Studies into the intestinal growth factor glucagon-like peptide-2

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Abstract

Glucagon-like peptide-2 (GLP-2) is a peptide hormone, secreted postprandially from enteroendocrine L-cells. GLP-2 has emerged as a central physiological mediator of intestinal growth and integrity and has recently shown promise as a therapeutic agent in patients with short bowel syndrome and inflammatory bowel disease.

Reliable methods for GLP-2 measurement are not widely available. In this work a radioimmunoassay (RIA), using the GLP-2 antiserum (FT-17) has been optimised and validated. Cross-reactivity with GLP-2 precursors and degradation products was investigated using column chromatography. It is shown that accurate estimations of active GLP-2 secretion in response to a physiological stimulus can be made using this RIA.

Plasma concentration of GLP-2 and other hormones, secreted from L-cells, were investigated in patients with short bowel syndrome (SBS), morbid obesity and in those following Roux-en-Y gastric bypass (RYGB) surgery. It is shown that, contrary to current thinking, SBS patients with a retained colon can be GLP-2 deficient despite increased L-cell activity as indicated by raised levels of PYY. Differential regulation of peptide generation in L-cells, depending on the residual intestinal anatomy, is a possible explanation. Secretion of PYY and glucagon-like peptides may also differ in obesity. Despite a previously documented attenuated postprandial PYY response in obese compared with normal weight subjects, no difference in glucagon-like peptide secretion was found in a series of test meals. In obese patients undergoing RYGB, an exaggerated postprandial GLP-2 response was noted as early as 2 days following the procedure and persisted for at least 2 years. In diabetic patients this adaptive response was delayed.

Enhancing endogenous GLP-2 secretion might serve as a therapeutic strategy for patients in whom intestinal mucosal regeneration is impaired. It is shown that bile acids and glutamine act as effective GLP-2 secretagogues in model L-cells and that diversion of bilio-pancreatic secretions to the distal ileum leads to increased plasma concentration of GLP-2 in rats.

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Abbreviations

ANOVA	Analysis of variance
AoV	Ampulla of Vater
AUC	Area under the curve
AVP	Arginin Vasopressin
	(also: Antidiuretic hormone)
BA	Bile acid
BMI	Body mass index
BSA	Bovine Serum Albumin
cAMP	Adenosine 3',5'-cyclic monophosphate
CCK	Cholecstokinin
CDCA	Chenodeoxycholic acid
CGRP	Calcitonin gene related peptide
CI	Confidence interval
CRF	Corticotropin releasing factor
DCA	Deoxycholic acid
DHCA	Dehydrocholic acid
DPPIV	Dipeptidyl peptidase IV
ELISA	Enzyme-Linked Immunosorbent Assay
GIP	Glucose-dependent insulinotropic peptide
	(Previously: Gastric inhibitory peptide)
GLP	Glucagon-like peptide
GLUT	Glucose transporter type
GPCR	G-protein coupled receptor
GRF	Growth Hormone releasing factor
HC	Healthy controls
HCl	Hydrochloric acid
HDCA	Hyodeoxycholic acid
HPLC	High Performance Liquid Chromatography
HPN	Home parenteral nutrition
IBMX	3-isobutyl-1-methylxanthine
IF	Intestinal Failure
IGF	Insulin-like growth factor

KGF	Keratinocyte growth factor
LCA	Lithocholic acid
LH-RH	Luteinizing Hormone Releasing Hormone
LREC	Local Research and Ethics Committee
MPGF	Major proglucagon fragment
MW	Molecular weight
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
NT	Neurotensin
NWC	Normal weight controls
PACAP	Pituitary adenylate cyclase-activating peptide
PCR	Polymerase chain reaction
PGDP	Proglucagon derived peptide
PHM	Peptide histidine-methionine amide
PMSF	Phenyl-methyl-sulphonyl-fluoride
PN	Parenteral nutrition
PYY	Peptide YY
RIA	Radioimmunoassay
RYGB	Roux-en-Y gastric bypass
SCFA	Short chain fatty acids
SEM	Standard error of mean
SEMF	Subepithelial myofibroblasts
SEP-PAK	Sample Enrichment and Purification
SPE	Solid phase extraction
SGLT	Sodium dependent glucose transporter
	(Mediates glucose and galactose entry through BBM)
tGLP-2-IR	Total GLP-2 immunoreactivity
TGF	Transforming growth factor
TPN	Total parenteral nutrition
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide

Chapter 1

Introduction

1.1 GLP-2- Physiology and clinical applications

1.1.1 Preface

Glucagon-like peptide-2 is an important growth factor secreted from the human intestine. Since its specific intestinotrophic properties were first described in 1996 by the Canadian research group around Prof. Daniel J. Drucker (Drucker et al. 1996), the body of research investigating GLP-2 has grown exponentially and there is now an increasing understanding of its role in intestinal physiology in health and disease. At the present moment it appears that GLP-2 may become a useful therapeutic adjunct in the treatment of short bowel syndrome and inflammatory bowel disease and first clinical trials in such patient groups are underway. Therapeutic application might however expand to other causes of intestinal injury like ischaemia, radiation or chemotherapy. Some nutrients, known to be important for intestinal integrity and growth, like glutamine and short chain fatty acids, might in fact mediate their effects via GLP-2. GLP-2 also appears to influence bone metabolism and could therefore provide a new treatment angle for patients with osteoporosis. Even though a major future impact on daily practice is not yet certain, GLP-2 is moving from the obscurity of research laboratories into the clinical environment. The introduction to this thesis is therefore dedicated to exploring our current knowledge of GLP-2 physiology and analysing its use in the clinical environment.

1.1.2 GLP-2 - Part of the proglucagon derived peptide family

Glucagon-like peptide-2 is a 33 amino acid peptide hormone with an estimated molecular mass of 3765.8 (Hartmann et al. 2000). It is formed through cleavage from a much larger peptide, proglucagon, a prohormone that is mainly expressed in pancreas, intestine and brain. In 1983 Bell and collegues first isolated and sequenced the human proglucagon gene (Bell et al. 1983). The mammalian gene is 9.4 kilobases in length (White et al. 1986; Irwin 2002), comprising 6 exons separated by 5 introns (White *et al.* 1986). The gene has been mapped to chromosome 2 (segment 2q36-2q37) (Schroeder et al. 1984; Tricoli et al. 1984). The initial translation product preproglucagon is a peptide of 21 kDa, containing 180 amino acids. It includes a 20-amino acid signal peptide that is cleaved to produce the 18-kDa proglucagon (Lopez et al. 1983).

Proglucagon contains three homologous hormonal sequences, glucagon, glucagon-like peptide-1, and glucagon-like peptide-2, separated by two intervening peptides, IP-1 and IP-2, and preceded by an N-terminal extension called glicentin related polypeptide (Rouille et al. 1995). Alternative, tissue specific splicing through prohormone convertases leads to the formation of additional, extended peptides. In intestine and brain ***GLP-1*** and ***GLP-2*** are produced together with ***glicentin*** and ***oxyntomodulin,*** which contain the glucagon sequence. In the pancreas, ***glucagon*** is secreted alongside the large ***major proglucagon fragment (MPGF),*** which combines the sequences of GLP-1, IP-2 and GLP-2 (Rouille et al. 1994; Rouille *et al.* 1995) (Figure 1-1).

In older literature, the terms ***enteroglucagon*** or ***glucagon-like immunoreactivity*** are commonly encountered. These in fact represent glicentin and oxyntomodulin. Because both peptides contain the entire glucagon sequence, they were detected when intestinal tissue was incubated with antibodies against glucagon.

The term *glucagon-like peptide* is derived from the observation that both GLP-1 and GLP-2 exhibit considerable sequence homology to glucagon (Figure 1-3). It is assumed that these three peptides originated by triplication of an exon that encoded a glucagon-like sequence (Irwin 2001). The GLP-2 peptide sequence has undergone significant evolutionary change in its amino acid composition and possibly also biological function. Within mammals the sequence is highly conserved (Irwin 2001); for example: human GLP-2 amino acid sequence differs only in one position from rat GLP-2 and in two positions from mouse GLP-2. Recent reports show that porcine GLP-2 comprises a 35 amino acid sequence with C-terminal Serine and Leucine, in contrast to the 33 amino acids of human, cow, rat and mouse GLP-2, although it retains similar effects in vivo and in vitro in spite of the structural differences (Pedersen et al. 2008) (Figure 1-4).

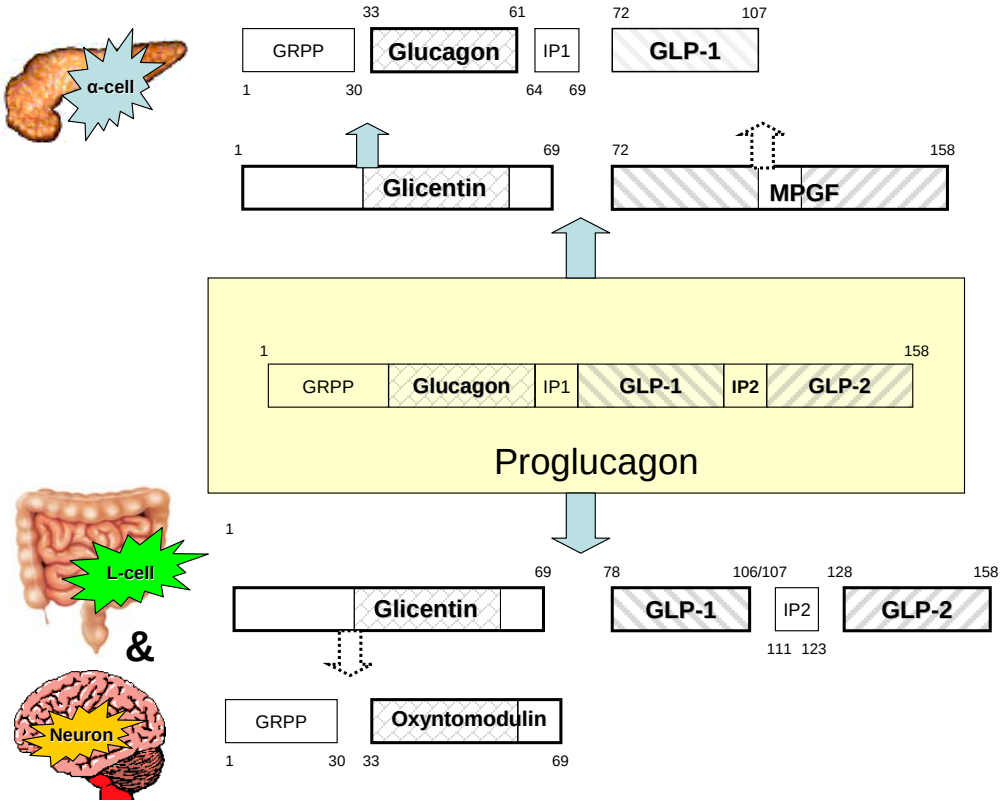


Figure 1-1: Differential processing of proglucagon in pancreas, intestine and CNS. (Partial processing is indicated by dashed arrows.)

Pancreatic α islet cell		
Glicentin related pancreatic peptide	PG 1-30	GRRP
Glucagon	PG 33-61	
Intervening peptide 1	PG 64-69	IP1
Major proglucagon fragment	PG 72-158	MPGF
Glucagon-like peptide 1 (1-37)	PG 72-107	GLP-1 (1-37)
Intestinal L-cell		
Glicentin related pancreatic peptide	PG 1-30	GRPP
Glicentin	PG 1-69	
Oxyntomodulin	PG 33-69	
Truncated Glucagon-like peptide 1 amide (7-36)	PG 78-106	GLP-1 (7-36)
Truncated Glucagon-like peptide 1 (7-37)	PG 78-107	GLP-1 (7-37)
Intervening peptide 2	PG 111-123	IP2
Glucagon-like peptide 2	PG 126-158	GLP-2

Figure 1-2: **Main products of proglucagon processing**.

Glucagon	**H**SQ**G**T**F**TSDY	SKY**L**DSRR**A**Q	D**F**VQ**WL**MNT	
GLP-1	**H**AE**G**T**F**TSDV	SSY**L**EGQA**A**K	E**F** I A**WL**VKGR	G
GLP-2	**H**AD**G**S**F**SDEM	NT I **L**DNLA**A**R	D**F** I N**WL** I QTK	ITD

Figure 1-3: **Sequence homology between Glucagon, GLP-1 and GLP-2.**
Underlined, bold: positions shared by all peptides.

Human	HADGSFSDEM	NT I LDNLAAR	DFI NWLIQTK	I TD	RK
Rat	* * * * * * * * * *	* * * * * * * * T *	* * * * * * * * * *	* * *	K*
Mouse	* * * * * * * * * *	S* * * * * * * T *	* * * * * * * * * *	* * *	K*
Cow	* * * * * * * * * *	* * V* * S* * T *	* * * * * * L* * *	* * *	**
Pig	* * * * * * * * * *	* * V* * * * * T *	* * * * * * LH* *	* * *	SL

Figure 1-4: **Sequence homology of proglucagon 126-160 between mammals**

(adapted from (Pedersen et al. 2008);

The last two amino-acids are the cleavage site (PG 159-169) which is altered in the pig sequence.

* Amino-acids identical to human GLP-2. 27 out of 235 amino-acids have been completely preserved among the 5 species.

