensity was used (0=no staining, 1=weak, 2=moderate, 3=strong expression).

5.3.4 Gastric Contractility Study

Detailed description on gastric contractility experimental methods are described in section 2.4. In the gastric tissue contractility experiments, fresh gastric tissue was obtained from tumour free margins during surgery. The gastric tissue is then transported to GSK laboratories and processed within 24 hours of collection. The tissues were mounted and tissue baths and tension measured.

Following this, longitudinal muscle strips were stimulated via two parallel platinum ring electrodes connected to a stimulator (STG2008, Scientifica Ltd, UK. After washing and in tissues not subjected to EFS, 10 μ M erythromycin was added for 15 min. As not all tissues responded to EFS or erythromycin, the data are expressed simply as the number of tissues which responded by contraction, or not.

For both longitudinal and circular muscle strips, a carbachol concentration-response curve (1nM – 10 μ M) was then generated, with each additional concentration being added a cumulative maximum maximum response was observed , usually between 2-8 min).

5.3.5 Data analysis

All data are expressed as Means \pm the Standard Error of the Mean (SEM), or as Mean values with ranges; n values are numbers of patient donors. In the contractility studies, any differences in responses to carbachol between the two treatment groups were analysed using a two-way ANOVA with Bonferroni post-hoc test. Data from the immunohistochemistry study was analysed using the Student's paired t test.

5.3.6 Compounds

All drugs were freshly prepared before use. Erythromycin (Sigma, Gillingham, Dorset, UK) was dissolved in 100% ethanol, with subsequent dilution in distilled water. Carbachol (Sigma, UK) was dissolved in distilled water.

5.4 Results

5.4.1 Immunohistochemistry

There were no clear differences in age for each of the patient groups. More males than females were recruited although the numbers are too small to observe meaningful differences (Table 5.4.1). All patients in the immunohistochemistry study had gastric or oesophageal adenocarcinoma

There was a marked decrease in expression of AChE (the scores were, respectively, 2.3 ± 0.7 , 0.5 ± 0.2 and 0 ± 0 in non-chemotherapy, oesophageal- and gastric-

chemotherapy groups ($P < 0.03$ each) compared with the non-chemotherapy tissues; Figures 5.4.1(a,b) and 5.4.2(a,b). By contrast, there was a tendency for ghrelin receptors and for motilin to increase (figure 5.4.2(c)), although this did not achieve statistical significance (ghrelin receptor: 0.7 ± 0.4 in non-chemotherapy vs 2.0 ± 0.4 and 1.2 ± 0.2 in oesophageal- and gastric-chemotherapy groups respectively, $P = 0.04$ and 0.2 ; motilin: 0.7 ± 0.5 vs 2.2 ± 0.5 and 2.0 ± 0.7 , $P = 0.06$ and 0.16 , respectively). There were no consistent changes in nNOS (1.2 ± 0.6 in non-chemotherapy vs 1.2 ± 0.6 and 0.5 ± 0.34 in oesophageal and gastric-chemotherapy respectively. $P > 0.3$ each).

Figure 5.4.1 (a)

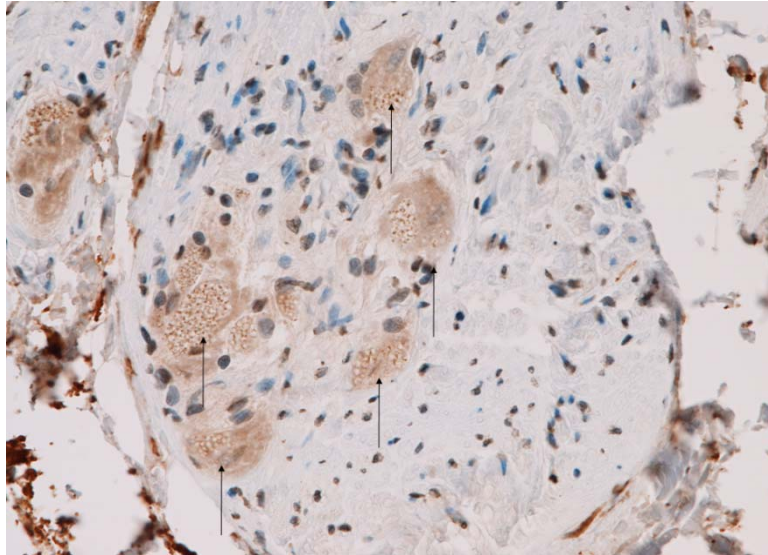


Figure 5.4.1 (b)

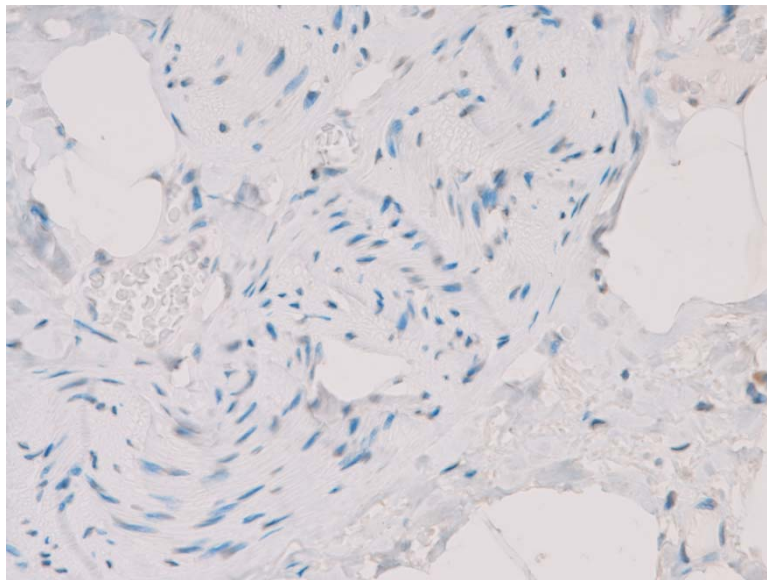


Fig 5.4.1(a) Gastric section from a non-chemotherapy patient. Acetylcholinesterase staining showing positive (brown) cytoplasmic staining within ganglion cells (arrows). Fig 5.4.1(b) Gastric section from a chemotherapy exposed patient. Despite an intensive search definite ganglion cells were not identified in this case, possibly because of degeneration from chemotherapy. The image shows a representative field illustrating negative staining in a neurovascular bundle. Original magnification x400.

Figure 5.4.2(a)

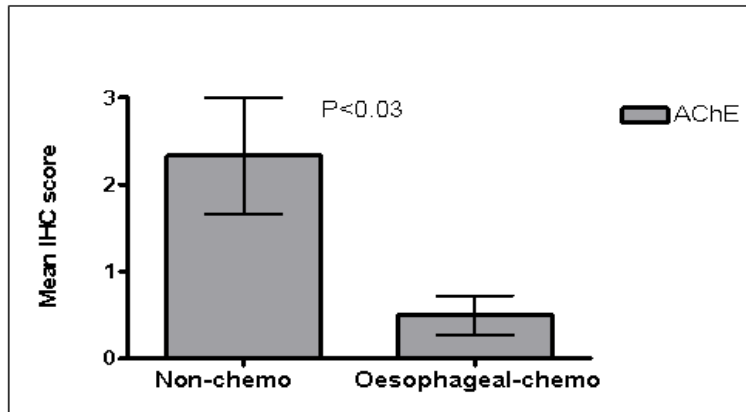


Figure 5.4.2(b)

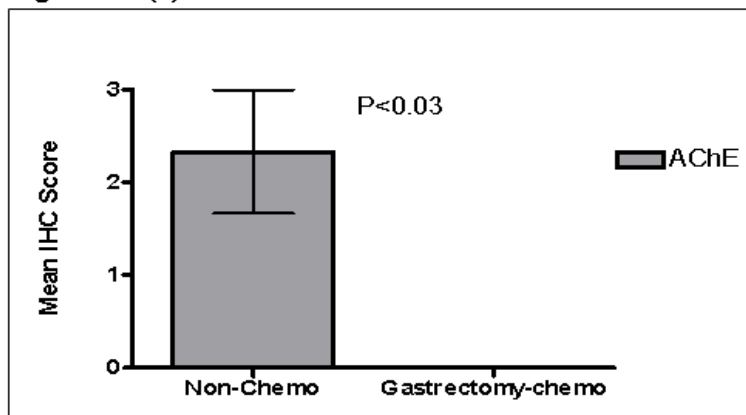


Figure 5.4.2(c)

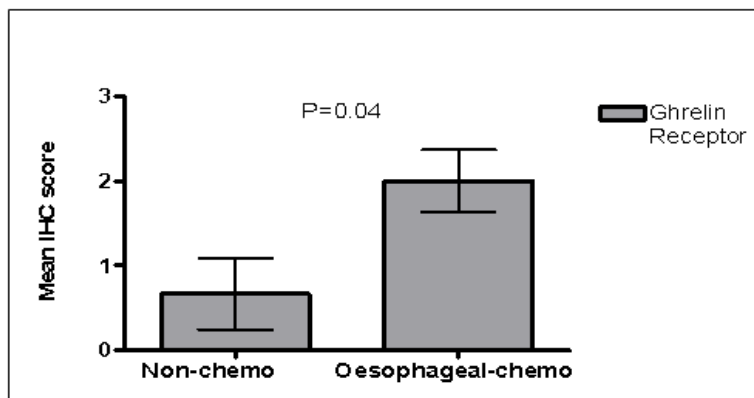


Figure 5.4.2 Immunohistochemistry (IHC) scores for AChE comparing non-chemotherapy and oesophageal-chemotherapy 5.4.2(a) and gastric-chemotherapy 5.4.2(b) exposed tissues ($P < 0.03$ using paired student's t-test). Figure 5.4.2(c) demonstrates the IHC scores for ghrelin receptor expression in oesophageal-chemotherapy group ($P < 0.04$).

5.4.2 Gastric contractility

Table 5.4.1 illustrates patient demographics. Again, there were more males than females recruited. Of the four patients receiving chemotherapy, three had gastric-chemotherapy and one had oesophageal-chemotherapy. The patient numbers were too small for subgroup analysis and hence, were analysed together as a single chemotherapy group. Longitudinal muscle preparations from either group did not consistently contract in response to EFS or erythromycin.

Contraction could be evoked by EFS in 2 of the 3 non-chemotherapy tissues (threshold 2-5 Hz) and in 1 of 4 chemotherapy tissues. Similarly, erythromycin 10 μ M increased basal tone or contractility in some tissues, but with no obvious difference between chemotherapy and non-chemotherapy tissues (4 of 8 non-chemotherapy tissues responded, compared to 2 of 4 tissues from patients receiving chemotherapy).

Application of carbachol 1 nM – 10 μ M caused a concentration-dependent contraction of both longitudinal and circular muscle tissues from both patient groups (Figure 5.4.3). However, there were clear differences between the groups of patients in terms of the force of contraction evoked by carbachol. The maximal contraction produced by 10 μ M carbachol in longitudinal muscle, for example, was 1.9 ± 0.8 g and 3.7 ± 0.7 g in chemotherapy and non-chemotherapy tissues respectively (n = 4 and 3, respectively, $P < 0.05$) and in circular muscle, these values were 1.6 ± 0.6 and 3.4 ± 0.4 g in chemotherapy and non-chemotherapy tissues respectively (n = 4 and 3 respectively, $P < 0.05$).

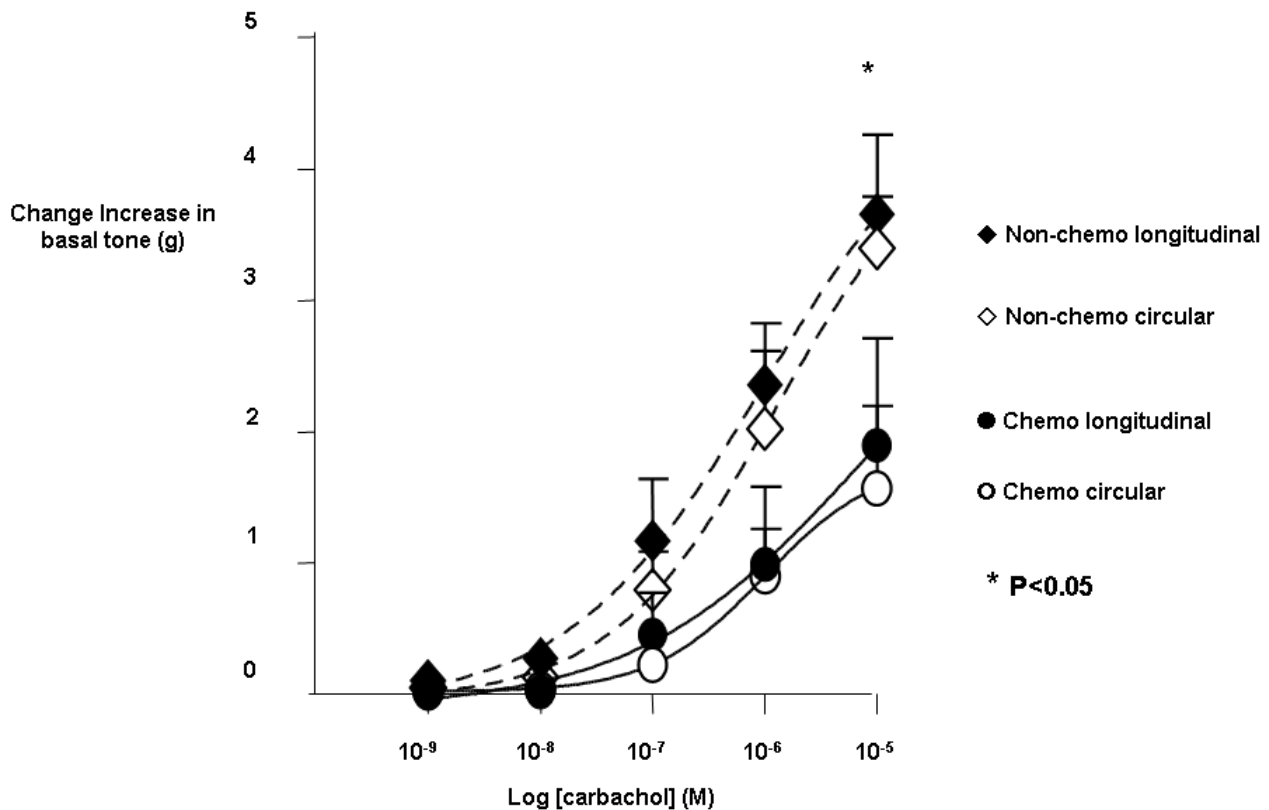


Figure 5.4.3 Concentration-response curves for carbachol-induced contraction of human isolated stomach. The contractions were compared using preparations obtained from patients treated with chemotherapy and from those who were not. * $P < 0.05$ (two way ANOVA with bonferroni post-hoc test) when the concentration-response curves were compared (chemo longitudinal muscle vs non-chemo longitudinal and chemo circular muscle vs non-chemo circular); $n = 4$ and 3 , respectively, for both longitudinal and circular muscle.