(See Appendix 3 for code of amino acids)

1.1.3 GLP-2 - synthesis

Under physiological conditions, intestinal GLP-2 is produced in **L–cells**, a subset of enteroendocrine cells. It is released synchronously and in equimolar amounts with GLP-1 (Orskov et al. 1986). The glucagon-like peptides are partly co-localized with GIP (“L/K cells”, primarily in the upper small intestine) and PYY (primarily in the lower small intestine and colon) (Eissele et al. 1992; Mortensen et al. 2003; Theodorakis et al. 2006). L-cells are the second most abundant population of endocrine cells in the human intestine, exceeded only by enterochromaffin cells. They are thought to arise from pluripotent stem cells in the crypts. Most of the L-cells reside in crypts of Lieberkühn, but a few cells can also be observed in the intestinal villi (Kieffer et al. 1999). L-cells are open type cells. Their microvilli reach into the intestinal lumen; the endocrine granules are located near the basement membrane. This design permits intimate contact with the luminal milieu, as well as proximity to the enteric nervous system and microvasculature and hence allows hormonal discharge in response to nutritional, neural and hormonal signals.

A high abundance of L-cells is present in the distal jejunum and ileum. Along the colon, the L-cell mass increases from the proximal to the distal segments with high concentrations found in the rectum (Eissele *et al.* 1992). The highest tissue levels of GLP-2 are found in the ileum. In the colon, the GLP-2 content increases from the ascending colon to a maximum concentration in the rectum (Caddy et al. 2003; Schmidt et al. 2005). Recently it was established that L-cells are also present in the human duodenum (Theodorakis *et al.* 2006).

The concept of distinct cell populations, dedicated to the synthesis and release of separate peptides, has been questioned lately. Instead, highly specialized enteroendocrine cells might originate from a multipotent cell population, able to produce peptides according to stimuli in the metabolic or biochemical milieu (Theodorakis *et al.* 2006).

1.1.4 GLP-2 - secretion

The complex mechanisms of glucagon-like peptide secretion from the L-cell are not fully understood as yet. Both GLP-1 and GLP-2 are produced simultaneously and co-secreted (Orskov *et al.* 1986; Theodorakis *et al.* 2006). Accordingly their concentration and diurnal profile are highly similar (Hartmann *et al.* 2000). This also means that data relating to GLP-1 synthesis and release is largely applicable to GLP-2 and vice versa. Basal GLP secretion appears to occur in a pulsatile manner, with 5-7 pulses per hour (Balks et al. 1997). Nutrient challenges produce a rapid rise of glucagon-like peptides (Balks *et al.* 1997). The postprandial profile seems to follow a biphasic pattern. A first peak occurs within 30-60 minutes and is followed by a second plateau within 90-150 minutes (dependent on the kind of nutrient ingested) (Balks *et al.* 1997; Xiao et al. 1999). It is believed that the initial rise of GLP-2 is caused by early indirect L-cell stimulation through neurohumoral signalling which is followed by later, direct stimulation through nutrients reaching the distal intestine (Brubaker et al. 2003).

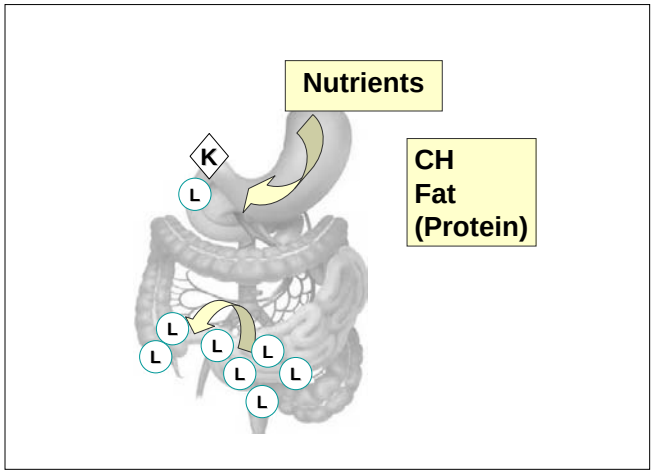
Nutrients play the predominant role in GLP secretion. Meal-related GLP secretion depends on nutrient composition and calorie content (Xiao *et al.* 1999). Luminal carbohydrates are well documented GLP secretagogues when given as a meal or intraduodenally (Unger et al. 1968; Kreymann et al. 1987; Elliott et al. 1993; Qualmann et al. 1995; Balks *et al.* 1997; Kong et al. 1999; Xiao *et al.* 1999; Feinle et al. 2002; Henriksen et al. 2003). Conversely, intravenous glucose administration has *no* effect on GLP secretion (Unger *et al.* 1968; Nauck et al. 1993; Herrmann et al. 1995). Lipids have also been established as potent GLP secretagogues in humans (Elliott *et al.* 1993; Layer 1995; Hartmann *et al.* 2000; Feinle *et al.* 2002; Feltrin et al. 2004; Feltrin et al. 2006). The effect of fatty acids appears to vary depending on chain length (Feltrin *et al.* 2004) and saturation (Brubaker et al. 1998; Feltrin *et al.* 2006). Recently, Hirasawa and colleagues have demonstrated in STC-1 cells that GLP-1 secretion in response to long-chain free fatty acids is mediated via the specific G-protein

coupled receptor GPR-120 (Hirasawa et al. 2005). A number of studies have demonstrated a positive effect of short chain fatty acids on proglucagon mRNA abundance (Tappenden et al. 1998; Drozdowski et al. 2002) and GLP-2 secretion (Tappenden 1998; Bartholome et al. 2004). The evidence for the role of protein in the regulation of GLP release is not conclusive. Xiao and colleagues have reported that levels of active GLP-2 do not increase following a protein meal (Xiao *et al.* 1999), other authors however found a positive response in GLP-1 (Elliott *et al.* 1993; Hall et al. 2003) and GLP-2 (Henriksen *et al.* 2003) levels. A recent report has indicated a strong stimulatory effect of glutamine on GLP-1 secretion (Reimann 2004). Even though **Cholecystokinin** (CCK) does not appear to influence GLP secretion (Brubaker 1991; Plaisancie et al. 1994), there is increasing evidence that bile acids play an important role in GLP release (Adrian et al. 1993; Plaisancie et al. 1995), possibly mediated through the novel bile acid receptor TGR5 (Maruyama et al. 2002; Katsuma et al. 2005).

The rapid postprandial rise of glucagon-like peptides is widely believed to be subject to intricate interaction of **neural and hormonal** mediators. Research in rodents led to the hypothesis of a proximal-distal neuroendocrine loop whereby nutrient induced release of gastric inhibitory peptide (GIP) initiates afferent vagal stimulation, which in turn leads to efferent signalling via the coeliac nerve to the ileum. In the enteric nervous system gastrin releasing peptide (GRP) is released via nicotinergic synapses, which in turn stimulates muscarinergic receptors on the L-cell to release GLP (Rocca et al. 1999; Anini et al. 2002). However; even though cholinergic transmitters are known to stimulate GLP secretion from L-cells (Brubaker 1991; Herrmann-Rinke et al. 1995; Brubaker *et al.* 1998; Dumoulin et al. 1998; Reimer et al. 2001; Anini *et al.* 2002; Anini et al. 2003), there is no conclusive evidence that efferent signalling through the vagus nerve is important for GLP secretion in pigs and humans (Wojdemann et al. 1999; Hansen et al. 2004). Instead it is possible that parasympathetic innervation mainly mediates *local* reflexes in the intrinsic enteric nervous system (Hansen *et al.* 2004). Equally, despite evidence of a direct stimulatory effect of GIP on L- cell models (Brubaker 1991; Brubaker *et al.* 1998; Damholt et al. 1998) and in rat intestinal explants

(Plaisancie *et al.* 1994; Herrmann-Rinke *et al.* 1995), its physiological role in GLP release in humans remains unclear (Kreymann *et al.* 1987; Nauck 1993; Nauck *et al.* 1993). Given the close proximity of K- and L-cells (Mortensen *et al.* 2003; Theodorakis *et al.* 2006) it is possible that, GIP related effects are mediated in a paracrine rather than hormonal manner. **Somatostatin**, which is produced in D cells throughout the intestinal tract, inhibits secretion of PGDPs in various animal models (Brubaker 1991; Hansen et al. 2000; Chisholm et al. 2002). Both GLP-1 and (to a lesser extend) GLP-2 have been shown to stimulate somatostatin secretion (Chisholm *et al.* 2002; Hansen et al. 2004), implying that L- cell secretion is subject to negative feedback mechanisms through glucagon-like petides themselves and via paracrine somatostatin activity.

A



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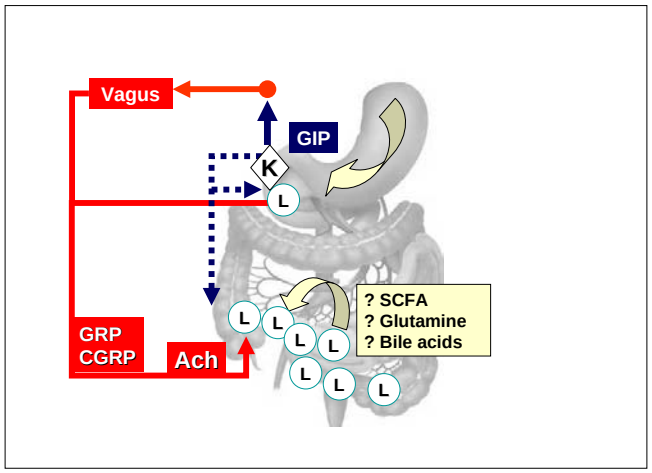

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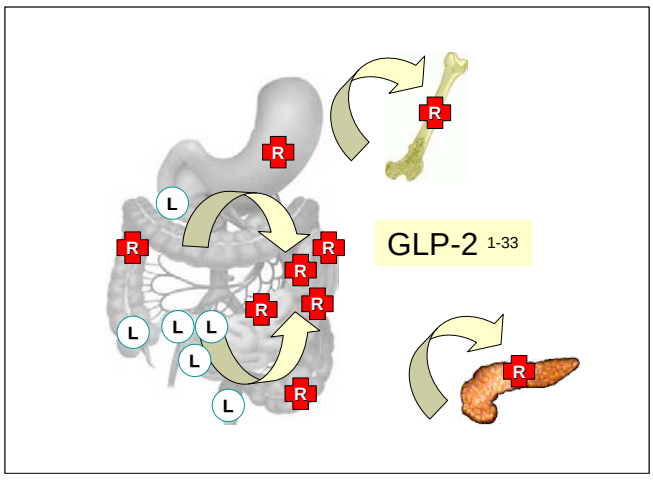


Figure 1-5: **Mechanisms of GLP-2 secretion**.

Enteroendocrine **L**-cells and **K**-cells are stimulated through different nutrients reaching the duodenum (A). Subsequently, L-cells in the distal ileum and colon are stimulated, either directly through nutrients and metabolites and/or neuroendocrine stimulators (B). GLP-2 is released from L-cells and stimulates GLP-2 **R**eceptor carrying cells, located mainly in the proximal small intestine but possibly also on osteoclasts and pancreatic islet cells (C).

1.1.5 GLP-2 - metabolism

In a similar fashion to GLP-1 and GIP, active GLP-2 (1-33) is degraded by the ubiquitously expressed proteolytic enzyme **dipeptidyl peptidase IV (DPPIV)** (also known as CD26). Cleavage of N-terminal histidine and alanine results in the formation of the GLP-2 degradation product: GLP-2 (3-33) (Brubaker et al. 1997; Tavares et al. 2000). In vivo half-life of intravenous GLP-2 (1-33) has been reported at 7 minutes, in vitro half-life is 8 hours in plasma and 3.3 hours in blood (Hartmann et al. 2000). Substitution of amino acid alanine with glycine at position 2, as in teduglutide (Gattex^{TM} NPS pharmaceuticals, Bedminster, New Jersey, USA), renders GLP-2 resistant to DPPIV-mediated degradation. This technique, or the adjuvant use of DPPIV inhibitors, extends the GLP-2 half-life (Thulesen et al. 2000; Jeppesen et al. 2005; Marier et al. 2008), and confers greater biological potency (Drucker et al. 1997; Hartmann et al. 2000; Jeppesen *et al.* 2005). **Renal excretion** plays a key role in GLP-2 clearance. Injection of I^{125} labelled GLP-2 has localized renal uptake to the proximal part of the tubules, consistent with glomerular secretion (Thulesen *et al.* 2000). Increased GLP-2 levels have been demonstrated in nephrectomized rats (Tavares *et al.* 2000) and humans with chronic renal failure (Brubaker *et al.* 1997). However, since some clearance can be observed in nephrectomized rats, other organs and mechanisms are suspected to contribute (Tavares *et al.* 2000). As uptake of injected I^{125} GLP-2 is also high in the liver (Thulesen *et al.* 2000), it is likely that this is a place of GLP-2 inactivation and possibly clearance.

1.1.6 GLP-2 - receptor and downstream mediators

GLP-2 acts through a specific receptor (GLP-2R) that belongs to “Family B” (also “Glucagon/Secretin family” or Class II”) of G-protein coupled receptors (GPCRs). In 1999 Munroe and colleagues were the first to clone and characterize the cDNAs encoding the rat and human GLP-2 receptor

(Munroe et al. 1999). The human GLP-2R gene has been mapped to Chromosome 17 (17p13.3). It encodes a polypeptide of 553 amino acids.

The highest GLP-2R concentrations have been described in the jejunum followed by duodenum, ileum, colon and stomach with undetectable levels in oesophagus, adipose tissue, heart, kidney, liver, skeletal muscle, spleen, testis and uterus (Munroe *et al.* 1999; Yusta et al. 2000). There have been recent reports of GLP-2 receptor presence on osteoclasts (Henriksen et al. 2004) and pancreatic islet cells (de Heer et al. 2007). Detection of GLP-2R in brain and lung tissue requires more sensitive techniques like real-time PCR. The relative abundance of GLP-2R expression in the jejunum is at least 20-30-fold higher than in the brainstem (Yusta *et al.* 2000). Small, focal areas of GLP-2R expression have also been detected in samples of human tumours including intestinal carcinoid tumours and colorectal carcinoma (Yusta *et al.* 2000; Koehler et al. 2008). Moreover, GLP-2R cDNA sequence tags have been detected in large cell lung- and cervical carcinoma cell lines, as well as in the normal human cervix (Koehler et al. 2005).

The precise location of the GLP-2R at a cellular level within the GI tract is the subject of ongoing research. Even though GLP-2 exerts strong stimulatory effects on epithelial proliferation, there is no apparent expression on crypt epithelial cells or enterocytes. Instead, the receptor has been demonstrated on enteroendocrine cells (Yusta *et al.* 2000), enteric neurons (Bjerknes et al. 2001; Guan et al. 2006) and subepithelial myofibroblasts (Orskov et al. 2005). This implies that intestinotrophic and other effects of GLP-2 are mediated through other downstream effectors (Figure 1-6). Insulin-like growth factor-1 (IGF-1), which is co-located with the GLP-2R in subepithelial myofibroblasts, appears to be essential for GLP-2 induced intestinal epithelial proliferation (Dube et al. 2008; Liu et al. 2008). Other growth factors, like vascular endothelial growth factor (VEGF) and transforming growth factor-ß (TGF- ß), have been linked to GLP-2 induced wound repair (Bulut et al. 2008). Anti-inflammatory effects appear to be mediated through release of vasoactive intestinal peptide (VIP) from enteric neurons, independent of

proliferative action (Sigalet et al. 2007). Nitric oxide (NO) might be a key mediator in GLP-2 induced up-regulation of intestinal blood flow (Guan *et al.* 2006).

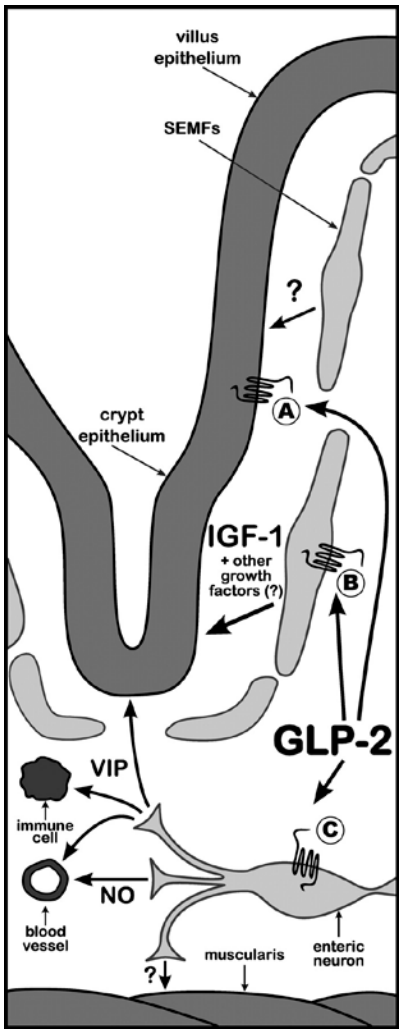


Figure 1-6: **Proposed model for the indirect mechanisms of GLP-2 action in the intestine.**

Expression of the GLP-2R in (A) enteroendocrine cells, (B) intestinal subepithelial myofibroblasts (SEMF) and (C) enteric neurons, suggests that GLP-2 acts indirectly to produce its diverse actions in the intestine. IGF-I is critical for the ability of GLP-2 to induce intestinal growth and activate crypt cell proliferation and ß-catenin signalling. Other SEMF-derived growth factors may induce complementary actions to those of IGF-I, such as keratinocyte growth factor (KGF) in the colon. GLP-2-mediated enteric neuronal signalling enhances intestinal blood flow through a mechanism involving NO production and has anti-inflammatory actions through vasoactive intestinal polypeptide (VIP), although the target of this VIP effect remains undefined. Neural GLP-2 receptor signalling may also influence the crypt epithelium and gastrointestinal motility.

Reprinted with permission (Dube et al. 2007)

1.1.7 GLP-2 - physiological role and pharmacological effects

GLP-2 influences multiple aspects of gastrointestinal physiology. Studies, utilizing relative GLP-2 deficiency models, have demonstrated that GLP-2 is one of the *causative* factors for regulation of intestinal growth (Hartmann et al. 2002; Perez et al. 2005; Shin et al. 2005). Recent studies suggest that GLP-2 plays a physiological role in control of local intestinal blood flow (Stephens et al. 2006; Bremholm et al. 2008) and bone homeostasis (Henriksen *et al.* 2003; Henriksen et al. 2004). GLP-2 may also have importance in foetal intestinal development (Amin et al. 2008).

In addition, there is a large amount of *correlative data* linking GLP-2 to intestinal growth and adaptation in various pathological conditions, like post-resection intestinal adaptation (Jeppesen et al. 2000; Ljungmann et al. 2001; Topstad et al. 2001; Dahly et al. 2003; Paris et al. 2004; Martin et al. 2005), coeliac disease (Kilander et al. 1984; Caddy et al. 2006), TPN induced intestinal atrophy (Burrin et al. 2000; Niinikoski et al. 2004) and inflammatory bowel disease (Schmidt et al. 2000).

Pharmacological administration of GLP-2 induces **mucosal growth** in small and large bowel (Drucker *et al.* 1996; Brubaker et al. 1997; Drucker et al. 1997; Tsai et al. 1997; Litvak et al. 1998; Kato et al. 1999; Ghatei et al. 2001). Intestinotrophic effects of GLP-2 are mediated not only through an increase of intestinal crypt cell proliferation but also through a reduction of villous cell apoptosis resulting in expansion of villous height (Drucker *et al.* 1996; Chance et al. 1997; Drucker *et al.* 1997; Tsai *et al.* 1997; Benjamin et al. 2000; Jeppesen *et al.* 2005) and crypt depth (Drucker *et al.* 1997; Ramsanahie et al. 2004; Jeppesen *et al.* 2005). Epithelial cells undergo morphological changes; become narrower and longer with elongated microvilli (Benjamin *et al.* 2000). The trophic effects are more pronounced in the proximal small intestine (Tsai *et al.* 1997; Scott et al. 1998; Hartmann *et al.* 2000). A single injection of GLP-2 appears sufficient to promote crypt cell division. On daily treatment, effects are persistent at 3 month; after treatment

cessation however, all changes of intestinal morphology rapidly reverse (Tsai *et al.* 1997).

The GLP-2 related increase of intestinal mass is accompanied by enhanced intestinal digestive and absorptive **functional capacity** as evident in enhanced expression and activity of brush border enzymes (Brubaker *et al.* 1997; Kato *et al.* 1999; Kitchen et al. 2000; Petersen et al. 2002) and absorption of nutrients (Cheeseman et al. 1996; Cheeseman 1997; Cheeseman et al. 1998; Kato *et al.* 1999; Au et al. 2002; Guan et al. 2003; Meier et al. 2006). The effect on glucose uptake is inhibited by L-NAME and therefore possibly dependent on nitric oxide (Guan *et al.* 2003). The rapidly increased hexose transport is facilitated through upregulation of membrane expression and activity of transporter proteins SGLT-1 (Cheeseman *et al.* 1996; Cheeseman 1997; Cheeseman *et al.* 1998; Au *et al.* 2002; Ramsanahie et al. 2003; Martin et al. 2004; Cottrell et al. 2005) and GLUT-2 (Au *et al.* 2002).

GLP-2 reduces **gastric acid** secretion (Wojdemann *et al.* 1999; Meier *et al.* 2006). In animal models GLP-2 inhibits **gastro-intestinal motility** (Wojdemann et al. 1998; Bozkurt et al. 2002); results of human studies are conflicting (Jeppesen et al. 2001; Schmidt et al. 2003; Nagell et al. 2004; Meier *et al.* 2006). GLP-2 stimulates **glucagon** secretion and inhibits the glucagonostatic effect of GLP-1 (Sorensen et al. 2003; Meier *et al.* 2006; de Heer *et al.* 2007)

GLP-2 has shown a beneficial effect on **intestinal barrier** function in rodent models of type-1 diabetes mellitus (Hadjiyanni et al. 2009), necrotising pancreatitis (Kouris et al. 2001), psychological stress (Cameron et al. 2005), food allergy (Cameron et al. 2003) and thermal injury (Chance et al. 2001).

GLP-2 infusion causes rapid up-regulation of portal **blood flow** (Guan *et al.* 2003). The increased portal flow mainly derives from a GLP-2 related perfusion increase in the proximal intestine and pancreas, whereas flow to the distal intestine, stomach, spleen and kidneys remains unchanged or is

decreased, indicating flow increase in the superior mesenteric artery (Guan *et al.* 2006; Stephens *et al.* 2006). The mean arterial blood pressure is not altered during GLP-2 infusion indicating that the vascular effect is locally restricted (Wojdemann *et al.* 1999; Guan *et al.* 2006; Stephens *et al.* 2006).

Nitric oxide (NO) appears to be an important mediator of the GLP-2 related vasoactive effects. The NO-synthase (NOS) inhibitor L-NAME suppresses GLP-2 increased portal blood flow (Guan *et al.* 2003). The GLP-2R has been localized in neurons also containing eNOS and GLP-2 infusion increases abundance of eNOS mRNA and protein. It is speculated that NO, following GLP-2 mediated release, diffuses locally to the vascular smooth muscle to activate cGMP synthesis and stimulate arteriole dilatation (Guan *et al.* 2006). The GLP-2R is also co-localized with VIP in enteric neurons and with 5HT in enteroendocrine cells (Guan *et al.* 2006). Both substances are known to have a potent vasoactive effect on mesenteric blood flow (Ganz et al. 1986; Hansen et al. 1998) and could represent downstream mediators of GLP-2 action.

In various animal models of inflammatory bowel disease GLP-2 improves outcome and reduces expression of inflammatory cytokines (L'Heureux et al. 2003; Ivory et al. 2008; Yazbeck et al. 2008). The **anti-inflammatory** effects appear to be independent of proliferative GLP-2 actions (Sigalet *et al.* 2007) and do not depend on Interleukin-10 (IL-10) (Ivory *et al.* 2008).

GLP-2 also affects **bone metabolism**. A dose dependent reduction of bone resorption marker s-CTX and attenuation of the physiological nocturnal s-CTX increase has been shown in healthy postmenopausal women. Moreover, the detection of GLP-2R on osteoclasts suggests that GLP-2 may form part of a “entero-osseous axis”, coordinating nutrient dependent bone turnover (Henriksen *et al.* 2003; Henriksen *et al.* 2004; Henriksen *et al.* 2004).

Glucagon-like peptides, including GLP-2, are also liberated in the **central nervous system** and are likely to play a regulatory role. This aspect of GLP-

2 action, not yet well explored, is an important area for future research but will not be covered in any further detail in this thesis.

For practical reasons, GLP-2 and other intestinal mediators are often studied in isolation. Undoubtedly however, GLP-2 secretion forms only a small part of a complex intestinal response to nutrient intake and will act in concert with other gut hormones (for instance GLP-1 and PYY which are also postprandially released from the L-cell).

A number of other factors are known to affect intestinal growth. Even though the individual growth promoting properties of these factors have been well documented, less is known about how they interact. Several, including epidermal growth factor (EGF), appear to act synergistically with GLP-2 (Drucker et al. 1997; Kitchen et al. 2005). Nutritional factors like glutamine and short chain fatty acids (SCFAs) might exert their growth enhancing properties by facilitating GLP-2 production and release (Bartholome *et al.* 2004; Reimann 2004), whereas keratinocyte growth factor (KGF) and insulin-like growth factor 1 (IGF-1) probably act as downstream mediators (Orskov *et al.* 2005; Dube et al. 2006).

Growth hormone (GH)
Glucagon-like peptide 2 (GLP-2)
Insulin-like growth factor 1 (IGF-1)
Epidermal growth factor (EGF)
Hepatocyte growth factor (HGF)
Transforming growth factor α (TGF-α)
Keratinocyte growth factor (KGF)
Neurotensin
Leptin
R-Spondin
Glutamine
SCFAs

Table 1-1: Intestinotrophic growth factors

1.1.8 GLP-2 - clinical applications

Short bowel syndrome (SBS) is a highly disabling condition that typically arises after extensive intestinal resection. When the remaining functioning intestinal mass is insufficient to maintain the nutritional needs of the individual, long term parenteral nutrition (PN) becomes mandatory. Although PN has revolutionized SBS treatment; it reduces quality of life and carries significant risks, mainly hepatic failure, central vein thrombosis and recurrent sepsis, which can reduce survival. SBS patients with end jejunostomy and no colon, known to have poor intestinal adaptation, have a markedly impaired postprandial GLP-2 response (Jeppesen et al. 1999). In contrast, patients with a preserved colon can exhibit increased fasting GLP-2 levels with a postprandial profile comparable to healthy controls (Jeppesen *et al.* 2000). These findings, combined with evidence of a correlation between GLP-2 and intestinal adaptation in animals (Scott *et al.* 1998; Ljungmann *et al.* 2001; Topstad *et al.* 2001; Dahly *et al.* 2003; Martin *et al.* 2005; Koopmann et al. 2008) have raised hopes that GLP-2 therapy might increase intestinal absorption and reduce PN dependence, through enhancement of post-resection intestinal adaptation.