5.5 Discussion

This study is the first to show that cisplatin-based chemotherapy causes marked changes in human gastric function that are sustained after chemotherapy has finished. In particular, the absence of staining for AChE and the significant reduction in contractility to carbachol suggests that cisplatin profoundly influences gastric cholinergic function. Such findings find consistency with the reported ability of cisplatin to affect cholinergic function in conscious rats (causing an increase in [^3H]-choline uptake into the cardiac region of the stomach), although not with the lack of change in gastric AChE staining, measured three days after treatment in the same study (Aggarwal *et al.* 1994). Differences in species, duration of chemotherapy and/or length of time after chemotherapy are likely to contribute to the differences observed.

The reduction in cholinergic function was not clearly accompanied by a change in nNOS expression, suggesting that any sustained influence of cisplatin on enteric nerve function is not uniform throughout this nervous system. The effects of chemotherapy on the nNOS isoform has not previously been studied, although in rats receiving cisplatin or taxol, reductions in expression of the inducible form of NOS expression are reported (Wang *et al.* 1997), along with increases in plasma nitric oxide (Nagahama *et al.* 2002). However, in another study there were no changes in NOS localisation or intensity of staining five days after dosing with cisplatin in rats (Jarve *et al.* 1997). Together these studies suggest the possibility that NOS expression may be affected by cisplatin treatment but that in our study, the changes are either too

subtle to be detected using the present methods and/or any changes occur immediately on treatment and recover thereafter. Further studies are required to clarify this.

The tendency for the expression of both ghrelin receptors and of motilin to increase up to six weeks after cisplatin treatment does not, by itself, indicate that the function of ghrelin and motilin are changed by chemotherapy. However, the observed trend does find at least some consistency with studies in rats. In another study, ghrelin receptor mRNA expression demonstrated in rat stomach was increased two days after treatment with cisplatin, at a time when the cisplatin-induced gastric stasis was at its greatest (Malik *et al.* 2008).

The observed up-regulation of ghrelin receptor expression suggested that ghrelin might be operating as part of a defence mechanism against the damage caused by cisplatin; the hypothesis was suggested because of the known ability of ghrelin to inhibit cisplatin-induced emesis, promote appetite and increase gastric emptying (Malik *et al.* 2008; Liu *et al.* 2006). However, the present findings, measured up to six weeks after discontinuation of treatment with cisplatin, suggest that if ghrelin were to play a similar defensive role in humans, any effects of ghrelin are likely to be overwhelmed by the more severe reduction in cholinergic function.

A similar defensive role for motilin has not previously been proposed and although tempting to speculate that the gastric prokinetic activity of motilin would serve this purpose, it would be inappropriate to place too much emphasis on the present data, which were not statistically significant. Interestingly, there is evidence that plasma

levels of motilin were transiently decreased during the day after a cisplatin-based treatment (Hursti *et al.* 2005).

In summary, this study suggests that neo-adjuvant chemotherapy can cause marked and enduring damage to gastric function. Most notable was the loss of AChE and reduction in contractility evoked by carbachol, providing a plausible explanation for some of the symptoms of gastric stasis reported by these patients long after completion of chemotherapy. In addition, the tendency for ghrelin receptors to increase may be consistent with studies in animals exposed to cisplatin and suggests an attempt to up-regulate compensating systems.

Chapter 6

Discussion

6.1 Discovery of Ghrelin

The discovery of ghrelin was an interesting journey. Although the mechanism of ghrelin has been studied for over 2 decades, it was only formally identified in 1999. In the beginning, the receptor for ghrelin was discovered prior to the actual ligand. It began in 1970 when an endocrine study group led by Cyril Bowers in New Orleans made a discovery of opioid based synthetic compounds which provoked the release of growth hormones. The receptor for this compound was later discovered to be distinct from Growth Hormone Releasing Hormone (GHRH) binding sites.

Further development of similar non-opioid compounds e.g. Growth Hormone Releasing Peptide-6 (GHRP-6) were collectively known as Growth Hormone Secretagogues (GHS). Consequently, the GHS receptor, a G protein coupled receptor was first identified in 1996 (Pong *et al.* 1996). However, the natural ligand for this receptor remained elusive.

To identify the ligand, a Japanese group produced a cell line expressing the GHS-R receptor and examined the intracellular calcium activity when exposed to various tissue extracts including the stomach (Kojima *et al.* 1999a). It was then evident that the highest activity seemed to be produced from the stomach extracts, from which a 28 amino acid peptide was purified. This was later named Ghrelin. The term arose from the Indo-European origins of which 'Ghre' means 'to grow' because of ghrelin's potent ability to stimulate the release of GH. Another possible origin for its name is the ability to secrete.

Although ghrelin is a potent GH stimulator, it was soon discovered that ghrelin also has orexigenic properties, modulating appetite and adiposity. This sparked interest in the scientific community as the developed world is now struggling with rising obesity. It would also be fitting for the stomach to be the major ghrelin secreting organ, functioning as the 'gatekeeper' for food intake.

There has been considerable interest in ghrelin since its discovery, with 4,888 publications to date (July 2011). In many research, it has raised more questions than answers about the physiological role of ghrelin. Since the discovery of ghrelin and its receptor, it has been established that many organs, tissues and cells express the receptor and secrete ghrelin, albeit in much smaller quantities in comparison to the stomach. This has left many questions about the role of ghrelin in non-GI tissue types. It is most likely that ghrelin is a part of a very complex signalling repertoire that maintains physiological function of a particular organ, tissue or cells. However, in diseased states, many studies have demonstrated dysregulation of ghrelin.

Ghrelin however, has an established central role in maintaining energy homeostasis, in balance with other energy signalling peptides. The balance of ghrelin and leptin is probably crucial in maintaining normal physiological balance (discussed in 1.2.1.1). It is generally accepted that target tissue resistance to leptin is a plausible cause for obesity. This however does not explain why in obesity ghrelin concentrations are decreased as well as increased concentrations of leptin. There are other hormonal influences that are involved in the regulation of not just energy intake but also energy expenditure which contributes to obesity.

The association of ghrelin and inflammation is still unclear. There are conflicting findings in the interplay of ghrelin and inflammation. Some studies have observed elevated ghrelin in some inflammatory diseases (Mafra *et al.* 2011; Koch *et al.* 2010; Daniel *et al.* 2010). There are many possibilities to this. Elevated ghrelin may be a defensive response to an inflammatory stimulus in attempt to dampen the inflammatory response. Another possibility may be dysregulation of ghrelin by other unidentified factors. Conversely, other studies have observed decreased ghrelin in diseased state (Carrero *et al.* 2010; Kim *et al.* 2010; Suzuki *et al.* 2004) . This has demonstrated that ghrelin dysregulation may be disease specific and interplay of other factors in certain diseases may affect ghrelin secretion.

Such conflicting data has made interpretation of the literature difficult. However, it is clear that inflammation alone is unlikely to be the only factor in regulating ghrelin secretion. Ghrelin therapy has been used by some investigators to address the relationship between ghrelin and inflammation. Current animal studies suggests that that ghrelin may have anti-inflammatory properties. Majority of the evidence are limited to animal studies as discussed in Chapter 1. However, there are also human studies which demonstrate the beneficial effect of ghrelin administration in inflammatory conditions (Moreno *et al.* 2010; Kodama *et al.* 2008; Li *et al.* 2004).

This suggests that ghrelin is likely to be a beneficial therapeutic agent, particularly in chronic inflammatory conditions which characteristically have lower ghrelin concentration when compared with healthy controls (Koca *et al.* 2008; Ukkola *et al.* 2006). Further studies involving human trials are warranted to investigate this. Understandably, there are some concerns regarding the use of ghrelin as a drug. Ghrelin itself has many physiological effects, which involve endocrine, cardiovascular, gastrointestinal and central nervous system. Administration of ghrelin at supra-physiological concentrations may profoundly affect these systems and manifest as side effects. There will be a need for further human studies to probe the anti-inflammatory effects and safety of ghrelin further before human trials of ghrelin as a drug can materialise.

In many therapeutic ghrelin studies described in Chapter 1, ghrelin was administered intravenously or subcutaneously. Development of an oral form ghrelin would be difficult due to poor bioavailability and short half-life. However, an oral form of ghrelin receptor agonist has been studied on healthy human subjects. EPO-1572 GHS receptor agonist has similar characteristics to ghrelin in inducing release of GH in healthy subjects (Broglia *et al.* 2002; Piccoli *et al.* 2007). The pharmacokinetics of this agonist has also been studied. Intra-duodenal administration of this compound appears to have higher bioavailability compared to identical oral dosing. In addition, it has been demonstrated that the half-life of the agonist ranged from 73 to 114 minutes. In addition, if this agonist is taken with a meal the bioavailability of this drug is reduced significantly (MacLean *et al.* 2009).

6.2 The Role of Ghrelin in Crohn's Disease

Crohn's disease can be a severe debilitating inflammatory condition affecting the whole luminal gastrointestinal tract. Crohn's disease consequently causes cachexia by reducing the physiological function of the gut to absorb nutrients, increase in inflammatory load, appetite suppression, and an increase in energy expenditure in a high catabolic state. With increase in inflammatory load and associated insulin resistance is likely to decrease ghrelin availability, hence the lack of desire to eat. Treatment of Crohn's disease involves the modulation of inflammation by immunosuppressive drugs, which in turn improves the patients' nutritional status. The accountable mechanism is likely to be the release of ghrelin suppression as demonstrated in chapter 2.

The central role of ghrelin in patients with Crohn's disease is unclear. Previous studies had compared fasting spot ghrelin measurements in patients with Crohn's disease with controls but did not find any significant difference (Nishi *et al.* 2005). In another study, ghrelin levels appear to be higher in patients with active IBD than patients in remission. Moreover, the author suggested that measuring ghrelin in these patients may be useful in determining disease activity (Ates *et al.* 2008). This study also suggested the dysregulation of adipocytokines (produced by adipose tissue) in inflammatory pathogenesis alongside ghrelin. However, adipokines e.g. leptin tend to be long term signals with little fluctuation over time in contrast to ghrelin. The findings in these studies should be treated with caution.

Ghrelin was also implicated in the pathogenesis of Crohn's disease. As study has demonstrated a significant increase in ghrelin mRNA expression on colonic tissue with Crohn's disease compared with controls (Hosomi *et al.* 2008). The investigators have also demonstrated increase in activated T-cells expressing GHSR-1a receptors but also an increase reactivity to ghrelin in secreting IL-4 and IL-13 when compared with controls

Ghrelin is a dynamic hormone which has a very short half life and serum plasma levels tend to fluctuate significantly hour by hour. A fasting spot sample may not be sufficient to justify their findings depending on whether the measurement is at the peak or trough level. To avoid this uncertainty, measurement of ghrelin performed as a profile over 2 hours, with introduction of the meal to ensure validity (by revealing a post-prandial decrease). The calculated AUC would be a more accurate reflection of ghrelin status and allow sensitive detection of change.

In Chapter 2 of this thesis investigates the relationship between inflammation in Crohn's disease and ghrelin. It became quite clear that modulating inflammation in Crohn's disease releases the inhibition of ghrelin production and produces plausible evidence that ghrelin is involved in the 'hunger balance' with TNF α (previously known as 'cachexin' (Beutler *et al.* 1985) because of its ability to suppress hunger and promote satiety). The decrease in circulating TNF α , following infliximab infusion improves the patients nutritional status (Wiese *et al.* 2008). We demonstrated a 25% increase in circulating ghrelin levels as a plausible mechanism for the improvement of nutritional status in these patients by modulating TNF α .

In this study, the patient results were compared before and after infliximab treatment hence they became their own controls, avoiding potential bias. Although majority of these patients have stable disease, evidenced by their low inflammatory markers, there was significant change in plasma ghrelin level. It would be expected that patients with higher initial inflammatory load would have a more profound effect.

The reciprocal relationship between TNF α and ghrelin is an interesting one and our findings are consistent with other studies (Jang *et al.* 2008; Endo *et al.* 2007; Birkas *et al.* 2005). This may represent a delicate balance of disease-inducing cachexia, regulation of inflammation or even combination of both. Ghrelin has been shown to be capable to reversing the effects of cachexia in animals (Wang *et al.* 2006; Garcia *et al.* 2008; DeBoer *et al.* 2007). Ghrelin has been used successfully in cancer patients (Neary *et al.* 2004) and also patients with chronic renal failure on peritoneal dialysis (Wynne *et al.* 2005) to treat disease-induced cachexia. This had led to much interest to many clinicians as disease induced weight loss and anorexia has been a significant challenge.