In small, uncontrolled, open label pilot studies of SBS patients, native GLP-2 and the long acting GLP-2 analogue teduglutide caused significant improvements in fluid absorption. Increases in energy absorption were relatively minor. Similar results were achieved in patients with and without preserved colon, implying that supra-physiological GLP-2 concentrations may exert additional benefit (Jeppesen *et al.* 2001; Jeppesen *et al.* 2005). Results of a phase 3 multi-centre randomized controlled study of teduglutide (Gattex™ NPS pharmaceuticals, Bedminster, New Jersey, USA) have been published in abstract form (O'Keefe et al. 2008). In 86 patients with stable post-resection SBS a reduction of parenteral nutrition requirement (> 20% of weekly requirements) was achieved significantly more frequently following 24 week daily treatment with teduglutide (0.05mg/kg) compared with placebo treatment (45.7% vs. 6.3% responders; p=0.007). Interestingly, this benefit was not seen in a parallel group who received teduglutide at a higher dose

(0.1mg/kg). Plasma citrulline, a sensitive marker for enterocyte mass and function (Papadia et al. 2007), increased with teduglutide treatment but not with placebo, indicating intestinotrophic effects (Messing et al. 2008). Accordingly, increased villous height was documented (Tappenden et al. 2008). A 28-week extension study established that long term treatment with teduglutide was safe and that parenteral nutrition reductions could be maintained for up to 1 year in the majority of patients. (Jeppesen et al. 2008), 4 subjects were able to be weaned off parenteral nutrition completely.

These results indicate that GLP-2 treatment can achieve clinically useful reductions of PN requirements in SBS patients, considered to be stable on optimal conventional management. However, SBS comprises a heterogeneous group of patients with regards to nature of the underlying illness and amount and quality of the residual intestine. These factors may influence treatment response and optimum treatment dose. It has been shown in rodents that GLP-2 augments post-resection adaptive growth predominantly in the proximal but not distal intestine (Koopmann *et al.* 2008). Timing of treatment initiation might also be of relevance. Recent studies suggest maximal effects of GLP-2 on post-resection intestinal adaptation, and in particular on intestinal stem cell expansion, in the early post-operative period (Garrison et al. 2008; Kaji et al. 2008). Post-resection intestinal adaptation involves alteration of other gut hormones, including GLP-1 and peptide YY, which have strong inhibitory effects on gastrointestinal motility and secretion. Combination treatments might provide synergistic benefit.

An 8 week, phase 2, placebo controlled clinical trial in 100 patients with moderate to severe **Crohn's disease** suggests a dose dependent benefit of teduglutide treatment; although the placebo response was high (55.6% of patients in the highest dose group vs. 33.3% of the placebo group went into remission) (Buchman et al. 2006). Further research must determine whether these results relate to symptomatic disease control or reflect decreases in inflammatory activity and improved mucosal healing.

The benefit of GLP-2 related reduction of bone resorption has been studied in SBS, because **metabolic bone disease** is highly prevalent in patients on long term PN. Initial results were promising. In a small group of SBS patients with jejunostomy, 35 day GLP-2-treatment led to an increase in bone mass and bone mineral density (BMD) (Haderslev et al. 2002). However, two recent studies could not replicate these findings. A single injection of GLP-2 reduced s-CTX in 15 healthy control subjects but not in 25 colectomy patients (13 ileostomy, 12 jejunostomy); whereas the postprandial decrease in s-CTX was preserved in all groups (Gottschalck et al. 2008). 14 day GLP-2 treatment decreased s-CTX in 13 ileostomy patients, but not in 8 jejunostomy patients. 56 day treatment did not change the BMD in the jejunostomy patients (Gottschalck et al. 2008). These findings make a direct effect of GLP-2 on bone cells less likely; instead it appears that the effect of GLP-2 on bone turnover requires an intact small bowel and might be mediated through other intestinal downstream effectors. Nonetheless GLP-2 might provide a new treatment modality for treatment of osteoporosis in patients with an intact intestine.

GLP-2 has positive effects in animals with chemotherapy- (Boushey et al. 2001), radiotherapy- (Torres et al. 2007) and ischaemia-reperfusion- (Zhang et al. 2008) induced mucosal injury. Improved outcome is thought to reflect reductions in apoptosis, stimulatory effects on splanchnic perfusion and enhancement of intestinal barrier function with subsequent reduction in bacterial sepsis. Preclinical trials of Teduglutide for the treatment of **chemotherapy induced mucositis** and **necrotising enterocolitis** are currently underway (NPS Pharmaceuticals press release).

The question, whether measurement of endogenous GLP-2 plasma concentration in SBS can be a useful **prognostic tool**, has so far only been addressed in one study of premature infants (Sigalet et al. 2004). Here, postprandial GLP-2 levels were shown to be significantly correlated with residual small intestinal length and macronutrient absorption. A significant difference in average GLP-2 level of infants, able to achieve TPN independence (45.3pmol/l) versus infants unable to achieve TPN

independence (2.25pmol/l) at 1 year follow up was demonstrated. Interestingly, and in contrast to findings by Jeppesen et al. (Jeppesen *et al.* 2000), some infants with severe short bowel but a residual colon had very low plasma GLP-2. Out of 4 patients with a GLP-2 level of less than 15pmol/l, 3 died, but none with a level above this threshold.

It is unlikely that results from an infant patient population can be directly applied to the adults in whom short bowel syndrome is caused by a completely different spectrum of diseases. However, defining a realistic prognosis in this patient group is crucial. Intestinal failure is a chronic condition that will require intensive treatment over many years. Accurate prognostication allows better management of patient expectations and allocation of resources.

1.1.9 GLP-2 - tolerability and safety of treatment

Human studies so far report good drug compliance, tolerance and safety of native GLP-2 and teduglutide with no significant increase of serious adverse events. Most commonly reported adverse events are abdominal pain, bloating, constipation, nausea, injection site erythema, lower limb oedema, stoma swelling, headache and nasopharyngitis.

The therapeutic use of GLP-2 may require lifelong administration. Because of concerns that permanent stimulation of epithelial proliferation might initiate or accelerate malignant growth, research into the role of GLP-2 in carcinogenesis is ongoing. GLP-2R expression has been demonstrated in cancer cell lines and human tumours including colonic adenocarcinoma (Koehler *et al.* 2005; Koehler et al. 2008). GLP-2 also exerts a direct proliferative effect on transformed cell lines despite apparent lack of a specific receptor (Rocha et al. 2004). Recently however, no GLP-2 dependent proliferation of GLP-2R transfected colon cancer cell lines could be demonstrated (Koehler *et al.* 2008). In vivo, GLP-2 treatment of tumour-bearing Apc Min/+ mice showed no increase of tumour burden (Koehler *et al.* 2008). However, in another mouse model, GLP-2 promoted the growth of chemically induced colonic neoplasms (Thulesen et al. 2004). Until a clear safety profile for GLP-2 is established, special vigilance is required. This should include screening of prospective patients for pre-malignant lesions (particularly colonic adenomas) as well as surveillance during treatment.

1.2 Hypotheses

The aim of my research was to establish and optimize a method for GLP-2 measurement and to contribute to the current understanding of GLP-2 physiology.

The hypotheses to be tested in this thesis are as follows:

- A departmentally generated GLP-2 antiserum (FT-17) can be utilized to develop a sensitive and specific radioimmunoassay.

- Measurement of GLP-2 and related L-cell secretions provides insights into the regulation of intestinal adaptation to intestinal failure secondary to short bowel syndrome.

- Alterations of GLP-2 and related L-cell secretions occur in the obese and changes of L-cell secretion are related to the adaptive response following obesity surgery.

- Endogenous GLP-2 secretion might be enhanced by bile acid or glutamine supplementation.

Chapter 2

Description and optimization of a glucagon-like peptide-2 radioimmunoassay

2.1 Introduction

2.1.1 Measurement of GLP-2

The importance of GLP-2 as a regulatory gut hormone is increasingly understood. Information about GLP-2 secretion in a variety of physiological or pathological states is therefore of great scientific interest and may also have clinical applications. The concentration of GLP-2 in tissue samples or bodily fluids can be determined in a **radioimmunoassay** (RIA). RIA is a highly sensitive technique, able to measure minute amounts of circulating peptide, which has revolutionized the field of endocrinology (Buchanan et al. 1981). The method is described in detail in chapter 2.1.2. Up until now, the RIA technique has been utilized in the majority of published GLP-2 research. An alternative method of GLP-2 measurement, the **enzyme-linked immunosorbent assay** (ELISA) technique, is described in chapter 2.1.3. The substitution of radioisotope by an enzyme label offers certain advantages over the RIA: like the lack of radioactive decay, which limits the shelf-life of RIA reagents, and the avoidance of the hazards associated with radioactive isotopes. The method requires less operator time and can be readily automated. However, sensitivity can be lower compared to the RIA technique. The greater precision of the RIA has ensured its survival where critical quantitative measurement is required (Zola et al. 1996). This might explain why so far only a few publications (Martin *et al.* 2004; Martin *et al.* 2005) have documented the use of ELISA techniques for the measurement of GLP-2. A Japanese research group has used a **biological GLP-2 assay** which comprises the measurement of serum-induced cAMP production in a COS-7 cell line, transfected with a recombinant GLP-2 receptor. This approach, that utilizes the fact that GLP-2 receptor stimulation leads to rapid cAMP accumulation (Munroe *et al.* 1999), detects full length, bioactive GLP-2 and has virtually no cross-reactivity with other glucagon-related peptides (Watanabe et al. 2005).

When measuring GLP-2, with either RIA or ELISA, the **specificity of the antiserum** used is of great importance. No cross-reactivity should occur with related gut hormones (e.g. glucagon, GLP-1, secretin and others). Additionally it has to be considered that biologically active GLP-2 (1-33), its largely inactive metabolite GLP-2 (3-33) and the pancreatic proglucagon product MPGF contain identical amino acid sequences. Therefore, only antibodies that specifically recognize the free N-terminus of GLP-2 are specific for the measurement of active GLP-2 (1-33). So called “side viewing” antibodies, raised against mid-sections of the GLP-2 peptide, will cross-react with N- and C- terminally modified forms of this peptide and therefore not only recognize active GLP-2 (1-33) but also its metabolites and precursors like proglucagon, GLP-2 (3-33) and MPGF. The resulting level is commonly referred to as **total GLP-2 immunoreactivity**.

It is possible to separate GLP-2 (1-33) and GLP-2 (3-33) with chromatographic techniques like reverse phase HPLC. However, this is technically difficult and laborious, requiring a relatively shallow gradient and very rapid fraction collection rate (Brubaker *et al.* 1997). For the measurement of active GLP-2 (1-33) a specific antiserum therefore seems preferable.

The main body of published research describes the use of two antisera. **Antiserum UTTH-7** was developed by the group of Prof Daniel J. Drucker at the University of Toronto, Canada (Brubaker *et al.* 1997). It was raised in rabbits against synthetic rat GLP-2 (1-33). The antigenic site recognized by UTTH-7 is localized to amino acids 25-30 of GLP-2. This means that this is a side viewing antibody with the limitations mentioned above. An N-terminal specific GLP-2 radioimmunoassay was developed by the group of Prof Jens J. Holst at the University of Copenhagen, Denmark (Hartmann *et al.* 2000). **Antiserum 92160** was raised in rabbits against synthetic GLP-2 (1-11). It is specific for active GLP-2 (1-33); only a minor cross-reaction of 5.6% $\pm$ 1.8% is reported with synthetic human GLP-2 (3-33). Neither of these antiserums is commercially available. **Antiserum FT-17** has been developed by Prof. Mohammed Ghatei at Imperial College London, UK. FT-17 is a polyclonal

antibody, raised in rabbits against synthetic human GLP-2 (1-33), (Bachem UK Ltd.) conjugated to BSA by glutaraldehyde. The antiserum cross-reacts 100% with human and rat GLP-2, less than 0.1% with GLP-1 and does not cross-react with any other known gastrointestinal or pancreatic hormone (see appendix 1). The antiserum is used at a final dilution of 1:500.000. This work aimed to characterise this antiserum further and to determine whether it may be suitable for determination of GLP-2 concentration in humans and specifically in patients with short bowel syndrome and related disorders.

2.1.2 Radioimmunoassay

The radioimmunoassay (RIA) of a peptide (or any other antigen) depends on the competition between radioactively-labelled and -unlabelled substrate for the specific binding site of an antibody. The amounts of antibody and labelled peptide are fixed. The only variable is the unlabelled peptide concentration. The higher the concentration of unlabelled peptide, the less radioactively labelled peptide will be bound to antibody. This can be measured after separating antibody-bound from free label (for example with charcoal or with a secondary antibody) and counting the radioactivity of either the bound fraction, the free fraction or both (Adrian 1982). By using a series of pure peptide standards, covering the full range of expected values, a standard curve is calculated from which the quantity of peptide in unknown samples can be deducted (Figure 2-1).

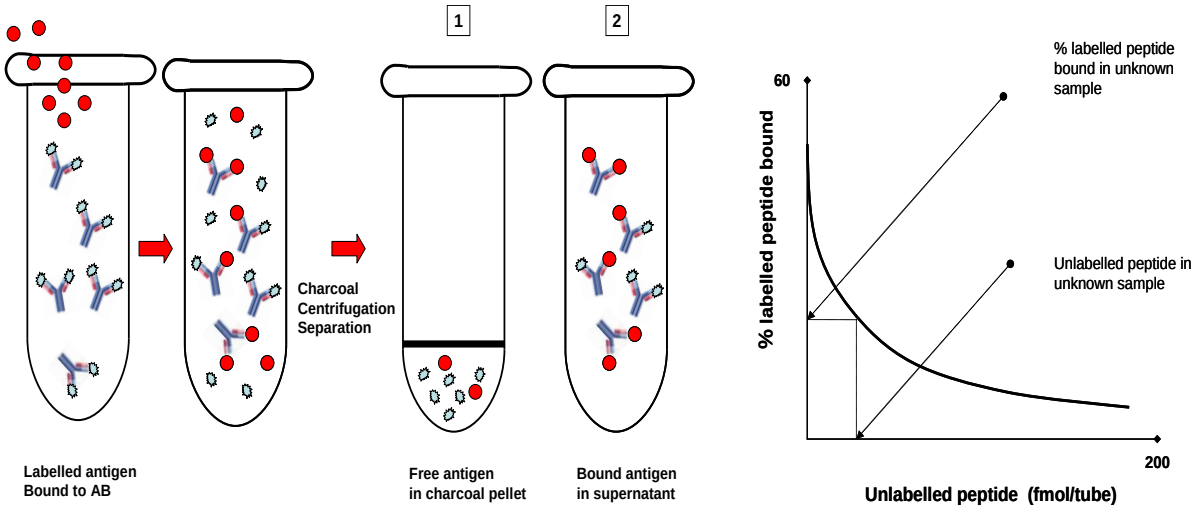


Figure 2-1: **Principle of RIA** (charcoal separation) and **RIA standard curve**.

Radioactively labelled and unlabelled antigen competes for the specific antibody binding sites. Centrifugation separates the unbound antigen (adsorbed to charcoal) from the bound antigen (in supernatant).

2.1.3 Enzyme-Linked Immuno-Sorbent Assay (ELISA)

An enzyme-linked immuno-sorbent assay (ELISA) is an immunological method that can detect and/or quantify the presence of an antibody or an antigen in a sample. In this work, the competitive ELISA technique was utilized to measure antigen (peptide GLP-2). A commercially available GLP-2 ELISA kit (Phoenix Pharmaceuticals, Inc.) was used. It contains a 96 well immuno-plate which is pre-coated with secondary antibody that binds to the Fc fragment of the primary (GLP-2) antibody, whose Fab fragment is competitively bound by either biotinylated GLP-2 and GLP-2 standard or GLP-2 in samples. The biotinylated peptide interacts with enzyme (streptavidin-horseradish peroxidise; SA-HRP) which catalyzes the TMB (tetramethylbenzidine) substrate solution and produces a measureable blue colour change. The reaction is terminated by addition of HCl solution which changes the solution colour from blue to yellow. The intensity of colour reaction is directly proportional to the amount of bound biotinylated peptide-SA-HRP complex and inversely proportional to the amount in standard solution and samples. A standard curve of known concentrations is established and concentration of peptide in samples extrapolated.

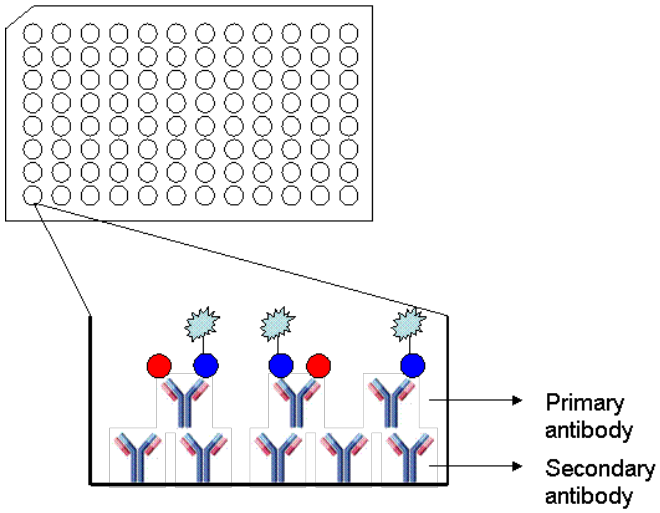


Figure 2-2: **Principle of competitive ELISA**.

Peptide and biotinylated peptide compete for binding sites on the primary (GLP-2) antibody.

2.1.4 Chromatography

The concentration of gut hormones in plasma is generally very low. The sensitivity of any measurement method might be enhanced if the peptide of interest is extracted and thereby concentrated. This will also limit non-specific interference from other plasma components and therefore improve accuracy and reproducibility of the measurements. Because minor variations in peptide length can have a profound effect on peptide function, as is the case with GLP-2, it may also be necessary to distinguish between various molecular forms of these hormones such as precursors, active peptide and degraded peptide.

These objectives can be achieved by using chromatography. This technique comprises a large group of analytical methods for the separation of mixtures. In principle, a mixture, containing various components (“solutes”), is carried in liquid or gas ("mobile phase"), through a solid or liquid column ("stationary phase"). Passage of the solutes through the stationary phase is modified through their specific interactions with the stationary phase, which leads to their separation. Different solutes may pass through the system at different rates and therefore become separated in time (e.g. size exclusion chromatography). Alternatively certain solutes may be retained in the solid phase and can then be selectively desorbed by washing with an appropriate solvent (e.g. solid phase extraction).

a) Solid phase extraction (SPE)

Solid phase extraction is a technique based on selective partitioning of one or more components between two phases one of which is a solid sorbent. The second phase typically is a liquid, but it may also be an emulsion, a gas, or a supercritical fluid. The components of interest may either preferentially adsorb to the solid phase, or they may remain in the second, non-solid phase. Once equilibrium has been reached, the two phases are physically separated by decanting, filtration, centrifugation or a similar process. If the

desired analytes are adsorbed on the solid phase they can be selectively desorbed by washing with an appropriate solvent. If the components of interest remain in the liquid phase they can be recovered via concentration or evaporation (McDonald 2001).

In this work, solid phase extraction was utilised to assess whether peptide concentration enhances the accuracy and diagnostic value of GLP-2 measurement. For this purpose, SepPak C18™ (**S**ample **E**nrichment & **P**urification) packed bed column cartridges were used. The solid phase packed into these cartridges consists of an 18-carbon hydrocarbon chain that is bound to silicate. The separation method is based on the interaction between this hydrophobic support and the hydrophobic areas located on the surface of proteins. The retained peptides can then be desorbed by washing with solvent (e.g. methanol) and analysed.

b) Gel permeation chromatography

Gel permeation chromatography, also known as size exclusion chromatography, separates molecules on basis of size. Columns are packed with porous beads, e.g. Sephadex (Pharmacia), with a specific pore size. Sample is loaded onto the column and a constant flow of buffer solution moves it through. Larger molecules that are excluded from the pores continue following the solvent flow, eluting quickly in what is designated the void volume, **Vo**. This can be determined experimentally by the use of a high-molecular weight component, such as dextran blue (MW $2x10^6$), which is easily visible. Smaller molecules however enter the porous media and thus attain a much larger volume of distribution. They are therefore retained longer and pass more slowly through the column. The elution volume for material included in the pores is designated the total volume, **Vt**. This represents the total liquid volume of the column and is often determined by small molecules, such as ^{125}I-Na which can be detected with a gamma counter.

The elution volume **Ve** for a given protein lies between Vo and Vt and can be determined with RIA. A partition coefficient can be determined for each protein as **Kav** (Laurent et al. 1964):

$$\mathbf{Kav = (Ve-Vo)/(Vt-Vo)}$$

Kav = partition coefficient
Vo = Void volume
Vt = Total volume
Ve = Elution volume of the analyte.

In size exclusion chromatography, the macromolecules are not physically retained, unlike adsorption techniques; therefore, the protein will elute in a defined volume between Vo and Vt. Elution of protein before the void volume (Ve<Vo) suggests channelling through the column; for example due to improper packing or operation of the column. If the protein elutes after the total volume (Ve>Vt), then some interaction must have occurred between the matrix and the protein of interest.

2.2 Aims and Methods

2.2.1 Specificity of antiserum FT-17

GLP-2 antiserum “FT-17” was raised by Prof Mohammed Ghatei (Department of Investigative science, Hammersmith hospital). His work has previously established that antiserum FT-17 has a high specificity for the detection of GLP-2. Apart from a weak (0.1%) cross-reactivity with GLP-1, no reaction occurs with other related gut hormones (laboratory data Prof. M. Ghatei, 1997; see Appendix 1). The Antiserum was used in a dilution of 1:500.000.

It was the purpose of this work to characterize and optimize a RIA utilizing FT-17 and to determine, whether this antiserum is specific for the detection of active GLP-2 (1-33); or whether cross-reaction occurs with other proglucagon derived peptides that contain the GLP-2 sequence.

a) Source of synthetic peptides

Synthetic human GLP-2 was used as peptide standard. Human carboxy-terminal GLP-2 (1-33) and GLP-2 (3-33) were obtained from the Wolfson Institute for biomedical research, University College London. Peptides had greater than 95% purity as determined by HPLC.

b) Preparation of standards

Standards were prepared gravimetrically (using an electronic microbalance). To maintain stability and solubility of the aliquotted peptides, standard vials were prepared in freeze-drying solution (appendix), lyophilised and stored in vacuo at -20°C.

c) Preparation of radioactive label

The synthesis of a radioactive peptide label is commonly performed through iodination of a tyrosine residue with ^{125}Iodine. Neither rat nor human GLP-2 contains a tyrosine residue for iodination; however a tyrosine extended analogue (Tyr-0 GLP-2) can be used for iodination without significant change in antigenicity. ^{125}I (Tyr0)-GLP-2 was prepared using iodogen method (Bolton 1989) and purified by HPLC. Due to departmental safety regulations, radioactive labelling was performed by Prof Ghatei. Test-assays to determine and monitor label quality were performed by me.

d) Peptide extraction from tissue biopsies

Terminal ileal and pancreatic tissue from a healthy adult Wistar rat was immediately placed in liquid nitrogen following resection. A tissue sample of 0.2 g was immersed in 0.5 ml of 0.5-molar acetic acid and boiled in a water bath for 15 minutes. The substrate was immediately put on ice and buffer solution added to a total volume of 1ml. To estimate peptide concentration, the extract was analysed with RIA in different dilutions (1:100; 1:10; 1:1) prior to chromatography.

e) GLP-2 RIA

Sample tubes were arranged in Perspex racks. Phosphate buffer solution (see Appendix 2), peptide standard, sample, labelled peptide and antibody were added with micropipettes according to protocol (Figure 2-3). Tubes for quality monitoring were included in every assay (Figure 2-4). Samples were set up in duplicates to increase precision and detect random error. For incubation of the reagents, the racks were covered in foil and placed in the cool room for 72 hours at 4°C. The cool temperature serves to minimize proteolytic degradation and evaporation. Following incubation, dextran coated charcoal solution was added with a repeating dispenser to adsorb the free hormone. The coating of dextran is thought to block the large pores in the charcoal and thus prevent adsorption of the antibody complex. Tubes were immediately centrifuged at 1600g for 20 min at 4°C. The liquid

supernatant was instantly separated from the solid phase (charcoal pellet) through aspiration using a Pasteur pipette and dispensed into a corresponding empty tube, which was subsequently sealed with heated paraffin wax. Radioactivity was counted for 180 sec in both the charcoal pellet (containing adsorbed free label) and supernatant (containing antibody bound label) in 4 multiple well counters (model NE1600, NE Technology, enabling simultaneous counting of 128 tubes). Peptide concentrations in the samples were calculated using a non-linear plot (RIA Software, NE Technology) and results calculated in terms of the GLP-2 standard preparations.

Tubes	Type		Buffer (µl)	AB (µl)	Label (µl)	Final Volume (µl)
1-2	Blank		600	0	100	700
3-4	1/2		550	100	50	700
5-6	2x		400	100	200	700
7-10	Zero		500	100	100	700
11-12	Standard 1µl	= 2fmol	500	100	100	700
13-14	Standard 2µl	= 4fmol	500	100	100	700
15-16	Standard 3µl	= 6fmol	500	100	100	700
17-18	Standard 5µl	= 10fmol	500	100	100	700
19-20	Standard 10µl	= 20fmol	490	100	100	700
21-22	Standard 15µl	= 30fmol	490	100	100	700
23-24	Standard 20µl	= 40fmol	480	100	100	700
25-26	Standard 30µl	= 60fmol	470	100	100	700
27-28	Standard 50µl	= 100fmol	450	100	100	700
29-30	Standard 100µl	= 200fmol	400	100	100	700
31-32	Zero		500	100	100	700
33- x	Samples 100 µl		400	100	100	700
y1-y2	XS			600	100	700

Figure 2-3: Typical set up of a RIA rack for determination of GLP-2 concentration in plasma samples

- **Blank:**
  - Contains all assay reagents except antibody
  - Evaluates non-specific binding of labelled hormone to plasma proteins
- **1/2:**
  - Half concentration of labelled hormone
  - Addresses whether greater sensitivity could have been achieved by adding a smaller amount of label
- **2x:**
  - Double concentration of labelled hormone
  - Addresses whether greater sensitivity could have been achieved by adding a greater amount of label
- **Zero:**
  - Contains no unlabelled hormone
  - Included at regular intervals (approximately every 100 tubes)
  - Detects any drift, e.g., due to reagent deterioration or syringe fatigue
- **XS:**
  - Excess antibody
  - Should bind the entire labelled hormone
  - Measure of the immunological integrity of the labelled hormone
  - Can be used to assess the quality of the label (once the label becomes older and starts to break down, the percentage of bound radioactivity in the “Blank” tube rises and in the “XS” tube falls)

Figure 2-4: Tubes for quality monitoring adapted from (Adrian 1982)

f) Gel permeation chromatography

A 0.9x60 cm column was prepared using pre-swollen Sephadex G-50 superfine (Pharmacia, Uppsala Sweden) and equilibrated overnight with column buffer (0.06 M phosphate buffer containing 0.2 M NaCl and 0.3% BSA). Caution was taken, not to disturb the gel once the column had set, as small surface disruptions are known to disturb flow speeds and may lead to complete disintegration of the column. To determine the partition coefficient K_{av}, dextran blue (50 µl, MW $2x10^6$ daltons) and ^{125}I-NA (30Bq) and horse heart cytochrome C (50 µl, MW 12 384 daltons) were added to each sample. All samples (as detailed below) were loaded onto the top of the column with a syringe at a total volume of 0.7ml. The remaining sample was saved to determine peptide recovery. Once the sample had completely entered the gel, the column was filled with buffer solution. Then the system was closed and connected to a pump, allowing constant flow of column buffer through the gel at 3.2 ml/hr. The eluent was collected in a rotating rack. Collection time was 15 minutes per fraction. Fractions of 0.8 ml were collected and RIA used to determine the elution profile of GLP-2-IR. Dextran Blue and ^{125}I-Na were used to mark the void volume and total column volume respectively. Elutes of synthetic peptide standard were analysed at a volume of 100µl. All other sample elutes were analysed at a volume of 300µl. Samples were analysed in groups, each containing a run of GLP-2 (1-33) standard. All samples within one group were analysed in a single RIA.