As for the modulation of inflammation, current evidence is largely based on animal and in-vitro studies. Indeed there are animal studies that showed efficacy of ghrelin as a therapeutic agent in inflammatory bowel disease (Gonzalez-Rey *et al.* 2006). In current human studies involving ghrelin administration, inflammatory parameters have not been studied. In our Crohn's disease cohort, the effects of ghrelin on cachexia and immune-modulation would be highly desirable, since weight loss and anorexia, induced by high inflammatory load, has posed a significant challenge to all gastroenterologists. Hence, future clinical trials of ghrelin on patients with

inflammatory conditions are warranted and will provide further insight into the benefits of its use.

6.3 Role of Ghrelin on Human Lymphocytes

The thymus is a critical gland for T-cell development and maturation. There is evidence that ghrelin may have protective effects on age-related thymic involution. A study on older mice showed that synthetic GHSR-1a agonist administration increases the cellularity of the thymic tissues (Koo *et al.* 2001; Dixit *et al.* 2007). This suggests that ghrelin may protect against age related degeneration of the immune system and promote lymphocyte development with a detectable increase in thymic progenitor cells and matured T-lymphocytes in the periphery.

If ghrelin has anti-inflammatory properties, it is not inconceivable that it would have a profound inhibition of immune cell activation which directly contributes to inflammation. There is currently one in-vitro study on human lymphocytes demonstrating apparent attenuation of cytokine production in activated lymphocytes. This has so far not been reproduced. The author has taken this further by speculating that the action of ghrelin may be mediated by the inhibiting NFκB mediated inflammatory pathway.

The study in Chapter 4 showed quite the contrary. NFκB is actually upregulated with octanoyl-ghrelin at concentrations of 1nM and 10nM but decreases with increasing concentration. The rise in NFκB did not translate to an increase in cytokine production. The reason for this is unclear. Firstly, although the primary function of B-lymphocyte is not the production of cytokines, our initial experiments suggest the

WILCL produces significant cytokines. Secondly, the characteristic of this cell line may have influenced the cytokine profile. Further studies involving the production of light chain immunoglobulins are warranted. Thirdly, another possible mechanism which drives the anti-inflammatory effects of ghrelin may be NFκB mediated cellular apoptosis. NFκB is indeed pivotal in cellular response to environmental stress, including cell death. It has been shown that viral induced apoptosis is mediated by NFκB (Kuhnel *et al.* 2000). Therefore, it possible that NFκB may be responsible for immune cell death resulting in overall immune-suppression. We concede that further studies are needed to investigate cellular apoptosis in response to ghrelin and NFκB, which unfortunately beyond the scope of this thesis.

The study in chapter 4 also demonstrated that lymphocytes in their activated state are minimally influenced by exposure to ghrelin. Certainly supra-physiological concentration of ghrelin has failed to alter NFκB activation in activated cells. This crucial finding suggest that ghrelin may prime the lymphocytes for apoptosis prior to activation or that the effects of ghrelin on the immune system is probably weak.

This study raises many important issues about the involvement of ghrelin in NFκB activation and lymphocyte response. Future studies are needed to investigate the effects of ghrelin on primary lymphocytes, cell apoptosis and also cytokine profile. This will provide further insight into the function of ghrelin in the immune process.

6.4 Role of Ghrelin in Gastric Motility

Ghrelin has a similar homology to motilin, a natural hormone which promotes gastrointestinal motility. It is then not conceivable that ghrelin may have effect on gastric motility and emptying. Previous animal studies supports have been reported to induce gastric emptying (Dornonville de la *et al.* 2004; Qiu *et al.* 2008). There are human studies demonstrating the increased rate of gastric emptying in healthy individuals when infused with ghrelin (Levin *et al.* 2006). Moreover, ghrelin has also been used successfully in diabetic patients with gastroparesis (Murray *et al.* 2005).

This effect is probably physiologically mediated by efferent vagal stimulation although another small study suggested that the motility action of ghrelin can be independent of vagus innervation (Binn *et al.* 2006). In addition, another animal study suggests that ghrelin may improve endotoxaemia induced motility disturbances (Chen *et al.* 2010). In addition to initiating peristalsis, ghrelin also has a role in regulating GI motility.

In Chapter 5 of this thesis, the study examines the effect of oesophageo-gastric cancer chemotherapy on the gastric pro-motility signals by immunohistochemistry and gastric contractility. The destruction of the acetylcholinesterase activity in the chemotherapy group is apparent. The study also revealed a compensatory upregulation of ghrelin receptor, presumably in response to cholinergic pathway damage.

The destruction of the cholinergic activity may also contribute to the acute and chronic emesis following chemotherapy administration, as part of the cancer-dyspepsia syndrome. It is widely accepted that cancer chemotherapy induces acute vomiting by acting on vagal afferents via 5-HT mechanisms and also by a direct effect on area postrema (Hesketh *et al.* 2008). There are 2 recognised vomiting syndromes following chemotherapy administration, acute (within 24 hours) and delayed emesis (occurring after 24 hours). Following this period, chemotherapy induced vomiting tends to subside.

It is possible that the destruction of cholinergic pathways may contribute to this initial acute vomiting and persists after chemotherapy treatment as destruction of cholinergic system is observed two weeks after completion of surgery. The upregulation of ghrelin may be compensatory as a result of cholinergic inactivity and help mask further symptoms.

This would have significant implications on future chemotherapy design and also ghrelin as a potential protector against chemotherapy-induced emesis. In addition, further studies involving ghrelin administration following chemotherapy will help further elucidate its effectiveness in prevention of vomiting. There may also be a role for ghrelin or its agonist in patients who have persistent ‘cancer-associated dyspepsia’ following treatment.

In summary, this thesis revealed that modulating TNF α in Crohn's disease can increase circulating ghrelin concentration, and suggests an immune-modulating role. In addition, ghrelin may also have a crucial role in managing disease induced cachexia by not just by reversing the cachectic effects of TNF α but also therapeutically inducing appetite and stimulate food intake. Ghrelin is also shown to upregulate NF κ B in human B-lymphocytes but did not translate to any meaningful cytokine production. This raises the possibility that NF κ B may have a different role e.g. Regulation of apoptosis in human lymphocytes. Thus, ghrelin has a complex immune-modulatory role which is still yet to be elucidated.

This thesis has also shown that ghrelin mechanisms are upregulated in response cancer chemotherapy induced damage to cholinergic activity, suggesting a protective role of ghrelin to regulate gastric motility. The findings also improves our understanding of the mechanisms that underpin post chemotherapy dyspepsia, and help designing new therapies to relieve the debilitating symptoms, that in the future we may be capable of improving the quality of life for these cancer patients.

6.5 Strengths and weaknesses of this thesis

This thesis is the result of 2 year research work undertaken at the Clinical Sciences Research institute at the University Hospital Coventry site without prior laboratory experience. All laboratory skills were acquired during time in laboratory.

6.5.1 Ghrelin in Crohn's disease

This is a novel study investigating the consequence of anti TNF α therapy on the ghrelin profile in patients with Crohn's disease. No similar studies have been published previously. This unique study demonstrates how modulating inflammation in Crohn's disease can alter a hunger mechanism. Previous studies measured spot ghrelin concentrations in this patients. In this study, ghrelin when measured as a profile over 2 hours provides a more accurate method of analyzing this very dynamic hormone with a short half life. The number of patients recruited were small due to time constraints as each patient was recruited over a 2 month period, in between anti-TNF α infusions. New commercially developed ELISAs and Bioplex assays were made available upon completion of patient recruitment. Significant data losses were observed in quantifying acylated and total ghrelin due to susceptibility of ghrelin to degradation. Based on previous experience of this research unit, aprotinin (Triasylol) was added to plasma samples on collection to reduce the degradation of ghrelin. It is possible that using a different protease inhibitor eg .Pefabloc (Roche) and acidification of the samples, as recommended by new ELISA assays, may have reduced data losses. Repeating the sample collection from all patients to assess the efficacy of the new protease inhibitors was not possible. It would be desirable to obtain data on calorie intake in these patients to demonstrate the effects of increased ghrelin concentrations after anti-TNF α treatment. Although food diaries have been sent to all participants, only one patient diary was returned partially completed which did not allow analysis.

6.5.2 Ghrelin, NFκB and Human lymphocytes

A published study suggest that ghrelin may attenuate pro-inflammatory cytokine production in human T-lymphocytes, and these findings were not substantiated by other studies. This is the first published experiment investigating the effects of ghrelin on NFκB upstream of cytokine production. Although this study established the characteristics of the B-lymphocyte cells in producing cytokines, it is not a primary function of B-lymphocytes. Analysis of κ-light chain production in the culture medium is also a possible endpoint although this was not an established characteristic of the cell line and not analyzed due to time constraints. It would be desirable to have replicated the experiments with the T-lymphocyte cell lines but this was not possible due to time constraints. A positive control would have been useful in validating the results of the NFκB activation. It is also accepted that the experiments should have been repeated more than 6 occasions and also assayed in triplicates.

6.5.3 Cancer chemotherapy and gastric motility

This study demonstrates the effect of cancer chemotherapy on gastric neurotransmitter expression and contractility. This unique study combines both immunohistochemistry work and also contractility experiments to explain the decreased gastric contractility. This highlights the toxicity of cancer chemotherapy to gastric function and may be important in future cancer chemotherapy design. The study numbers are unfortunately small, particularly in the gastric contractility study. There were far fewer patients undertaking surgery without chemotherapy which resulted in small patient numbers in this group. Participation in this study was frequently declined as these patients were

experiencing anxious and worrying times in the face of major, potentially complicated surgery. Unfortunately this study is not sufficiently powered to differentiate the 2 different cancer chemotherapy used (oesophageal and gastric chemotherapy) due to small numbers. These patients were grouped collectively as cisplatin-based chemotherapy as many animal studies have demonstrated cisplatin has potent influences on gastric dysmotility.

The three experiments described in this thesis are novel studies not previously reported and it provides an insight into the undiscovered characteristics of ghrelin function. Many limitations of this thesis work is attributable to time and cost constraints.

Appendix

All experiments described in this thesis were undertaken between May 2006 to May 2008, during my tenure as clinical research fellow at Warwick University and the University Hospital Coventry. This work was supervised by Professor Chuka Nwokolo and Dr. Paul O'Hare. Most laboratory work was based at the Clinical Sciences Research Institute based at the university hospital, in collaboration with the Diabetes and Metabolism group led by Dr. Philip McTernan. The research was funded by Professor Nwokolo's research funds and a research grant from GlaxoSmithKline. As part of my collaborative work with GSK, fresh stomach and colonic tissues extracted from patients undergoing cancer surgery were couriered to GSK laboratory for motility studies. During our collaborative work, it seemed that tissues that have been exposed to cancer chemotherapy had poor motility response to stimulation. The collaborative work with GSK neurogastroenterology unit facilitated the completion gastric contractility experiment in chapter 5.

Publications

There are 2 papers published from the work of this thesis:

- 1. Increased plasma ghrelin following infliximab in Crohn's disease**
Sung E, DA Silva NF, Goodyear S, McTernan PG, Sanger GJ, Nwokolo CU.
Aliment Pharmacol Ther; Volume 29, Issue 1, January 2009, 83–89
- 2. Ghrelin Promotes Nuclear Factor Kappa-B in Human B-Lymphocyte Cell Line**
Sung EZH, Da Silva NF, Goodyear SJ, McTernan PG, Nwokolo CU
Mol Bio Rep 2010 Dec 5 (Epub ahead of print)
- 3. Long- term consequences of neo-adjuvant chemotherapy for oesophago-gastric cancer on gastric contractility and innervations**
Sung EZH, Jarvie EM, James S, Goodyear SJ, Borman RA, Snead D, Sanger GJ, Nwokolo CU (In press, accepted for publication in Molecular Biology Reports)

During my time in research, I also co-authored the following in collaborative work:

- 1. Dysregulation of plasma ghrelin in alcoholic cirrhosis**
Stephen J Goodyear, Marcus Mottershead, Edmond ZH Sung, Ling S Wong, Philip G McTernan, Sudhesh Kumar, Chuka U Nwokolo
Clin Endocrinol (Oxf) 2010 Sep; 73(3):323-9 Epub 2010 Feb 23
- 2. Activation of prostaglandin EP receptors by lubiprostone in rat and human stomach and colon**
Bassil AK, Borman RA, Jarvie EM, McArthur-Wilson RJ, Thangiah R, Sung EZ, Lee K, Sanger GJ.
Br J Pharmacol. 2008 May;154(1):126-35.
- 3. Altered mRNA expression of telomere binding proteins (TPP1, POT1, RAP1, TRF1 and TRF2) in ulcerative colitis and Crohn's disease**
Da Silva N, Arasaradnam R, Getcliffe K, Sung E, Oo Y, Nwokolo C
Dig Liver Dis 2010 Aug;42(8):544-8. Epub 2010 Jan 12

Presentations at meetings

The work from this thesis has been presented in the following meetings :

1. Midlands Gastroenterological Society (June 2007)
Increased plasma ghrelin following infliximab in Crohn's disease (Oral presentation)
2. British Society of Gastroenterology (March 2008)
Ghrelin increases nuclear factor kappa-B expression and activity in human lymphocyte cell line (Oral presentation, Gut 2008; 57:A1-A172)
3. Digestive Disease Week (May 2008)
Increased plasma ghrelin following infliximab infusion in Crohn's disease (Gastroenterology 2008; Vol 134, Issue 4, Supplement 1, Page A-667)
4. Digestive Disease Week (June 2009)
Long term consequences of neo-adjuvant chemotherapy for oesophago-gastric cancer on gastric contractility and innervation (Gastroenterology 2009; Vol. 136, Issue 5, Supplement 1, Page A-420)

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Published work related to this thesis

Increased plasma ghrelin following infliximab in Crohn's disease

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SUMMARY

Background

Ghrelin, a potent orexigenic peptide produced by the stomach, may be affected by circulating inflammatory mediators.