Samples analysed with gel permeation chromatography:

Synthetic human GLP-2 (1-33)	1.5 pmol in 1ml buffer
Synthetic human GLP-2 (1-33)	1.5 pmol in 1ml plasma
Synthetic human GLP-2 (3-33)	37.5 pmol in 1 ml buffer
Human plasma (fasting)	1.1ml
Human plasma (postprandial)	1.1ml
Human plasma postprandial (SepPak)	Extract of 2ml, freeze-dried (Reconstituted in 1.2 ml buffer)
Rat pancreas extract	500µl in 300µl buffer (Estimated GLP-2 conc. 1.5 pmol)
Rat terminal ileum extract	375µl in 425µl buffer (Estimated GLP-2 conc. 1.5 pmol)

2.2.2 Optimization of assay conditions

a) Albumin concentration

Antibody-antigen interaction may be influenced by the presence of proteins and other constituents of plasma. Antiserums vary in their susceptibility to these non-specific effects (Adrian 1982). Ideally, the standard curve of a plasma assay should always be set up with an equal volume of hormone-free plasma in place of the sample to minimize any residual error; but this is not always available. Other measures known to reduce non-specific plasma effects include reducing the proportion of plasma in the assay tube and hormone extraction. Bovine serum albumin (BSA) can be added to the buffer solution as a simple measure to adjust for this confounding factor and in order to prevent “non-specific sticking” of the peptide to the test tube.

To investigate the impact of varying BSA concentration in the assay buffer, three series of standards (0-100fmol) were prepared in phosphate buffer containing 0.3%; 1% and 3% BSA and analysed according to protocol.

b) Separation method

The ability to separate antibody-antigen complexes from free antigen reliably, consistently and rapidly is essential for any good radioimmunoassay (Bretheron-Watt 1991). Separation can be achieved in various ways such as through immuno-precipitation with a secondary antibody, chemical precipitation (alcohol, polyethylene glycol or ammonium sulphate) or adsorption (with cellulose, talc or charcoal) (Adrian 1982). Charcoal separation is a relatively uncomplicated and commonly used method. To examine whether immuno-precipitation with a secondary antibody enhances assay sensitivity, two series of standards (0 -100fmol) were prepared. After a 4 day incubation period samples were separated by means of:

1. Charcoal adsorption: Phosphate buffer 100 ml
Charcoal 2.4 g,
Dextran 0.24 g
(250 µl of solution added to sample immediately prior centrifugation.)

2. Immunoprecipitation: Anti-rabbit antibody (sheep) (100µl),
Triton X (500µl)
(Samples are incubated with the antibody for 1hour at room temperature. Triton X is added and samples immediately centrifuged)

2.2.3 Optimization of sampling conditions

a) Protease inhibitor

In view of the rapid degradation of GLP-2 through the serine protease DPPIV, enzyme inhibitors are commonly added to the collection system to prevent peptide loss. The protocols differ amongst laboratories, most likely due to local experience and availability of the reagents. The most commonly inhibitors used are **Trasylol**, a non-specific protease inhibitor, phenyl-methyl-sulphonyl fluoride (**PMSF)**, a serine protease inhibitor and **Diprotin A**, a specific inhibitor of DPPIV. These inhibitors are used either alone or in combination. In this laboratory, Trasylol is used routinely when collecting blood samples for gut hormone analysis. To assess whether, through addition of a specific serine protease inhibitor, peptide loss can be reduced when measuring GLP-2, PMSF was added to selected plasma samples.

Pre- and postprandial (60, 120, 180 and 240 minutes) samples of 6 healthy volunteers were obtained. 10ml venous blood was drawn from a peripheral vein and transferred into Lithium Heparin tubes, containing either Trasylol® (0.2ml) alone or a combination of Trasylol® and PMSF (200 mM, 40µl). Subsequently blood samples were processed in the usual manner and GLP-2 IR measured in an RIA.

GLP-2 immunoreactivity in the samples collected at different time points, with and without addition of PMSF was compared, using the paired t-test and ANOVA. A p-value of less than 0.05 was regarded as statistically significant.

b) Peptide extraction

Assay sensitivity may be enhanced by removing interference from other plasma components through extraction of peptides. To assess whether result interpretation could be aided by extracting GLP-2 prior analysis, solid phase extraction with SepPak C18 ™ cartridges was performed for selected

samples. Peptide recovery was assessed by extracting peptides in plasma samples with known quantity of synthetic GLP-2 standard (100fmol, n=8 and 300fmol, n=8). Fasting and post-prandial blood samples of 8 healthy subjects were obtained and GLP-2-IR in unextracted samples was compared with results in corresponding extracted plasma samples.

For the extraction process, SepPak C18 cartridges were pre-conditioned with 10 ml of methanol, followed by 20 ml of water. The prepared cartridges were then stored in 4% acetic acid to prevent drying out. 1ml of plasma sample was mixed with 1ml of 0.1M hydrochloric acid (HCl) and drawn up into 2ml syringes. The mixture was then “loaded” by gently pushing through the cartridge into an empty syringe at constant speed. This process was repeated ten times to ensure maximum adsorption of the peptide. The remnant plasma was discarded. The cartridges were washed with 10ml of 4% acetic acid, to flush out any interfering compounds. The cartridge was placed on top of an eluting tube. 1.5ml methanol was filled into an empty syringe without plunger and the solvent was left to slowly filter through the cartridge. The eluent was collected and freeze dried and stored. Prior to analysis it was dissolved in 100µl of water, left for 60 min at room temperature and then re-suspended with 400µl of buffer.

For the comparison of the fasting values with different postprandial time points as well as for comparison of neat versus concentrated samples the paired student t-test was used. For the evaluation whether the percentage change from baseline to a number of subsequent times varied between measurements of non-extracted vs. extracted plasma, analysis of 2-factor ANOVA was performed**.** A p-value of less than 0.05 was regarded as statistically significant.

c) Freeze-thaw cycle

The stability of peptides is dependent on amino acid composition and sequence. Peptide degradation can occur through hydrolysis, deamidation,

oxidation and racemization (loss of chiral integrity) of the amino acid or peptide. Lyophilized peptides are generally more stable than peptides in solution. If the peptide is in solution, freeze-thaw cycles should be avoided but this is not always achievable in practice.

To determine whether repeat thawing and re-freezing influences the measurement of GLP-2-IR, a post-prandial plasma sample was frozen in 2ml aliquots. Aliquots were defrosted 1x, 2x, 3x, 4x, 5x and 10x and GLP-2-IR analysed in a single assay (n=12 for each aliquot to determine intra-assay variability). Statistical differences between the mean GLP-2-IR concentrations in the groups were examined with single factor analysis of variance. A p-value of less than 0.05 was regarded as statistically significant.

d) Haemolysis

Haemolysis is understood as the release of haemoglobin and other intracellular components from erythrocytes to the surrounding plasma, following damage or disruption of the cell membrane. It may occur either in vivo (due to pathological conditions, such as autoimmune haemolytic anaemia or transfusion reaction) or in vitro (due to improper specimen collection, processing, or transport). Haemolysis may interfere with laboratory testing due to leakage of haemoglobin and other intracellular components into the surrounding fluid, which induces false elevations of some analytes or reductions through dilution effects (Lippi et al. 2006). Serum does not look haemolysed until the concentration of haemoglobin reaches 0.2 g/L (Kroll et al. 1994). Products of haemolysis may interfere with immunoassays. However not all peptides appear to be affected to the same extend and the severity of haemolysis might also be of relevance (O'Rahilly et al. 1987).

To determine the influence of haemolyses on the measurement of GLP-2-IR, 20ml of venous blood was separated into 10ml aliquots. 10ml were immediately spun down at 1600g at 4°C and 10ml were first frozen at -20°C,

then defrosted and spun down, causing severe haemolysis of the sample. Defined amounts of haemolytic plasma were added to the non-haemolytic sample and GLP-2 immunoreactivity measured. Differences between the groups were determined by single factor analysis of variance and Student-Newman-Keuls test (post hoc ANOVA analysis for pair-wise comparisons). A p-value of less than 0.05 was regarded as statistically significant.

In a separate experiment, synthetic GLP-2 (1-33) peptide standard was added and standard curves obtained for different concentrations of haemolysed plasma. Difference between the standard curve values was determined by student T-test and two factor analysis of variance. A p-value of less than 0.05 was regarded as statistically significant.

2.2.4 Quality control

a) Sensitivity

Assay sensitivity is the lowest hormone concentration that can be reliably detected. It is determined by the steepness of the dose-response curve (which depends on the antibody-antigen affinity) and the error of a given value on the standard curve. The main causes of error of a radioimmunoassay occur through counting error and pipetting error (mostly during separation) (Sarson 1982). The maximum sensitivity of each assay was calculated as the smallest standard curve value, 2 standard deviations from the zero standard (95% confidence interval).

b) Reproducibility

Many factors cause variation in assay performance including deterioration of the radioactive label, low antiserum affinity, errors of technique and inefficient equipment (Sarson 1982). Comparative data are needed to evaluate the quality of a single assay as well as assay performance over time. Quality control tubes were included in every assay to determine the non-specific binding of the labelled antigen and the quality of the label (Figure 2-4). Zero hormone standard tubes were included at various points within each assay to allow detection of any drift in measurement. Reproducibility controls were prepared by adding synthetic GLP-2 (1-33) to pooled human plasma such that 100 µl of plasma would read off the GLP-2 standard curve in the range of “high”, “medium” or “low”. Plasma was aliquoted, lyophilised and stored in vacuo at -20°C. Inter-assay variation was determined by adding quality controls to consecutive assays (n=5); intra-assay variation was determined by inclusion of replicates in a single assay (n=8). In addition, aliquoted fresh plasma from a single subject was also frozen and stored at -20°C and inter-assay variation (n= 5) and intra-assay variation (n=10) determined.

To determine reproducibility of GLP-2-IR in physiological samples, pre-and postprandial GLP-2-IR concentration of 8 subjects was analysed at 10 time points over 270 minutes in 2 sequential RIAs (see also 2.2.5). Pearson coefficient of correlation was determined between the duplicate samples within in each assay and between the means of the duplicates for the two RIA-runs.

c) Sample carryover

Separation of bound and unbound fractions following centrifugation is performed with a single pipette. Carryover of analyte from one sample into one or more following samples can falsely increase the measured analyte concentration. To determine whether this carryover effect is a relevant source of error, 100 µl of plasma was analysed as per protocol ("low concentration" sample). Synthetic GLP-2 standard (30fmol) was added to selected tubes ("high concentration" sample). Sample position within the assay was chosen, so that separation would occur in the order of: low-high-high-low concentration sample. Samples were analysed in duplicates.

2.2.5 Postprandial GLP-2-IR profile in healthy humans

Nutrients are the main stimulants of GLP-2 secretion. A valid assay should therefore be able to demonstrate a postprandial secretory response. It has been demonstrated that the postprandial GLP-2 secretion depends on nutrient composition and calorie content of an ingested meal (Xiao *et al.* 1999). It is well documented that in particular lipids and carbohydrates lead to a substantial rise in GLP-2 plasma levels (Xiao *et al.* 1999; Hartmann *et al.* 2000). Reports of response to protein-rich meals vary (Xiao *et al.* 1999; Henriksen *et al.* 2003; Feltrin *et al.* 2006). A test-meal, evoking a reliable GLP-2 response, should therefore be energy-dense and have high lipid- and/or carbohydrate content. Other aspects to consider are consistent and reproducible nutrient composition, ease of preparation and, last but not least, palatability. Commercial ice-cream fulfils most of these criteria and was therefore used as a test-meal in healthy volunteers (Table 2-1). It was postulated that the available assay will be able to demonstrate a significant postprandial rise of plasma GLP-2.

Test meal:	**Ice cream (chocolate flavour, Sainsbury's Finest™)**	
Test meal composition:	Volume	500 ml
	Energy content	920 kcal
	Total fat	50g
	Saturated fat	30.8g
	Total carbohydrates	127.5g
	Sugar	101.1g
	Protein	14g
	Fibre	6g

Table 2-1: **Test meal composition**
Nutrient and energy content as per product label

Ethical approval for this procedure was obtained from the local research ethics committee (REC 04/Q0405/83).

8 healthy volunteers (6 male, 2 female; median age 43.4 years; range 32-66 years) were asked to consume 500ml of ice cream, served in semi-liquid form at 4°C, over a time period of 15-20 minutes. Venous sampling was undertaken at 2 time-points prior and at half hourly intervals (up to 240 min) after the meal. Blood was collected in 10ml lithium-heparin tubes containing 0.2ml Trasylol. Samples were immediately spun down in a refrigerated centrifuge at 4°C for 10 min at 1600g. Plasma was collected and stored at -70°C until analysis. All samples were analysed in a single RIA. For the comparison of the fasting values with different postprandial time points the paired t-test was used. A p-value of less than 0.05 is regarded as statistically significant.

2.2.6 Correlation to antiserum 92160

An N-terminal-specific GLP-2 radioimmunoassay (92160) was developed by the group of Jens J. Holst. 92160 is the only antiserum in published literature that is specific for the measurement of biologically active GLP-2 (1-33) and RIAs using this antiserum could therefore be considered as the “gold standard” of GLP-2 measurement. Antibody 92160 was raised in Danish white rabbits against synthetic PG 126–136 coupled to bovine serum albumin with carbodiimide. It shows no cross-reaction with GLP-1 (1-36NH2), GLP-1 (7-36NH2), glicentin, oxyntomodulin, glucagon, pituitary adenyl cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP), growth hormone-releasing factor (GHRF), gastric polypeptide (GIP), secretin and peptide histidine isoleucine amide (PHI) in concentrations up to 5 nmol/l. Experimental detection limit is 5pmol/l, intra-assay coefficient of variation has been quoted as 2.3% at 40pmol/l (Wojdemann *et al.* 1998).

Venous blood samples from 14 healthy volunteers were collected in the fasted state and 60 and 120 minutes following a standard meal as described in section 3.3.2. Plasma samples were frozen in 1 ml aliquots and both analysed with RIA using antiserum FT-17 and also sent to the University of Copenhagen and analysed by Dr. Jens J. Holst using antiserum 92160. None of the samples had been defrosted prior to measurement. GLP-2 concentrations in plasma were measured after extraction of plasma with 70 % ethanol.

2.2.7 Correlation to ELISA

The measurement of GLP-2 with radioimmunoassay is labour- and time intensive, depends on the availability of a specific laboratory and involves the use of radioactive substances. Measurement of GLP-2 using ELISA technique might be a useful alternative which could be applied in wider laboratory settings. GLP-2 RIA (FT-17) measurements were therefore compared with results from a commercially available ELISA kit (Phoenix Pharmaceuticals, Inc). Usual assay performance (as per company information) indicates a sensitivity of 0.1ng/ml, intra-assay variation <5%, inter-assay variation: <14%, detectable range: 0-100ng/ml and linear Range: 0.1-1.72 ng/ml. Cross-reactivity is reported as 100% with human and rat proglucagon (126-159) which corresponds to GLP-2 (1-33)-Arg). There is 6% cross-reactivity with human GLP-1 (7-36), 5% with human GLP-1 (7-37) and no cross-reactivity with Glucagon, GIP, VIP or Insulin.

Venous blood samples from 14 healthy volunteers were collected in the fasted state and 60 and 120 minutes following a standard meal as described in section 3.3.2. Plasma samples were frozen in 1 ml aliquots and analysed with RIA using antiserum FT-17 and also processed with the commercial ELISA kit as described below. None of the samples had been defrosted prior to measurement. Samples were analysed in duplicate.

The GLP-2 ELISA was performed as per manufacturers' instruction. Peptide standard solutions were prepared. 50µl of standard or sample, 25µl primary antiserum and 25µl biotinylated peptide was added to the wells. The immunoplate was sealed and incubated at room temperature for 2 hours on an orbital plate shaker at 400rpm. The immunoplate was washed 5 times with 350µl/well of buffer. 100µl/well of SA-HRP solution was added. The immunoplate was incubated as above for 1 hour. The immunoplate was washed 5 times with 350µl/well of buffer. 100µl/well of substrate solution was added. The immuno-plate was incubated as above for 1 hour. 100µl/well of

2N HCl was added to terminate the reaction. Absorbance (O.D.) was read at 450nm and concentrations extrapolated from the standard curve.

2.3 Results

2.3.1 Specificity of antiserum FT-17

Interlocked RIA of GLP-2 (1-33) and (3-33) standard (Figure 2-5): Within normal standard curve concentrations (2-200fmol/tube) there was minimal antibody displacement by GLP-2 (3-33) at 200fmol. Analysis of 1, 10 and 100 pmol GLP-2 (3-33) quantified 4% cross reactivity with antiserum FT-17 (1pmol GLP-2 (3-33) resulted in 28% binding of GLP-2 (1-33) standard (equals 40 fmol).

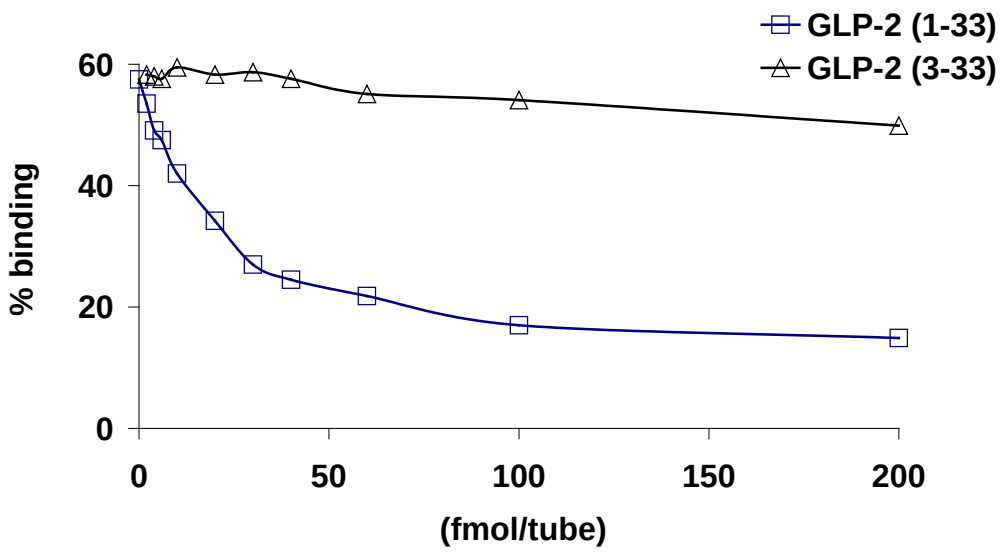


Figure 2-5: **Cross reactivity of FT-17 with GLP-2 metabolite (3-33)**

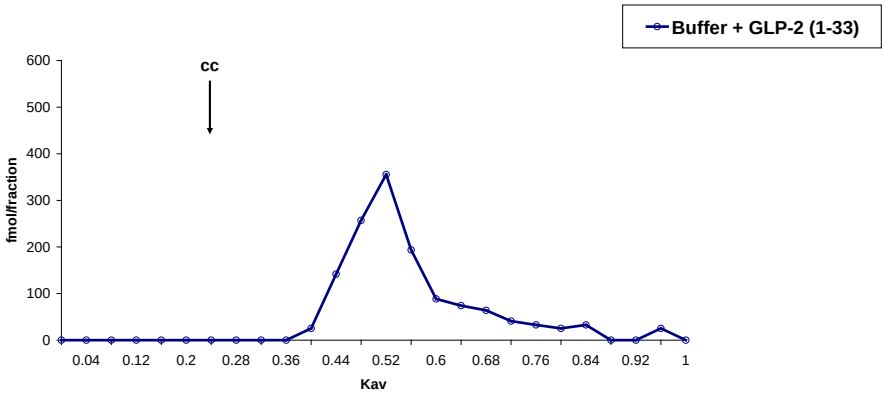
Displacement of antiserum FT-17 with standard concentrations of synthetic GLP-2 (1-33) and synthetic GLP-2 (3-33)

GLP-2-IR elution profile of synthetic GLP-2 standards (Figure 2-6):
The elution profile of synthetic GLP-2 (1-33) standard identified GLP-2-IR in 5 adjacent collection fractions with a maximum peak at K_{av} of 0.52. Recovery of peptide standard was 60%. The elution profile of truncated GLP-2 (3-33) shows an identical peak to GLP-2 (1-33) at K_{av} 0.52. When GLP-2 (1-33) standard was diluted in plasma instead of column buffer, GLP-2-IR appeared to elute earlier (peak K_{av} 0.44). As in some of the chromatography runs, plasma or tissue samples were compared with GLP-2 standard diluted in buffer, this finding had to be taken into consideration for the result analysis.

GLP-2-IR elution profile in rodent tissue extracts (Figure 2-7):
The elution profile of rat terminal ileal extract shows a single peak corresponding to elution of GLP-2 (1-33) standard. Rat pancreatic tissue extract shows 2 large peaks of GLP-2-IR material with maximum K_{av} at 0.15 and at 0.56-0.63. There is no obvious peak corresponding to the elution point of GLP-2 standard (K_{av} 0.48).

GLP-2-IR elution profile in unextracted plasma samples (Figure 2-8):
Elution profiles of pre- and post-prandial human plasma samples were obtained and representative profiles are shown (Figure 2-8 B and C). In the fasting sample, GLP-2-IR mainly consists of larger molecular weight material (max Kav peaks at 0.24 and 0.29). There is only a very small peak corresponding to the elution of GLP-2 (1-33) standard, which is outside of the sensitivity limitations of the FT-17 assay. In the postprandial plasma, overall GLP-2-IR is increased. There is a discernable peak, corresponding to the elution point of GLP-2 standard (25% of total GLP-2-IR).

GLP-2-IR elution profile in extracted plasma samples (Figure 2-9):
The GLP-2-IR elution profile of plasma following peptide extraction with a SepPak cartridge shows a reduction in overall GLP-2-IR compared to unextracted plasma. However it appears that, following the removal of most large molecular weight material in the extracted plasma, a small peak corresponding to the elution point of GLP-2 (1-33) standard can be shown (43% of total GLP-2 IR).



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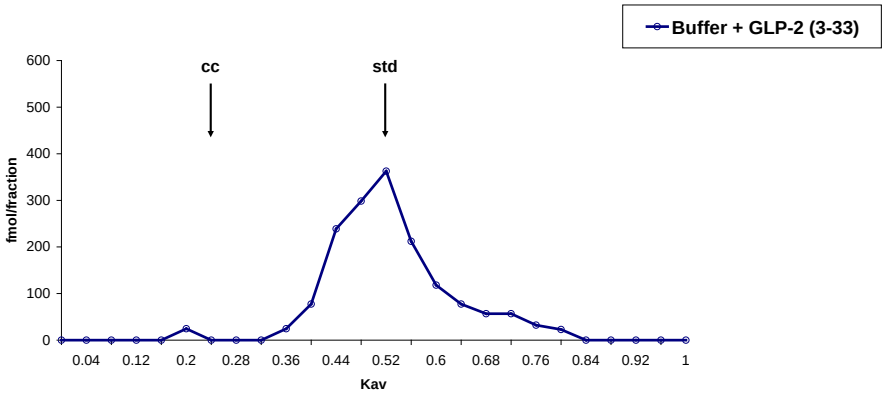

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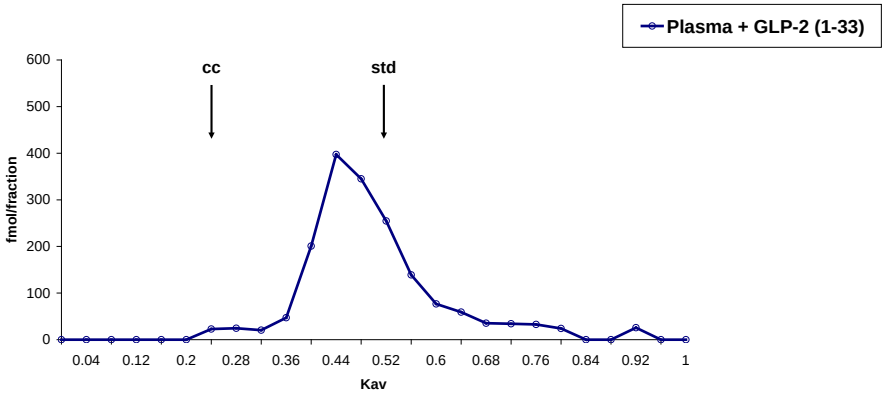
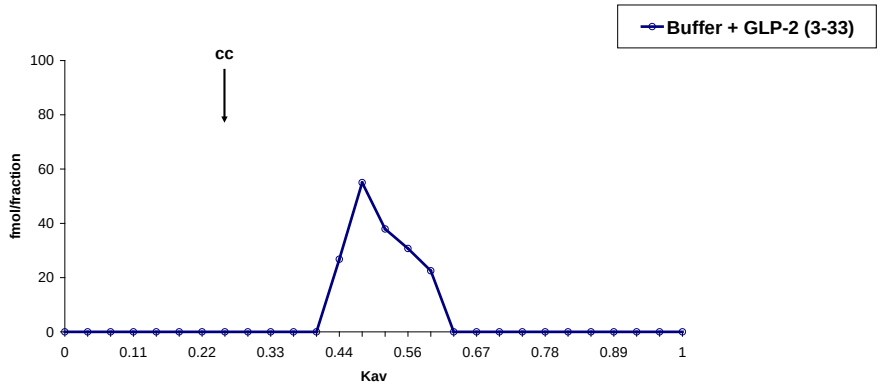


C

Figure 2-6: GLP-2-IR elution profiles with Sephadex G50 chromatography.

A) GLP-2 (1-33) standard in column buffer.
B) GLP-2 (3-33) standard in column buffer.
C) GLP-2 (1-33) in plasma.

Std indicates the elution of GLP-2 (1-33) standard in buffer, cc indicates elution of cytochrome C (MW 12384). Dextran Blue (MW >2,000 000) elutes at Kav = 0 and Na 125 I at Kav = 1.