Aim

To assess the effect of an anti-TNF α antibody on ghrelin in patients with Crohn's disease (CD).

Methods

Fifteen patients with Crohn's receiving infliximab were studied before and 1 week after infusion. Following an overnight fast, blood was sampled before a meal and then every 20 min for 2 h. Total ghrelin and CRP were measured using ELISA. Acylated ghrelin and TNF α , IFN γ , IL-1 β and IL-6 were measured with bioplex. Harvey Bradshaw Activity Index was assessed.

Results

Median (95% CI) 2-h integrated plasma total ghrelin increased from 162 (99–311) before infliximab to 200 (128–387) pg/mL h, ($P = 0.02$) after. Following infliximab, 20 min postmeal, median acylated ghrelin decreased from 50.3 (24–64) to 38.6 (26–82) pg/mL, ($P = 0.04$) thus reverting to a traditional meal related ghrelin curve. Median (range) disease activity decreased from 5 (2–28) before to 3 (0–22), ($P = 0.0001$) and Median (95% CI) TNF α decreased from 2.8 (1.89–4.48) to 1.31 (0.73–2.06) pg/mL ($P = 0.002$).

Conclusions

Infliximab increases circulating total ghrelin by 25% in CD and restores the postprandial response of acylated ghrelin to food intake. Acylated and de-sacyl ghrelin remain unchanged, suggesting that an alternate isoform could be affected by infliximab.

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INTRODUCTION

Ghrelin is a novel 28 amino acid peptide first discovered in 1999¹ and is the natural ligand for the growth hormone secretagogue receptor (GHS-R).² The stomach produces two-thirds of circulating ghrelin, demonstrated by the decrease in plasma ghrelin concentration following gastrectomy.³ In humans, ghrelin is encoded in chromosome 3.⁴ It is initially translated into a precursor, preproghrelin and subsequent cleavage produces two main forms. The first is acylated ghrelin, a 28-amino acid peptide with post-translational *n*-octanoylation of its serine at position 3, which is regarded as the 'active' isoform. The octanoylation process is essential for its biological activity. The second form of ghrelin is the des-[Gln¹⁴] formed from alternate splicing of the ghrelin gene,⁵ known as des-acyl ghrelin. The biological role of des-acyl ghrelin remains unknown with conflicting reports regarding its function.^{6, 7} However, this form of ghrelin can also undergo octanoylation and become biologically active.

The GHS-R receptor for ghrelin is found in abundance in the arcuate nucleus of the hypothalamus.⁸ Ghrelin is the most potent stimulator of growth hormone release in humans.⁹ Ghrelin also stimulates hunger, initiates feeding¹⁰ and increases food intake in animals¹¹ and humans.¹² It is regarded as the only known circulating 'hunger hormone' in intact mammals and is thought to play an important role in energy balance. In humans of normal or low body mass index (BMI), ghrelin concentrations are high during fasting and decrease immediately after a meal is eaten. This effect is blunted in obese individuals, suggesting a dysregulation of ghrelin in these individuals.¹³

There is emerging evidence that ghrelin may have immune-modulatory effects. In rats with pancreatitis, it attenuates pancreatic damage by decreasing the release of pro-inflammatory IL-1 β cytokine.¹⁴ Ghrelin, when infused into rats, protects against ethanol induced gastric ulcers.^{15–17} A ghrelin agonist ameliorates experimentally induced inflammatory arthritis by decreasing the production of IL-6.¹⁸

Growth hormone secretagogue receptors are expressed in human immune cells suggesting that ghrelin may play a role in the function of the lymphocytes.¹⁹ Studies have shown that ghrelin decreases the release of proinflammatory cytokines (TNF α , IL-1 β and IL-6) by activated human monocytes and lymphocytes. This anti-inflammatory action is opposite to that reported for leptin.²⁰ Further studies on human

endothelial cells suggest that ghrelin may inhibit nuclear factor kappa-B activation, decrease TNF α -induced proinflammatory cytokine production and thus protect against the development of atherosclerotic disease. On this basis, it has been suggested that the accelerated atherosclerosis in obesity may be in part because of low levels of circulating ghrelin observed in these individuals.²¹ There are no studies on humans investigating the link between ghrelin and the immune system.

Crohn's disease (CD) is characterized by chronic inflammation involving the gastrointestinal tract. While the cause remains unknown, there are several contributing factors, including genetic, immunological, infective and environmental factors. It commonly affects the small intestines, but can affect any part of the gastrointestinal tract from the mouth to anus, with transmural inflammation and fistula formation being its important hallmarks. Weight loss and malnutrition are commonly reported as resulting from multiple factors including poor appetite. Infliximab, a chimeric antibody active against TNF α , is an effective treatment for moderately severe CD.²²

We hypothesized that circulating ghrelin concentration may change when TNF α activity is altered by infliximab in patients with CD. In this study, we assessed the effect of infliximab infusion on the total, acylated and desacyl isoforms of ghrelin in patients with CD and related this to changes in biochemical and clinical measures of inflammation in CD.

METHODS

Patient characteristics

Fifteen patients with CD receiving infliximab were recruited. Patients with gastrectomy or gastric by-pass surgery were excluded. Mean age for all patients was 31 (range 21–50) and 10 were male. Mean BMI was 23 (range 19–34). Table 1 shows demographic and clinical characteristics of each patient. There was no change in drug treatment during the study.

Protocol

Each patient was studied on two occasions, immediately before and 1 week after an infusion of infliximab (5 mg/kg). All studies were started between 08:00 and 10:00 h. In five patients, the study conducted '1 week after' infliximab was performed first followed by the

Table 1. Demographic and clinical data for 15 patients with Crohn's disease

Patient	Age	Gender	BMI	Disease site	Immunomodulation	Previous surgery
1	27	F	22	Perianal, colonic	Azathioprine	No surgery
2	22	M	34	Ileal	Azathioprine	No surgery
3	21	M	21	Ileal, colonic	Azathioprine	No surgery
4	50	M	24	Perianal	Azathioprine	No surgery
5	27	M	22	Colonic	Azathioprine	No surgery
6	23	M	23	Ileal, colonic	Azathioprine	No surgery
7	30	M	22	Ileal, colonic	Azathioprine	Panproctocolectomy
8	39	F	21	Ileal, colonic, perianal	None	Panproctocolectomy
9	29	F	24	Ileal, perianal	None	Right hemicolectomy
10	45	M	21	Colonic, perianal	Azathioprine	No surgery
11	30	M	21	Colonic, perianal	Azathioprine	No surgery
12	23	M	19	Ileal, colonic	Azathioprine	Right hemicolectomy
13	49	F	25	Ileal, colonic	Azathioprine	No surgery
14	34	F	24	Ileal	Azathioprine	Right hemicolectomy
15	24	M	21	Colonic	Azathioprine	No surgery

BMI, body mass index.

'immediately before' study performed just before the next infusion, usually 7 weeks later. This allowed us to exclude any effect that could be attributed to the order in which the studies were conducted. Following an overnight fast, an 18 G cannula was inserted into a forearm vein. A 20 mL sample of blood was collected in a lithium heparin tube containing 20 U/mL of aprotonin. A 450 cal meal consisting of a cereal bar, a slice of buttered toast, a glass of orange juice (250 mL) and a cup of tea with milk (180 mL) was eaten. Following this, further samples were taken every 20 min for 2 h. Blood samples were centrifuged immediately, plasma extracted and stored at -80°C for later analysis. Assays were performed for plasma total ghrelin, acylated ghrelin, desacyl ghrelin, C-reactive protein and a panel of cytokines. The Harvey Bradshaw Activity Index (HBAI) was calculated for each patient on each study occasion.

Assays

Ghrelin. All samples were assayed in duplicate. Total ghrelin, was measured by ELISA (Catalogue DSL-10-33700; Diagnostics Systems Laboratory, Webster, TX, USA). Inter-assay variation was 7%. Acylated ghrelin was assayed using Linco Gut Panel Multiplex (Catalogue HGT-68K; Linco Research, St. Charles, MO, USA). Inter-assay variation was 2.06%. Desacyl ghrelin was assayed using an EIA kit (Catalogue A05119; SPI-

BIO, Montigny le Bretonneux, France). Inter-assay variation was 6.6%. The Tecan GENios plate reader with Magellan ver.4 software (Tecan Group Ltd., Mannedorf, Switzerland) was used to analyse the results.

C-reactive protein (CRP). C-reactive protein was measured by ELISA using the High Sensitivity C-Reactive Protein EIA (Catalogue 2210; Life diagnostics Inc., West Chester, PA, USA).

Cytokines. $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-6 and $\text{IFN-}\gamma$ were measured using the High Sensitivity Human Cytokine Lincplex Kit (Catalogue: HSCYTO-60SK; Linco Research) with the Bio-Plex system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Results were generated using Bio-Plex Manager software ver.4 (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Data processing

The principles of data processing were agreed by investigators before the study and adhered to strictly during data analysis. Data loss was defined as failure of an assay to record a value for a time point. As assays were performed in duplicate when data were unavailable for one or both wells, this was regarded as data loss for that time point. If a single time point is lost, a value is calculated which is the average of the

values of the two adjacent time points. If the lost time point is either the first or last time point in the profile, then the single adjacent value was assigned to that time point. If data are lost for two adjacent time points in a 2-h profile, such a profile was declared invalid and the patient was excluded from the final analyses.

Statistics

For each patient, a 2-h profile of ghrelin concentration before and after infliximab was available. From these data, a 2-h integrated ghrelin concentration or area under the curve (AUC) for the study period was calculated using the trapezoid rule.

C-reactive protein, cytokines and glucose and insulin were measured in fasting samples taken before and after infliximab.

Data are described in nonparametric terms and as all comparisons were paired, the Wilcoxon's matched pair nonparametric test was used throughout. Graphpad Prism software was used for the analysis.

Ethical considerations

The study was approved by Coventry Research Ethics Committee and the R & D Department of the University Hospital of Coventry. The patients gave written informed consent.

RESULTS

Following adjustment for data loss, data were available for 11, 9 and 15 patients in the total ghrelin, acylated ghrelin and des-acyl ghrelin groups respectively. The assay for acylated ghrelin was subject to most data loss partly because acylated ghrelin is a small fraction of all circulating ghrelin isoforms.

Ghrelin

Total ghrelin ($n = 11$). Figure 1 shows that following infliximab infusion, there was a higher median concentration of total ghrelin during the 2-h study period. It also shows a typical postprandial decrease in plasma ghrelin in both profiles.

Figure 2 shows that plasma integrated total ghrelin for the 2-h study period increased in nine of the 11 patients following infliximab. Figure 3 and Table 2 show that median (95% CI) plasma integrated total ghrelin for the 11 subjects increased by 25% from 162

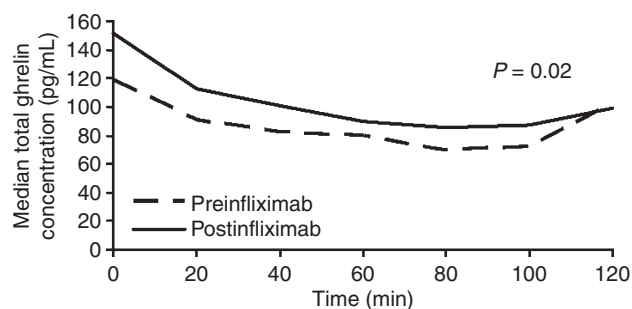


Figure 1. Median plasma total ghrelin (pg/mL) concentration profiles for 11 patients pre- and postinfliximab infusion. A 450 cal meal was eaten immediately after the fasting blood sampling at $t = 0$. Both curves show the typical postprandial decrease in total ghrelin.

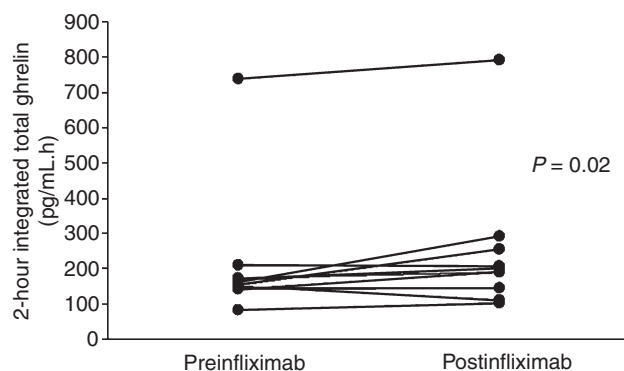


Figure 2. Two-hour integrated plasma total ghrelin (pg/mL h) for 11 patients pre- and postinfliximab infusion. Integrated ghrelin is increased in nine of 11 patients after infliximab.

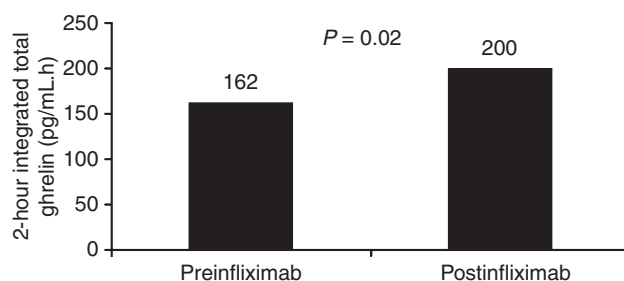


Figure 3. Median 2-h integrated plasma total ghrelin area under the curve (pg/mL h) for 11 patients pre- and postinfliximab infusion. Median (95% CI) 2-h integrated total ghrelin increased by 25% from 162 (99–311) pg/mL h before to 200 (128–387) pg/mL h 1 week after infliximab infusion ($P = 0.02$).

Table 2. Summary of ghrelin data

	Pre-infliximab (95% CI)	Post-infliximab (95% CI)	P-value
Total ghrelin AUC (pg/mL h)	162 (99–341)	200 (128–387)	0.02
Acyated ghrelin (pg/mL) at $t = 20$ min	50.3 (24–64)	38.6 (26–82)	0.04
Acyated ghrelin AUC (pg/mL h)	91 (49–142)	102 (47–139)	0.38
Des-acylated ghrelin AUC (pg/mL h)	397 (356–648)	423 (331–651)	0.63

AUC, area under the curve.

P-value is derived from comparison between paired pre- and postinfluximab studies using the Wilcoxon Rank Sum Test.

(99–341) pg/mL h before infliximab to 200 (128–387) pg/mL h ($P = 0.02$) 1 week after infliximab.

Acyated ghrelin ($n = 9$). Figure 4 shows the profiles of median plasma acyated ghrelin concentration before and after infliximab. A normal postprandial decrease in ghrelin is absent in the before infliximab profile. However, in the after infliximab profile, at 20 min postmeal, median (95% CI) acyated ghrelin concentration decreased below fasting concentration (Table 2) and was also significantly lower than the same time point in the before infliximab profile [38.6 (26–82) pg/mL vs. 50.3 (24–64) pg/mL; $P = 0.04$]. Thus, in the after infliximab profile, the traditional meal-related ghrelin curve with its postprandial dip is

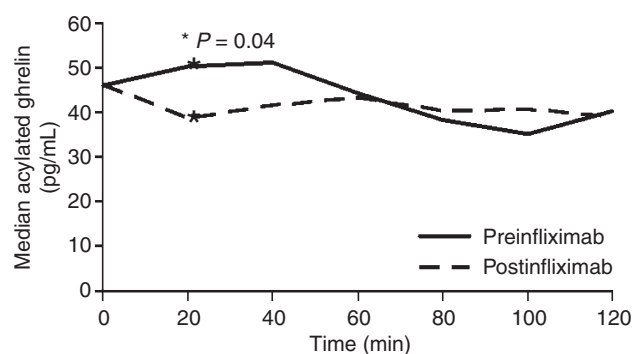


Figure 4. Median plasma acyated ghrelin concentration profiles for nine patients pre- and postinfluximab infusion. Unlike the pre-influximab profile, the post-infusion shows a typical postprandial decrease in acyated ghrelin with median (95% CI) plasma acyated ghrelin concentration at $t = 20$ min being significantly lower than the same time point in the pre-influximab curve [38.6 (26–82) pg/mL vs. 50.3 (24–64) pg/mL; $P = 0.04$].

restored. Over the 2-h study period, integrated acyated ghrelin increased nonsignificantly from 91 (49–142) pg/mL h before infliximab to 102 (47–139) pg/mL h after infliximab (Table 2), a trend similar to that observed for total ghrelin.

Des-acylated ghrelin ($n = 15$). There was no statistical difference in median (95% CI) integrated des-acylated ghrelin, which was 397 (356–648) pg/mL h before infliximab and 423 (331–651) pg/mL h after (Table 2).

Cytokines (Table 3)

TNF α . Median (95% CI) TNF α concentration decreased significantly from 2.8 (1.89–4.48) pg/mL before infliximab to 1.31 (0.73–2.06) pg/mL ($P = 0.002$) after infliximab.

IL-1 β , IL-6, and IFN- γ . Before and after infliximab median (range) IL-1 β values were 0.7 (0.55–1.76) and 0.89 (0.69–1.96) respectively, median (95% CI) IL-6

Table 3. Summary of cytokine data

	Pre-infliximab (95% CI)	Postinfluximab (95% CI)	P-value
TNF α (pg/mL)	2.8 (1.89–4.48)	1.31 (0.73–2.06)	0.002
IL-1 β (pg/mL)	0.7 (0.55–1.76)	0.89 (0.69–1.96)	0.16
IL-6 (pg/mL)	20.6 (13.0–29.9)	20.7 (12.5–27.9)	0.10
IFN γ (pg/mL)	10.8 (4.7–22.3)	7.3 (4.6–23.9)	0.70

P-value is derived from comparison between paired pre- and postinfluximab studies using the Wilcoxon Rank Sum Test.

values were 20.6 (13.0–29.9) and 20.7 (12.5–27.9) respectively and median (95% CI) IFN- γ values were 10.8 (4.7–22.3) and 7.3 (4.6–23.9) respectively. There was no significant difference.

C-reactive protein

C-reactive protein concentration decreased nonsignificantly from 2.1 (0.57–7.44) mg/mL before infliximab to 0.7 (0.11–3.28) mg/mL after infliximab.

Harvey Bradshaw Activity Index

Median (range) HBAI decreased from 5 (2–28) before infliximab to 3 (0–22) after infliximab, ($P = 0.0001$).

DISCUSSION

Our study design extending over a 2-h period took account of the pulsatile nature of ghrelin secretion in humans. The eating of a meal was also included in the study period to test ghrelin regulatory mechanisms. We were able to report and compare data as 'area under the curve' and 2-h concentration profiles immediately before and 1 week after the infusion of infliximab. Our study provides novel evidence that systemically altering an important inflammatory mediator like TNF α can lead to changes in circulating ghrelin. The infusion of infliximab led in CD patients to a 25% decrease in total ghrelin concentration assessed during a 2-h period after a meal. As expected, there was a significant decrease in TNF α and CD activity. A similar trend was observed for CRP. The relatively low concentrations of TNF α and CRP reflect that our study patients, the majority in maintenance infliximab programmes, were in clinical remission. It would be of interest to study patients who start with a higher inflammatory burden who, we believe, would show an exaggeration of the changes in ghrelin observed in our study. It is possible that ghrelin producing cells can sense and respond to changes in circulating TNF α . This mechanism may operate at the cellular level with circulating TNF α exerting a direct effect on gastric ghrelin producing cells. Another hypothesis is that TNF α might act centrally and modulate circulating ghrelin through the efferent vagal nerves to the stomach. In a recent study, rats that were rendered hypophagic by TNF α administration were found to have

decreased gastric and circulating ghrelin leading to the conclusion that some of the anorexic effects of TNF α are mediated via ghrelin. In that study, the effects of hypophagia induced by TNF α could be reversed by administration of ghrelin.²³

Circulating acylated ghrelin is often referred to as 'active' ghrelin. This isoform stimulates hunger and growth hormone release by acting on hypothalamic nuclei. In our Crohn's patients, at the time of maximum inflammatory activity, the acylated ghrelin concentration at the 20-min time point is higher than the preceding fasting value. There was no postprandial decrease in acylated ghrelin concentration, which is an accepted feature of a normal ghrelin curve. Similar abnormalities in postprandial ghrelin profiles have been reported in obesity and non-alcoholic steatohepatitis, two diseases that have a significant inflammatory contribution to their pathophysiology.^{13, 24} Following infliximab infusion in our patients, the acylated ghrelin curve was normalized and the 20-min ghrelin concentration is lower than the preceding fasting value. Thus, inflammation in general and perhaps TNF α specifically affect not only total ghrelin production but may also modify the mechanisms that regulate ghrelin production in the stomach.

Our study also showed that des-acyl ghrelin, an isoform that still has an uncertain physiological role, was not affected either in terms of its profile or AUC by infliximab.

In conclusion, this study provides novel evidence that altering TNF α with a biological agent can affect circulating ghrelin in humans. It adds further evidence that ghrelin and inflammatory mediators interact in inflammatory diseases like CD pointing to a possible immuno-modulatory role. Our study suggests that neither des-acyl nor acylated ghrelin mediates this role raising the possibility that a yet unrecognized biological isoform could be involved. We acknowledge that inter-assay variability could explain the difference in results seen with the ghrelin isoforms. An infliximab-induced increase in circulating plasma ghrelin, if accompanied by increased food intake, suggests another mechanism by which this drug might improve well-being in CD patients.

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Ghrelin promotes nuclear factor kappa-B activation in a human B-lymphocyte cell line

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Abstract Ghrelin, an orexigenic hormone of gastric origin that stimulates growth hormone secretion, may modulate inflammation. This experimental study examines the effect of ghrelin on NF κ B (p65 subunit), a transcriptional factor involved in inflammation on a human B-lymphocyte cell (WILCL). After confirming the expression of ghrelin receptor protein using western blotting the cells were transferred to wells maintaining a density of 1×10^6 cells per ml and a proportion activated with phytohaemagglutinin. Activated and resting cells were exposed to octanoyl-, desoctanoyl ghrelin and a non-peptide ghrelin agonist (Pfizer CP-464709) in increasing concentrations for 6 h. Cell protein extracts were analyzed for NF κ B activation using Trans AM NF κ B p65 assay. IL-6, IL-8, IL-10, IL-13 and TNF α were measured in the media using Lincoplex human cytokine assay. In octanoyl ghrelin treated resting cells, NF κ B activity (Optical Density OD_{450nm}) (mean \pm SEM) in control cells was 0.42 ± 0.10 and increased to 0.61 ± 0.20 ($P = 0.044$), 0.54 ± 0.10 ($P = 0.043$), 0.52 ± 0.08 at 1, 10 and 100 nM concentrations respectively. No effect was detected with desoctanoyl ghrelin or ghrelin agonist and no specific change in cytokine production. In conclusion, Octanoyl ghrelin increased NF κ B activation by up to 50% in a B-lymphocyte cell line suggesting an effect on the inflammatory process.

Keywords Nuclear factor kappa-B · Lymphocyte · Ghrelin · Ghrelin receptor · Human

Abbreviations

NF κ B	Nuclear factor kappa-B
I κ B	Inhibitory kappa-B
IL	Interleukin
TNF	Tumour necrosis factor
GHSR	Growth hormone secretagogue receptor
PHA	Phytohaemagglutinin

Introduction

Ghrelin is a 28 amino acid signaling peptide produced mainly by the stomach [1] but also in smaller quantities by other organs. It is the natural ligand for the growth hormone secretagogue receptor, GHSR-1a [2], now recognized widely as the ghrelin receptor. There are two main isoforms of ghrelin, octanoyl ghrelin which is thought to be the biologically active and desoctanoyl ghrelin, an isoform of which the primary function is still undetermined. Ghrelin is a potent stimulant of growth hormone release and also has an established role in the control of hunger and energy hemostasis [3]. Recent studies suggest that it may have an immunomodulatory role. In animal studies ghrelin seems to protect against inflammatory damage [4–6]. The presence of ghrelin receptors on human immune cells [7] and the suppression of inflammatory cytokine (IL-1 β , IL-6 and TNF α) secretion by these cells when exposed to ghrelin in vitro suggests a possible immuno-modulatory role. This effect appears to be opposite to that of leptin [8].

A nuclear factor kappa B (NF κ B) a nuclear transcriptional factor discovered in 1986 [9], is involved in the

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cellular responses to an environmental stress, by inducing the production of protective signals. NF κ B is held inactive in cellular cytoplasm by inhibitory kappa B (I κ B). NF κ B activation involves dephosphorylation of I κ B and releasing NF κ B [10]. The conserved N-terminus of NF κ B then binds to the DNA and promotes the transcription of a variety of pro-inflammatory signals. In B-lymphocytes, NF κ B plays a significant role in immunoglobulin production. The gene enhancer for κ light chain is activated by NF κ B and found to be crucial for B-cell maturation and function against pathogens [11]. More recently, NF κ B is demonstrated to play an important role in driving and coordinating the innate and adaptive immune response to an antigen [12]. NF κ B has various roles in the inflammatory process, particularly in uncontrolled lymphocyte activation, and has been a subject of interest as a therapeutic target [13]. In addition, NF κ B activation is also thought to be involved in cellular apoptosis [14] and also implicated in tumour invasion [15].

The objective of this study is to investigate the effect of ghrelin on NF κ B activation and inflammatory cytokine production in human B-lymphocyte cell line. If ghrelin has immunomodulatory properties, we hypothesize that ghrelin may decrease NF κ B activation in a B-lymphocyte cell line. Although immunoglobulin production is a measure of primary B-lymphocyte cell function, it is uncertain if this characteristic is replicated in the cell line we used. We therefore focused our enquiry on cell activation and NF κ B expression both upstream of immunoglobulin production but established characteristics of the cell line we studied.

Materials and methods

Cell line selection and screening experiments

Four cell lines, 2 B-lymphocyte (WILCL, ECACC Cat: 89120565; DAUDI, ECACC Cat: 85011437) and 2 T-lymphocyte (JM, ECACC Cat: 86010201; HUT-78, ECACC Cat: 88041901) were tested for expression of the ghrelin receptor. Briefly cells were cultured in appropriate medium, collected and lysed. Total protein was extracted and quantified. Using western blotting techniques, the GHSR 1a (now known as the ghrelin receptor) and the dormant GHSR 1b receptor was detected using commercially available antibodies from Phoenix Pharmaceuticals Inc, CA, USA (GHSR 1a, Cat G-001-62; GHSR 1b, Cat G-001-61) (Fig. 1). The WILCL cell line, a normal Caucasian human B-cell lymphoblast immortalized by the Epstein-Barr virus expressed ghrelin receptors and was used for the rest of the study.

Phytohaemagglutinin (PHA) was used for lymphocyte activation. The optimal concentration of PHA was estimated using trypan blue staining for cell viability. PHA concentration of 0.5 mg/ml was optimal for WILCL cell

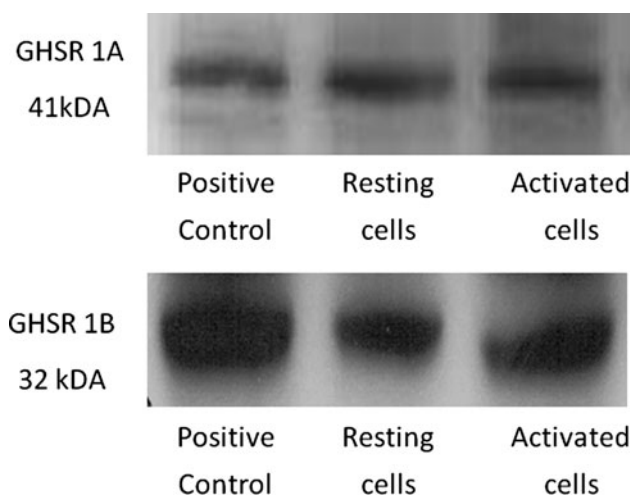


Fig. 1 Western blots of the ghrelin receptor protein in WILCL human B-lymphocyte cell line. Human adipose tissue protein extracts which has been confirmed to express both ghrelin and GHSR-1b receptors are used for positive controls. The ghrelin receptors (42 kDa) and GHSR 1b (32 kDa) are demonstrated in both resting and activated lymphocyte cells

activation. We assessed the effect of the compounds on activated and resting cells.

To assess the effect of ghrelin isoforms and a non-peptide ghrelin agonist on the cell line, each treated well was compared to control wells with no additions.

A screening experiment (performed in triplicate) using time points of 6, 24 and 48 h were performed using octanoyl ghrelin on resting and activated cells (Table 1). A response was detected at 6 h to PHA activation and octanoyl ghrelin. In all subsequent experiments 6 h incubation duration was applied.

Cell resuscitation and culture

Cells were resuscitated from liquid nitrogen storage in a warm water bath (37°C), added to a flask containing 5 ml of pre-prepared media and were grown in RPMI 1640 media (Invitrogen, UK) enriched with L-glutamine (200 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml) and 10% fetal bovine serum (Invitrogen, UK). Cell density was maintained at $3\text{--}5 \times 10^6$ /ml using a haemocytometer. They were cultured in an incubator maintaining an environment of 5% CO₂ and 95% air. Media was changed every 2–3 days and cell subculture was performed to maintain cell density.

Cells cultured in flasks were centrifuged (150 g for 10 min) and resuspended in fresh media. 1×10^6 /cells were added to each well of a 6-well culture plate.

The lymphocytes were activated by PHA (Sigma, UK) as described earlier and exposed to 3 different compounds namely; octanoyl ghrelin (Phoenix, CA, USA; Cat. 031-39), des-octanoyl Ghrelin (Phoenix, CA, USA; Cat. 031-32) and

Table 1 Results of the screening experiments (N = 3) in cells dosed with octanoyl ghrelin. There was a trend towards increased NFκB activation in the resting group at T = 6 h

	Mean and SEM	<i>P</i> value		Mean and SEM	<i>P</i> value
NFκB activation in WILCL at 6 h					
Control	0.301 ± 0.044		Control and PHA activation	0.311 ± 0.083	
1 nM	0.29 ± 0.05	0.42	1 nM and PHA activation	0.351 ± 0.10	0.1
10 nM	0.371 ± 0.12	0.26	10 nM and PHA activation	0.341 ± 0.13	0.09
100 nM	0.321 ± 0.063	0.34	100 nM and PHA activation	0.321 ± 0.062	0.39
NFκB activation in WILCL at 24 h					
Control	0.311 ± 0.04		Control and PHA activation	0.291 ± 0.05	
1 nM	0.301 ± 0.04	0.28	1 nM and PHA activation	0.291 ± 0.05	0.15
10 nM	0.311 ± 0.04	0.32	10 nM and PHA activation	0.311 ± 0.04	0.18
100 nM	0.281 ± 0.04	0.03	100 nM and PHA activation	0.321 ± 0.04	0.1
NFκB activation in WILCL at 48 h					
Control	0.431 ± 0.10		Control and PHA activation	0.411 ± 0.06	
1 nM	0.371 ± 0.05	0.17	1 nM and PHA activation	0.371 ± 0.04	0.26
10 nM	0.421 ± 0.11	0.43	10 nM and PHA activation	0.431 ± 0.09	0.3
100 nM	0.381 ± 0.065	0.17	100 nM and PHA activation	0.451 ± 0.08	0.14

a non-peptide ghrelin agonist (Pfizer CP-464709) [16, 17] which has been used in motility studies. These compounds were added to the wells with resting and PHA activated cells to make up concentrations of control cells (no addition), 1, 10 and 100 nM. For the ghrelin receptor agonist and des-octanoyl ghrelin that are not natural ligands of the ghrelin receptor, we widened the concentration range to include 0.1 and 1000 nM. The cells were then washed, collected and frozen to -80°C . The cell pellets were later lysed and protein content were extracted and quantified.

NFκB assay

NFκB expression was analysed using Active Motif TransAM NFκB p65 (Cat: 31102, CA, USA) ELISA-based assay. In brief, this assay consists of a 96 well plate each coated with an oligonucleotide containing the NFκB consensus region (5'-GGGACTTTCC-3') which the active form of NFκB from cell lysates bind. The primary antibody only binds to the epitope on p65 only when NFκB is activated and bound to its target. A secondary HRP-conjugated antibody binds to the primary complex which then produces a signal quantifiable by spectrophotometry (measurable by optical density at 450 nm wavelength). The cell culture medium was analysed for IL-6, IL-8, IL-10 and TNFα using a high sensitivity human cytokine Lincoplex assay (Linco Research, Cat. HSCYTO-60SK, St. Charles, MO, USA) using the Bio-Plex system (Bio-Rad Laboratory Inc., Hercules, CA, USA). Inter-assay variability ranged between 2 and 15% for all cytokines measured. Cells exposed to ghrelin and its agonists were compared with resting and activated controls.

Statistical analysis

Data is reported as mean ± SEM. A Student's *t*-test was used to compare data from cell groups using GraphPad Prism software.

Results

Octanoyl-ghrelin (N = 6)

In the resting state, NFκB activity in the control cells was 0.42 ± 0.10 and increased to 0.61 ± 0.20 , 0.54 ± 0.10 , 0.52 ± 0.08 when exposed to 1, 10 and 100 nM octanoyl ghrelin respectively (Fig. 2a). The increase in NFκB activity in 1 and 10 nM was statistically significant ($P = 0.044$ and $P = 0.043$ respectively). NFκB activity increased by 25–50% in the octanoyl ghrelin treated cells when compared to control cells.

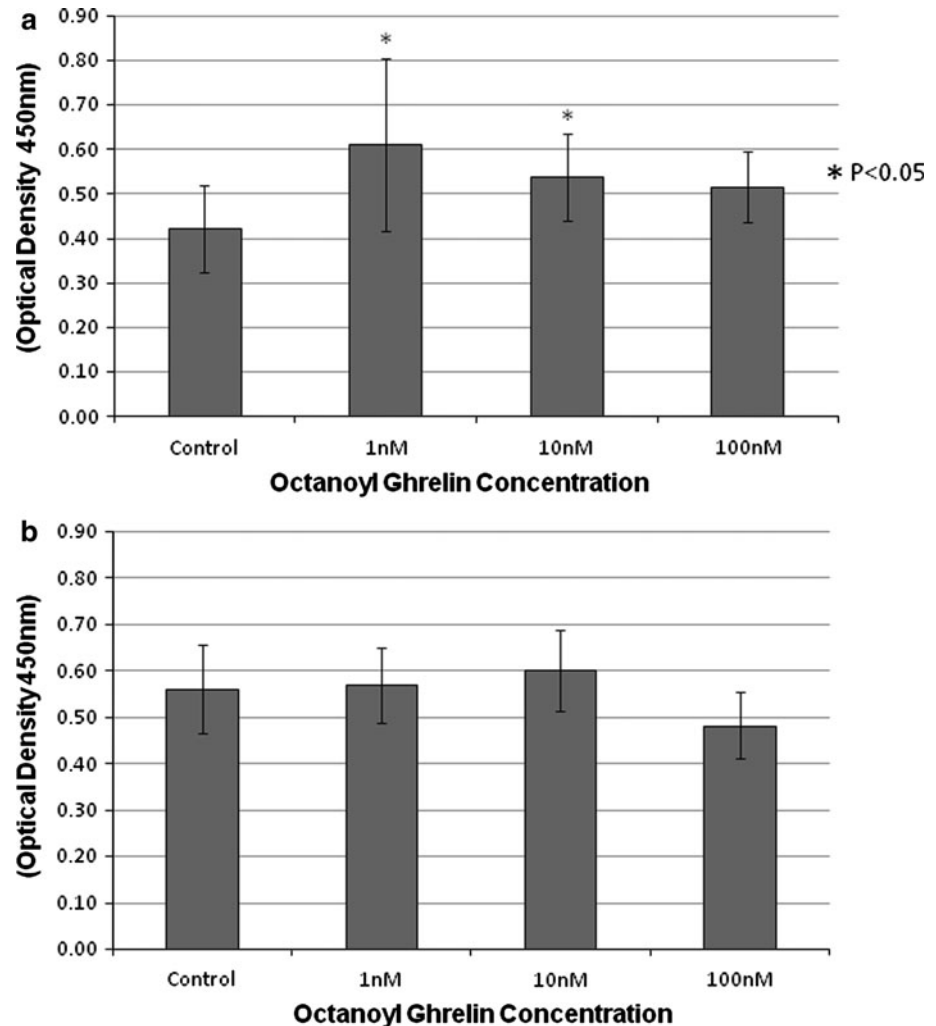
In the activated state, NFκB activity was 0.56 ± 0.10 in control cells and remained unchanged at 0.57 ± 0.08 , 0.60 ± 0.09 , 0.48 ± 0.07 when exposed to 1, 10 and 100 nM octanoyl ghrelin respectively (Fig. 2b).

Desoctanoyl ghrelin (N = 6)

In the resting state, the NFκB activity in the control cells was 0.35 ± 0.03 and remained unchanged at 0.36 ± 0.04 , 0.39 ± 0.04 , 0.36 ± 0.04 , 0.36 ± 0.03 when exposed to 0.1, 1, 10 and 100 nM des-octanoyl ghrelin respectively.

In the activated state, NFκB activity in the control cells was 0.38 ± 0.04 and remained unchanged at 0.38 ± 0.03 ,

Fig. 2 a, b NF κ B activation in resting and activated WILCL human B-lymphocyte cell line with increasing doses of octanoyl ghrelin. Data is the average of six experiments. The Student's *t*-test was used to compare NF κ B activation in increasing concentration of octanoyl ghrelin and controls. **a** The NF κ B profile in resting cells and demonstrates a 25–50% increase in NF κ B activation at 1 and 10 nM concentrations of octanoyl ghrelin. **b** The NF κ B activation in activated cells and showed no significant change with increasing concentrations of octanoyl ghrelin



0.41 ± 0.04 , 0.39 ± 0.04 , 0.39 ± 0.04 when exposed to 0.1, 1, 10 and 100 nM des-octanoyl ghrelin respectively.

Ghrelin receptor agonist (N = 6)

In the resting state, NF κ B activity in control cells was 0.30 ± 0.04 and remained unchanged at 0.29 ± 0.03 , 0.31 ± 0.04 , 0.31 ± 0.05 , and 0.29 ± 0.04 when exposed to 1, 10, 100 and 1000 nM ghrelin receptor agonist respectively.

In the activated state, the NF κ B activity in control cells was 0.31 ± 0.04 and remained unchanged at 0.31 ± 0.04 , 0.33 ± 0.05 , 0.32 ± 0.05 and 0.31 ± 0.04 when exposed to 1, 10, 100 and 1000 nM ghrelin receptor agonist respectively.

Cytokines (N = 6)

In resting and activated cells exposed to all concentrations of octanoyl ghrelin, no significant change in TNF α IL-6,

IL-8, IL-10 concentration in the media was demonstrated (Table 2). Table 3 showed that the non-peptide ghrelin agonist significantly suppressed lymphocyte IL-13 production in a dose dependent manner particularly in resting lymphocytes. This effect was not associated with any changes in NF κ B. IL-13 is an anti-inflammatory cytokine and its suppression suggests that the non-peptide ghrelin agonist could be pro-inflammatory acting via a pathway independent of NF κ B. This effect is not seen with the biological ligand octanoyl ghrelin suggesting that it may be a non-specific effect of the synthetic agonist.

Discussion

After a series of screening experiments we selected an immortalized B-lymphocyte cell line expressing ghrelin receptors as the target of investigation. Contrary to our initial hypothesis, this study shows that physiological concentrations of octanoyl ghrelin increased NF κ B

Table 2 Cytokine concentration in culture media of lymphocytes dosed with octanoyl ghrelin (N = 6)

	Non-PHA	<i>P</i> value	PHA	<i>P</i> value
IL-6				
Control	0.23 ± 0.03		0.31 ± 0.10	
1 nM	0.24 ± 0.04	0.729	0.21 ± 0.03	0.2673
10 nM	0.22 ± 0.03	0.252	0.27 ± 0.07	0.190
100 nM	0.19 ± 0.04	0.101	0.23 ± 0.05	0.188
IL-8				
Control	1.12 ± 0.93		1.40 ± 1.17	
1 nM	1.97 ± 1.54	0.33	1.36 ± 1.12	0.68
10 nM	1.47 ± 1.12	0.16	1.28 ± 1.09	0.11
100 nM	1.58 ± 1.22	0.16	1.28 ± 1.08	0.15
IL-10				
Control	1033.15 ± 270.57		1095.37 ± 246.01	
1 nM	1018.35 ± 232.24	0.88	1056.51 ± 249.85	0.58
10 nM	1031.57 ± 251.83	0.48	1130.65 ± 283.10	0.36
100 nM	966.62 ± 232.18	0.17	1067.75 ± 241.99	0.31
IL-13				
Control	3.83 ± 0.68		4.10 ± 0.50	
1 nM	3.91 ± 0.65	0.80	4.06 ± 0.64	0.86
10 nM	3.48 ± 0.60	0.13	4.07 ± 0.73	0.47
100 nM	3.55 ± 0.52	0.22	3.72 ± 0.54	0.06
TNFα				
Control	27.93 ± 678		35.54 ± 9.63	
1 nM	33.58 ± 12.31	0.44	31.37 ± 8.63	0.03
10 nM	30.40 ± 9.13	0.24	32.46 ± 7.98	0.12
100 nM	30.25 ± 9.22	0.27	30.19 ± 7.24	0.10

activation by up to 50% when applied to this cell line. This effect was not dose dependent but peaked at 1 and 10 nM and seemed to decline at 100 nM concentration. As expected this effect was not seen with the non-peptide ghrelin receptor agonist and des octanoyl ghrelin; neither are the natural ligands for the ghrelin receptor. The effect of octanoyl ghrelin was detected only when the cells were in a resting state and absent when activated with PHA. In the active state lymphocytes are probably at a state of maximal NFκB activation and additional stimulation via the ghrelin receptor is unable to increase this measurably.

Previous studies have demonstrated ghrelin receptors on human immune cells and also a decrease in cytokine production in T-lymphocytes and monocytes treated with ghrelin but not in B-lymphocytes. As expected, our in vitro model detected no consistent change in the panel of cytokines occurring in parallel with the changes in NFκB. We also detected an NFκB-independent non-specific effect of the non-peptide ghrelin agonist which suppressed IL-13 production in both resting and activated lymphocytes. However we believe the effect of octanoyl ghrelin on

Table 3 Cytokine concentration in culture media of lymphocytes dosed with the non-peptide ghrelin agonist (N = 6)

	Non-PHA	<i>P</i> value	PHA	<i>P</i> value
IL-6				
Control	0.25 ± 0.06		0.26 ± 0.05	
1 nM	0.24 ± 0.05	0.04	0.25 ± 0.04	0.30
10 nM	0.27 ± 0.03	0.16	0.27 ± 0.05	0.45
100 nM	0.25 ± 0.04	0.11	0.25 ± 0.06	0.05
1000 nM	0.28 ± 0.06	0.36	0.26 ± 0.06	0.47
IL-8				
Control	12.18 ± 3.86		11.86 ± 3.71	
1 nM	12.45 ± 4.19	0.34	11.85 ± 3.77	0.49
10 nM	11.81 ± 3.38	0.28	11.84 ± 3.14	0.49
100 nM	11.03 ± 3.35	0.05	13.35 ± 5.07	0.17
1000 nM	12.40 ± 14.46	0.38	11.74 ± 3.93	0.38
IL-10				
Control	790.84 ± 126.17		835.59 ± 134.23	
1 nM	764.81 ± 122.30	0.26	775.24 ± 138.14	0.04
10 nM	844.94 ± 195.24	0.27	770.74 ± 134.49	0.06
100 nM	723.14 ± 121.23	0.10	812.52 ± 131.26	0.20
1000 nM	759.59 ± 127.19	0.21	753.79 ± 127.14	0.05
IL-13				
Control	5.88 ± 0.67		5.96 ± 0.68	
1 nM	5.29 ± 0.65	0.01	5.60 ± 0.73	0.14
10 nM	5.08 ± 0.82	0.00	5.40 ± 0.70	0.06
100 nM	4.88 ± 0.56	0.01	5.35 ± 0.55	0.05
1000 nM	4.97 ± 0.63	0.00	4.55 ± 0.42	0.00
TNFα				
Control	99.68 ± 5.29		113.49 ± 7.43	
1 nM	95.33 ± 7.33	0.24	116.70 ± 7.31	0.25
10 nM	99.24 ± 8.86	0.46	108.70 ± 7.21	0.18
100 nM	88.17 ± 5.19	0.02	110.74 ± 8.15	0.30
1000 nM	92.64 ± 8.18	0.13	107.25 ± 8.36	0.15

NFκB detected only in resting lymphocytes is more biologically plausible.

We recognize that cytokine production is not a primary function of B-lymphocyte. Our experiment explored the interaction between B-lymphocyte cell activation and NFκB expression both known characteristics of this cell line. If this was a primary B lymphocyte cell line function would have been best assessed by immunoglobulin production but we were unable to confirm that this cell line had this characteristic. We also had more experience with PHA in our laboratory.

NFκB is known to regulate the development and maturation of lymphocytes [18]. The uncontrolled activation of NFκB has been implicated in many inflammatory diseases and cancer. This has driven much interest in NFκB as a therapeutic target in many diseases. There are studies that suggest an anti-inflammatory effect of ghrelin on human

and animal lymphocytes (8, 19) and although our data points to a contrary conclusion it provides further evidence that ghrelin may be part of a complex human immune regulatory repertoire and raises the possibility that its receptor could be a target for immune-modulatory therapy. Indeed there has been contrary evidence that ghrelin increases NF κ B activation and promote inflammation in the human colon [20] This is the first study to demonstrate the effects of ghrelin on a human B-lymphocytes in vitro model. Further evaluation and understanding on how NF κ B regulates the immune system will be important in understanding a possible role of ghrelin.

Conflict of interest None.

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Effects of neo-adjuvant chemotherapy for oesophago-gastric cancer on neuro-muscular gastric function

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Abstract Delayed gastric emptying symptoms are often reported after chemotherapy. This study aims to characterise the effects of chemotherapy on gastric neuro-muscular function. Patients undergoing elective surgery for oesophago-gastric cancer were recruited. Acetylcholinesterase, nNOS, ghrelin receptor and motilin expressions were studied in gastric sections from patients receiving no chemotherapy ($n = 3$) or oesophageal ($n = 2$) or gastric ($n = 2$) chemotherapy. A scoring system quantified staining intensity (0–3; no staining to strong). Stomach sections were separately suspended in tissue baths for electrical field stimulation (EFS) and exposure to erythromycin or carbachol; three patients had no chemotherapy; four completed cisplatin-based chemotherapy within 6 weeks prior to surgery. AChE expression was markedly decreased after chemotherapy (scores 2.3 ± 0.7 , 0.5 ± 0.2 and 0 ± 0 in non-chemotherapy, oesophageal- and gastric-chemotherapy groups ($p < 0.03$ each) respectively. Ghrelin receptor and motilin expression tended to increase (ghrelin: 0.7 ± 0.4 vs 2.0 ± 0.4 and 1.2 ± 0.2 respectively; $p = 0.04$ and $p = 0.2$; motilin: 0.7 ± 0.5 vs 2.2 ± 0.5 and 2.0 ± 0.7 ; $p = 0.06$ and $p = 0.16$). Maximal contraction to carbachol was 3.7 ± 0.7 g and 1.9 ± 0.8 g (longitudinal muscle) and 3.4 ± 0.4 g and 1.6 ± 0.6 (circular) in

non-chemotherapy and chemotherapy tissues respectively ($p < 0.05$ each). There was loss of AChE and reduction in contractility to carbachol. The tendency for ghrelin receptors to increase suggests an attempt to upregulate compensating systems. Our study offers a mechanism by which chemotherapy markedly alters neuro-muscular gastric function.

Keywords Acetylcholinesterase · Ghrelin · Motility · Stomach · Chemotherapy · Cisplatin

Abbreviations

EFS Electrical field stimulation
AChE Acetylcholinesterase
nNOS Neuronal nitric oxide synthase

Introduction

Neoadjuvant chemotherapy for oesophageo-gastric cancers prior to surgery is now common practice following clinical trials that showed survival improvement [1, 2]. However, following completion of chemotherapy, these patients frequently continue to experience distressing symptoms resembling dyspepsia and attributed to a disorder in gastric emptying [3]. Riezzo et al. [4] suggested that gastric dysmotility-like symptoms (susceptibility to nausea, early satiety, post-prandial fullness) and tachygastria were common in patients seven days after completion of chemotherapy, during which emesis was well controlled. Such studies raise the possibility that the anti-cancer chemotherapy may somehow induce a gastric dysmotility which outlasts the treatment and which may lead to prolonged symptoms of dyspepsia. For this to occur, it is certainly not

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inconceivable that chemotherapy directly affects the integrity of the neuromuscular functions of the stomach. In rat gastro-duodenal muscle, for example, a reduction in the presence of the Ca^{2+} -calmodulin complex has been observed three days after a single treatment with cisplatin [5].

To test the possibility that certain anti-cancer chemotherapy treatments may cause long-term damage to gastric function, we have looked at the effects of cisplatin-based treatments on the function and integrity of human stomach. For this purpose we examined the ability of human isolated stomach to contract in response to carbachol and other stimuli, following removal at surgery from patients who had undergone chemotherapy up to six weeks previously, compared to patients who had not undergone chemotherapy. The integrity of the motor nerve system was also examined by immunohistochemical staining for acetylcholinesterase (AChE) and for the neuronal isoform of nitric oxide synthase (nNOS). In addition, we looked for changes in expression of the gastric motility stimulant motilin [6] and the receptors for ghrelin. The latter was included because of an association between ghrelin and changes in appetite and gastric motility [7] and also because of a previous study in rats, which showed that the ghrelin receptor may be up-regulated following administration of cisplatin [8].

Methods

All patients enrolled were diagnosed with resectable oesophageo-gastric cancers. The patients were staged by computed tomography (CT) scan, endoscopy including endoscopic ultrasound and laparoscopy. All patients were staged as TxN0(or x)M0 by the TMN staging system and were assessed to be suitable for surgery. The study was approved by the local Coventry research ethics committee and written informed consent was obtained from all patients.

Patients with gastric adenocarcinomas ($n = 2$) received three courses of epirubicin (50 mgm^{-2}), cisplatin (60 mgm^{-2}) and capecitabine (625 mgm^{-2}). Patients with oesophageal cancers ($n = 2$) received two courses of cisplatin (80 mgm^{-2}) and 5-fluorouracil (1 gm^{-2}). Patients who did not undertake chemotherapy ($n = 3$) were physically frail, deemed at risk of severe side effects of chemotherapy or undergoing Whipple's procedure for pancreatobiliary tumours. All patients underwent surgical resection within a 2 week window of 4–6 weeks following completion of chemotherapy regime, based on local cancer pathway.

For the histological studies, paraffin-embedded samples were used for staining and immunohistochemistry. In the

gastric tissue contractility experiments, gastric tissue was obtained from tumour free margins during surgery. Samples were then stored at 4°C in Gey's balanced salt solution (Sigma, Gillingham, Dorset, UK) which was pre-bubbled with 95 % O_2 /5 % CO_2 . These tissues were processed within 24 h of collection (see below).

Immunohistochemistry and histological staining

Histological paraffin embedded tissue blocks showing normal tissue adjacent and distant from the tumour site were selected. $5 \mu\text{m}$ thick sections were baked onto 3-aminopropyltriethoxy-silane coated slides. Specific antibodies were used to look for the expression of motilin, the ghrelin receptor, AChE and nNOS, in sections of gastric tissues taken from patients who did not have chemotherapy and in those receiving oesophageal-chemotherapy (cisplatin + 5-fluorouracil) or gastric chemotherapy (epirubicin, cisplatin and capecitabine) ($n = 6$ each).

Antigen retrieval was carried out by pressure cooking in Tris EDTA buffer pH 7.8 for 80 s. Vector Universal Elite ABC kit (Catalogue Number PK-6200; Burlingame, CA, USA) with a diaminobenzidine tetrahydrachloride visualisation agent (Vector ImPACT DAB substrate, Cat. No. SK-4105, Burlingame, CA, USA) was used for visualization of bound antibody. Optimised antibody dilutions were determined for motilin receptor (MBL International Corp, Woburn, MA, USA, Cat. No. LS-A134), ghrelin receptor (Chemicon, Billerica, MA, USA, Cat. No. AB9543), nNOS (Abcam, Cambridge, UK, Cat. No. ab40662) and AChE (Abcam, Cambridge, UK, Cat. No. ab2803). Haematoxylin was used as a counter stain, dehydrated, cleared and mounted. The intensity and distribution of peroxidase staining was examined and scored by a histopathologist who was blinded to the patient groups. For all procedures, a semi-quantitative scoring system for staining intensity was used (0 = no staining, 1 = weak, 2 = moderate, 3 = strong expression).

Gastric contractility

Specimens of stomach were placed in oxygenated (95 % O_2 , 5 % CO_2) Gey's salt solution overnight. In some cases, on the following morning the tissues were placed in fresh Krebs solution, bubbled and then transported to the laboratory. On arrival the specimens were placed in fresh, oxygenated Krebs solution (containing in mM NaCl 121.5, CaCl_2 2.5, KH_2PO_4 1.2, KCl 4.7, MgSO_4 1.2, NaHCO_3 25, glucose 5.6) which had been equilibrated with 5 % CO_2 and 95 % O_2 . The mucosa was removed and strips ($2\text{--}4 \times 10\text{--}15 \text{ mm}$) were cut parallel to the longitudinal or the circular muscle. The strips (2–4 from each patient) were mounted in tissue baths (10 ml) containing Krebs solution at 37°C and gassed with 5 % CO_2 in O_2 . Tension

was measured using Dynamometer UF1 force–displacement transducers (Pioden Control Ltd., UK). Data acquisition and analysis were performed using MP100 hardware and AcqKnowledge software (Biopac Systems Inc., USA). Tissues were initially suspended under 2–3 g tension and allowed to equilibrate for at least 45 min during which time bath solutions were changed every 15 min. During this time, muscle tension stabilised at ~ 1 g.

At the end of the equilibration period, some longitudinal muscle strips were stimulated via two parallel platinum ring electrodes connected to a stimulator (STG2008, Scientifica Ltd, UK). The stimulation parameters were 50 V (~ 200 mA), 0.5 ms bipolar pulse duration, applied for 10 s, every 1 min. The frequency was changed every 3 min to produce a frequency–response curve, using frequencies of 1, 2, 5, 10, 15, and 20 Hz. After washing and in tissues not subjected to EFS, 10 μ M erythromycin was added for 15 min. As not all tissues responded to EFS or erythromycin, the data are expressed simply as the number of tissues which responded by contraction, or not. The tissues were washed and left for 30 min. For both longitudinal and circular muscle strips, a carbachol concentration–response curve (1 nM–10 μ M) was then constructed, with each additional concentration being added after a maximum response was observed to the previous, usually between 2 and 8 min). Changes in muscle tension (g) evoked by each carbachol concentration were expressed in grams.

Data analysis

All data are expressed as means \pm the standard error of the mean (SEM.), or as mean values with ranges; n values are numbers of patient donors. In the contractility studies, any differences in responses to carbachol between the two treatment groups were analysed using a two-way ANOVA with Bonferroni post hoc test. Data from the immunohistochemistry study was analysed using the Student's paired t test.

Compounds

All drugs were freshly prepared before use. Erythromycin (Sigma, Gillingham, Dorset, UK) was dissolved in 100 % ethanol, with subsequent dilution in distilled water. Carbachol (Sigma, UK) was dissolved in distilled water.

Results

Histology and immunohistochemistry

There were no clear differences in age for each of the patient groups. More males than females were recruited

although the numbers are too small to observe meaningful differences (Table 1).

All patients in the immunohistochemistry study had gastric or oesophageal adenocarcinoma. There was a marked decrease in expression of AChE (the scores were, respectively, 2.3 ± 0.7 , 0.5 ± 0.2 and 0 ± 0 in non-chemotherapy vs oesophageal- and gastric-chemotherapy groups ($p = 0.03$ and $p < 0.001$ respectively)—each compared with the non-chemotherapy tissues; Figs. 1 and 2. By contrast, there was a tendency for ghrelin receptors and for motilin to increase, although this did not always achieve statistical significance (ghrelin receptor: 0.7 ± 0.4 in non-chemotherapy vs 2.0 ± 0.4 and 1.2 ± 0.2 in oesophageal- and gastric-chemotherapy groups respectively, $p = 0.04$ and $p = 0.2$; motilin: 0.7 ± 0.5 vs 2.2 ± 0.5 and 2.0 ± 0.7 , $p = 0.06$ and $p = 0.16$, respectively). There were no consistent changes in nNOS (1.2 ± 0.6 in non-chemotherapy vs 1.2 ± 0.6 and 0.5 ± 0.34 in oesophageal and gastric-chemotherapy $p = 0.3$ respectively).

Gastric contractility

Table 1 illustrates patient demographics. As before, there were more males than females. Of the four patients receiving chemotherapy, three had gastric-chemotherapy for gastric adenocarcinoma and one had oesophageal-chemotherapy for squamous cell carcinoma of oesophagus. The patient numbers were too small for subgroup analysis and hence, were analysed together as a single chemotherapy group. Longitudinal muscle preparations from either group did not consistently contract in response to EFS or erythromycin. Contraction could be evoked by EFS in two of the three non-chemotherapy tissues (threshold 2–5 Hz) and in one of four chemotherapy tissues. Similarly, erythromycin 10 μ M increased basal tone or contractility in some tissues, but with no obvious difference between chemotherapy and non-chemotherapy tissues (four of eight

Table 1 Patient demographics in the immunohistochemistry and the contractility study

Immunohistochemistry study			
	Non-chemotherapy	Oesophageal-chemotherapy	Gastric-chemotherapy
Female:male ratio	1:5	1:5	2:4
Mean age	76	71	63
Contractility study			
	Non chemotherapy	Chemotherapy	
Mean age	74	63	
Female:male ratio	2:1	0:4	

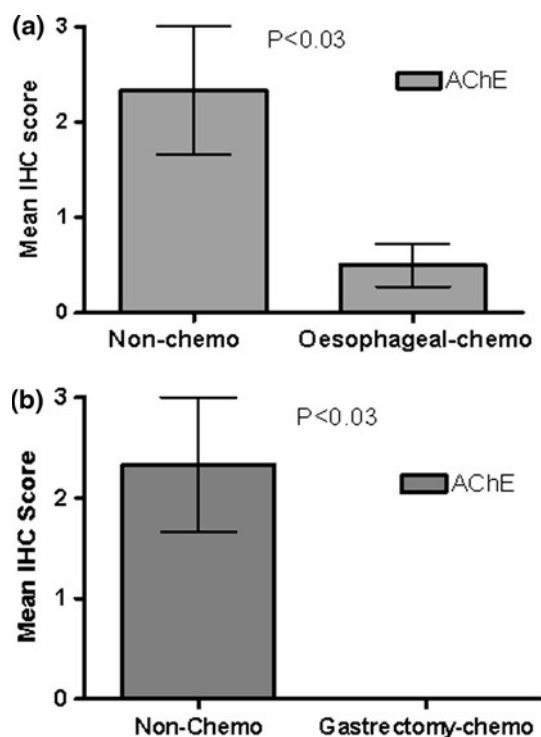


Fig. 1 Immunohistochemistry (IHC) scores for AChE comparing non-chemotherapy and oesophageal-chemotherapy (a) and gastric-chemotherapy (b) exposed tissues. Mean IHC scores were compared using the Student's paired *t* test. $p < 0.03$ when comparing AChE scores in non-chemotherapy and chemotherapy groups

non-chemotherapy tissues responded, compared to two of four tissues from patients receiving chemotherapy).

Application of carbachol 1 nM–10 μ M caused a concentration-dependent contraction of both longitudinal and circular muscle tissues from both patient groups (Fig. 3). However, there were clear differences between the groups of patients in terms of the force of contraction evoked by carbachol. The maximal contraction produced by 10 μ M

carbachol in longitudinal muscle, for example, was 1.9 ± 0.8 g and 3.7 ± 0.7 g in chemotherapy and non-chemotherapy tissues respectively ($p < 0.05$) and in circular muscle, these values were 1.6 ± 0.6 and 3.4 ± 0.4 g in chemotherapy and non-chemotherapy tissues ($p < 0.05$).

Discussion

Our preliminary results indicate, for the first time, that marked changes in the contractility and integrity of the human stomach are caused by cisplatin-based chemotherapy.

The absence of staining for AChE and the significant reduction in contractility to carbachol suggests that cisplatin profoundly influences gastric cholinergic function. These findings are consistent with the reported ability of cisplatin to affect cholinergic function in conscious rats (causing an increase in [3 H]-choline uptake into the cardiac region of the stomach), although not with accompanying change in gastric AChE staining, measured three days after treatment in the same study [9]. Differences in species, duration of chemotherapy and or length of time after chemotherapy are likely to contribute to the differences observed.

The reduction in cholinergic function was not clearly accompanied by a change in nNOS expression, suggesting that any sustained influence of cisplatin on enteric nerve function is not uniform throughout this nervous system. The effects of chemotherapy on the nNOS isoform has previously not been studied in humans, although in rats receiving cisplatin or taxol, reductions in expression of the inducible form of NOS expression are reported [10], along with increases in plasma nitric oxide [11]. Conversely Jarve et al. [5] showed no changes in NOS localisation or intensity of staining five days after dosing with cisplatin in

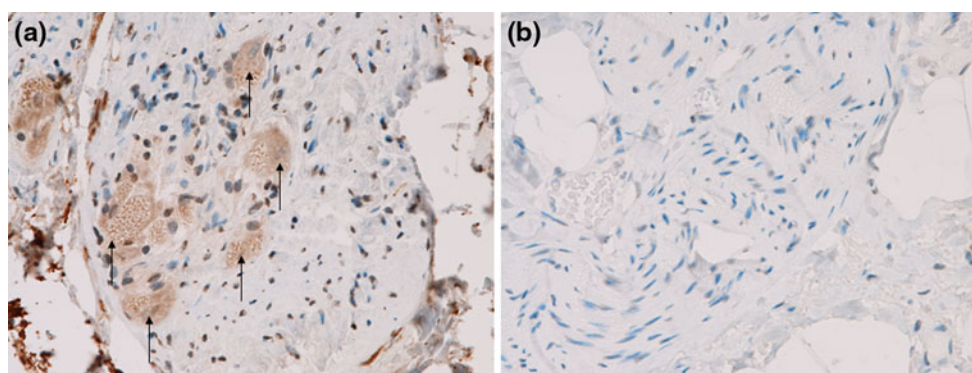


Fig. 2 a Gastric section from a non-chemotherapy patient. Acetylcholinesterase staining showing positive (brown) cytoplasmic staining within ganglion cells (arrows). b Gastric section from a chemotherapy exposed patient. Despite an intensive search definite ganglion

cells were not identified in this case, possibly because of degeneration from chemotherapy. The image shows a representative field illustrating negative staining in a neurovascular bundle. Original magnification $\times 400$. (Color figure online)

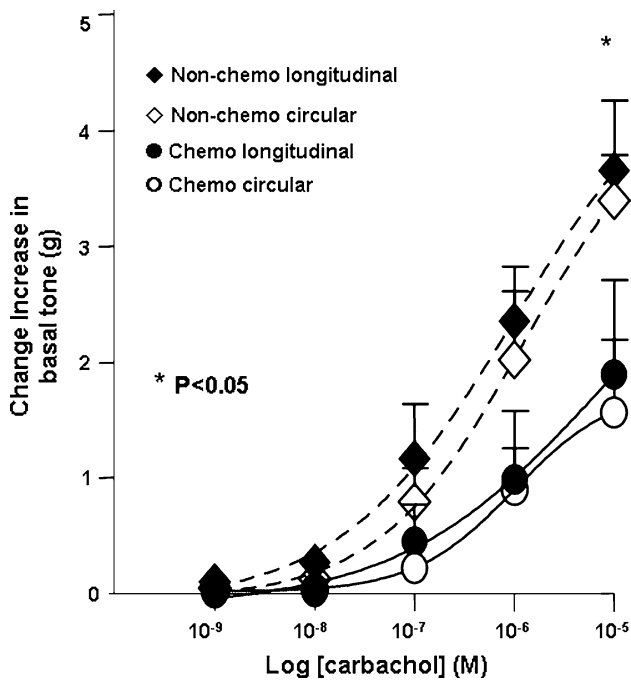


Fig. 3 Concentration-response curves for carbachol-induced contraction of human isolated stomach. The contractions were compared using preparations obtained from patients treated with chemotherapy and from those who were not. * $p < 0.05$ (two way ANOVA with bonferroni post hoc test) when the concentration-response curves were compared (chemo longitudinal muscle vs. non-chemo longitudinal and chemo circular muscle vs. non-chemo circular); $n = 4$ and 3 , respectively, for both longitudinal and circular muscle

rats. Taken together, these studies suggest the possibility that NOS expression may be affected by cisplatin treatment but in our study, the changes are either too subtle to be detected using the present methods or perhaps changes occur immediately on treatment and recover thereafter.

The tendency for the expression of both ghrelin receptors and of motilin to be increased up to six weeks after cisplatin treatment does not, by itself, indicate that the functions of ghrelin and motilin are changed by chemotherapy. However, the observed trend is consistent with rodent studies. For example, Malik et al. [8] found that ghrelin receptor mRNA expression in rat stomach was increased two days after treatment with cisplatin, at a time when the cisplatin-induced gastric stasis was at its greatest; there were no changes in ghrelin mRNA expression. This up-regulation of ghrelin receptor expression suggested that ghrelin might be operating as part of a defence mechanism against the damage caused by cisplatin; this hypothesis is proposed because of the known ability of ghrelin to inhibit cisplatin-induced emesis, promote appetite and increase gastric emptying [8, 12]. However, the present findings, measured up to six weeks after discontinuation of treatment with cisplatin, suggest that if ghrelin were to play a similar defensive role in humans, any effects of ghrelin are likely

to be overwhelmed by the more severe reduction in cholinergic function. A similar defensive role for motilin has not previously been proposed and although tempting to speculate that the gastric prokinetic activity of motilin would serve this purpose—the small numbers and lack of statistical significance limits our interpretation. Interestingly, Hursti et al. [13] found that plasma levels of motilin were transiently decreased 4 days after a cisplatin-based treatment.

In summary, our study suggests that neo-adjuvant chemotherapy can cause marked and enduring damage to neuro-muscular gastric function. Most notable was the loss of AChE and reduction in contractility evoked by carbachol, providing a plausible explanation for some of the severe symptoms of gastric stasis reported by patients long after completion of chemotherapy. In addition, the tendency for ghrelin receptors to increase may be consistent with studies in animals exposed to cisplatin and suggests an attempt to up-regulate compensating systems. The consequences of these changes merits further assessment in the clinical setting.

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Conflicts of interest The authors state no conflicts of interest.

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