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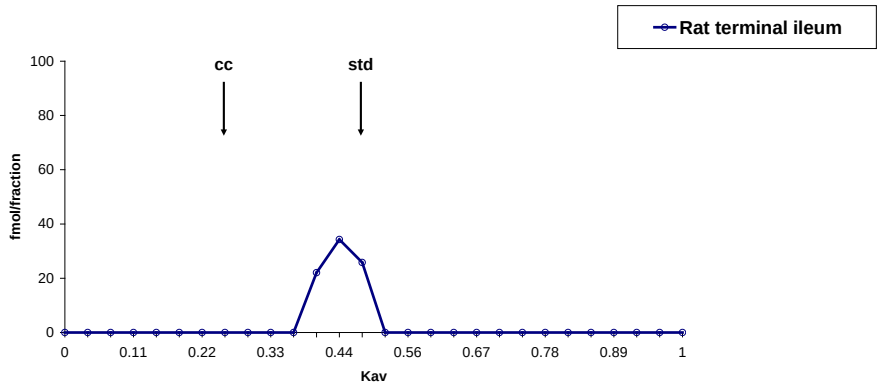

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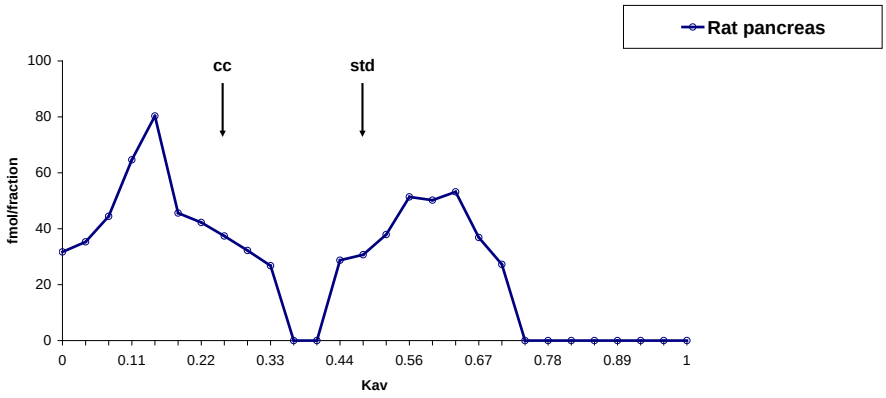
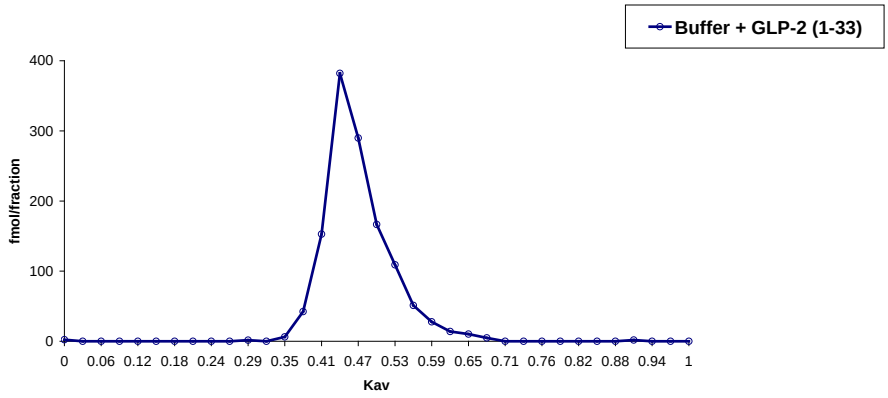


C

Figure 2-7: Elution profile of GLP-2-IR using Sephadex G50 chromatography.

A) GLP-2 standard in buffer.
B) Rat intestinal extract
C) Rat pancreatic extract

Std indicates the elution of synthetic GLP-2 (3-33) in buffer, cc indicates elution of cytochrome C (MW12 384). Dextran Blue (MW >2,000 000) elutes at Kav = 0 and Na 125 I at Kav = 1.



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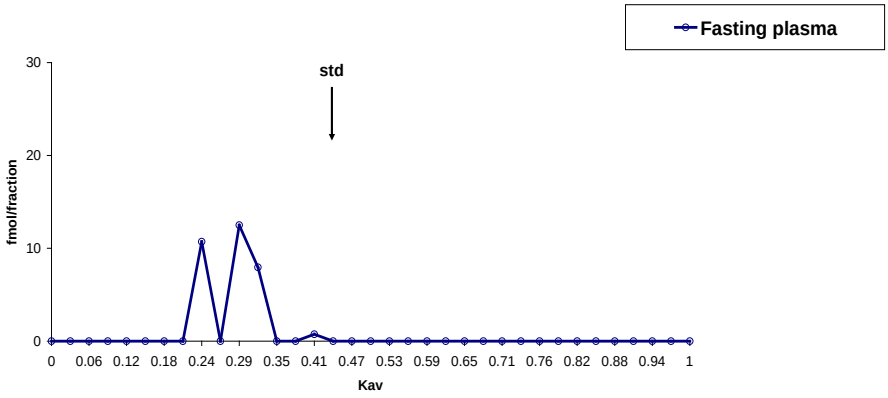

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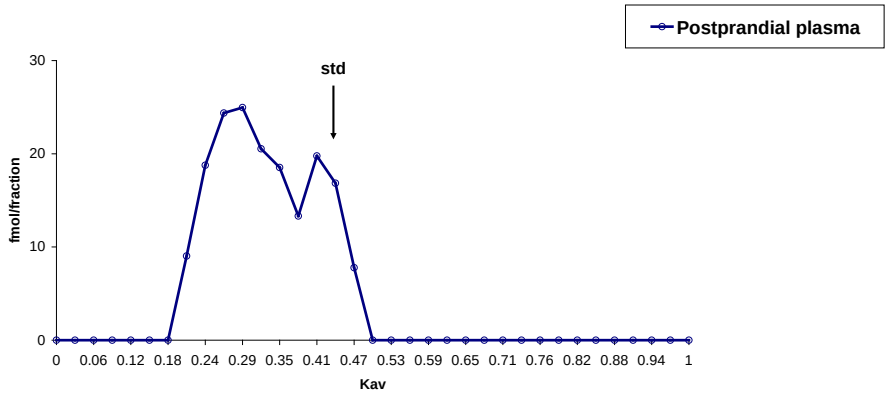
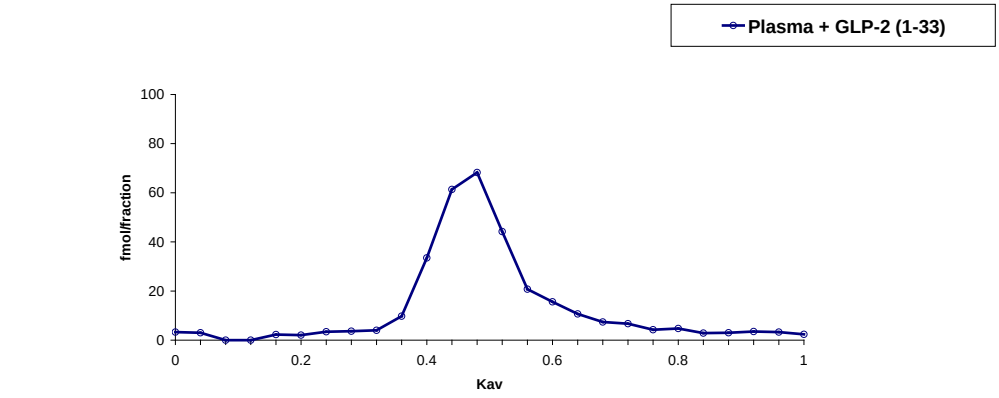


C

Figure 2-8: Elution profile of GLP-2-IR using Sephadex gel G50 chromatography.

A) Synthetic GLP-2 (1-33) in column buffer.
B) Non-extracted human plasma (fasting).
C) Non-extracted human plasma (postprandial).

The arrow indicates the elution of GLP-2 (1-33) standard in buffer.
Dextran Blue (MW >2,000 000) elutes at Kav = 0 and Na 125 I at Kav = 1.



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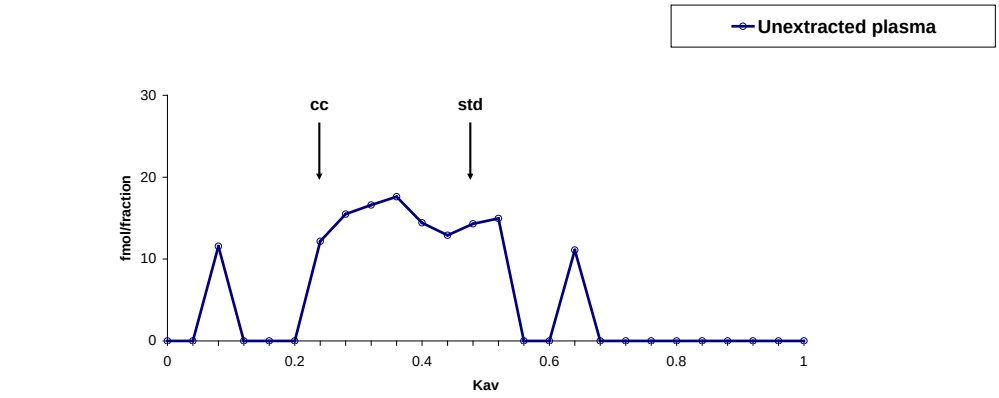

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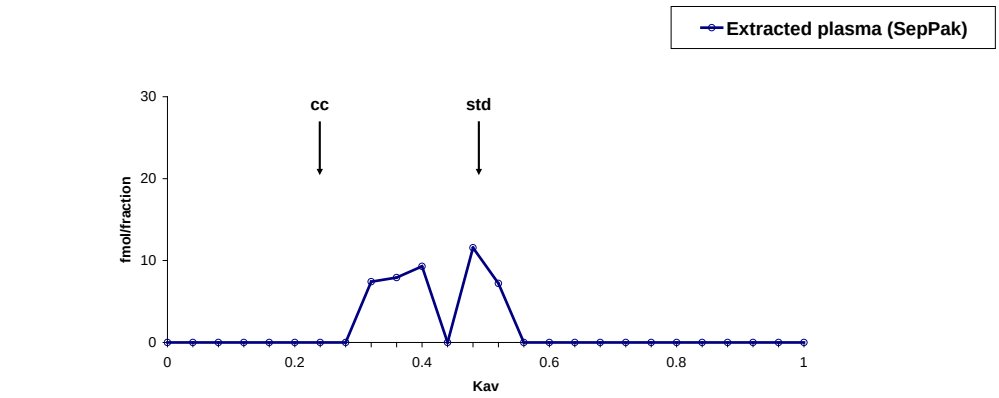


C

Figure 2-9: Elution profile of GLP-2-IR using Sephadex G50 chromatography.

A) Synthetic GLP-2 (1-33) standard in plasma.
B) Unextracted postprandial plasma sample.
C) Postprandial plasma sample following peptide extraction (SepPak).

Std indicates the elution of synthetic GLP-2 (1-33) in buffer, cc indicates elution of cytochrome C (MW12 384). Dextran Blue (MW >2,000 000) elutes at Kav = 0 and Na 125 I at Kav = 1.

2.3.2 Optimisation of assay condition

a) Albumin concentration

Variation of bovine serum albumin (BSA) concentration in the assay buffer caused no difference in antibody displacement by peptide standard, resulting in overlapping standard curves.

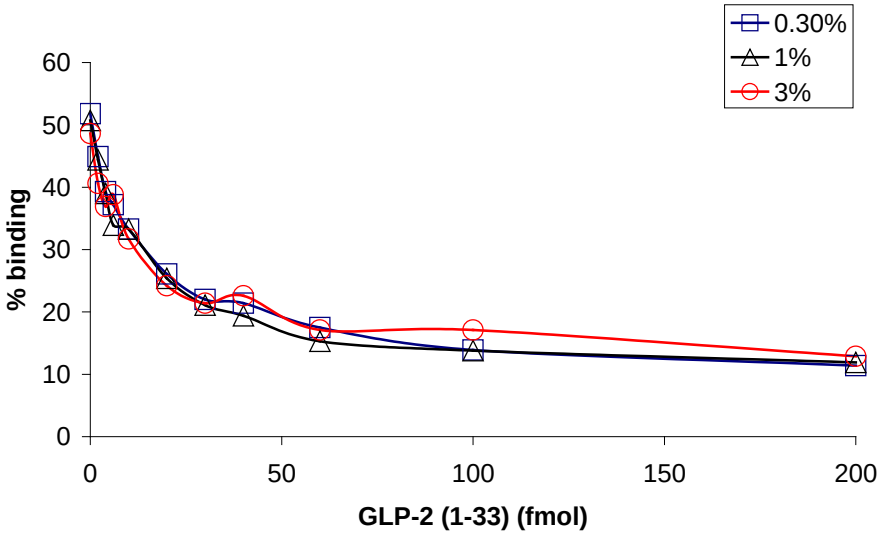


Figure 2-10: **Variation of the BSA concentration** (0.3%, 1%, 3%) in the buffer solution had no apparent impact on the binding characteristics of the GLP-2 assay, resulting in overlapping standard curves.

b) Separation method

Both, charcoal separation and immunoprecipitation with secondary antibody produced acceptable standard curves (Figure 2-11). There was a small difference in assay sensitivity (2fmol/tube in charcoal vs. 4fmol/tube in secondary antibody separation). This would result in a detection limit of 20 and 40pmol/l (on the basis of a 100µl sample).

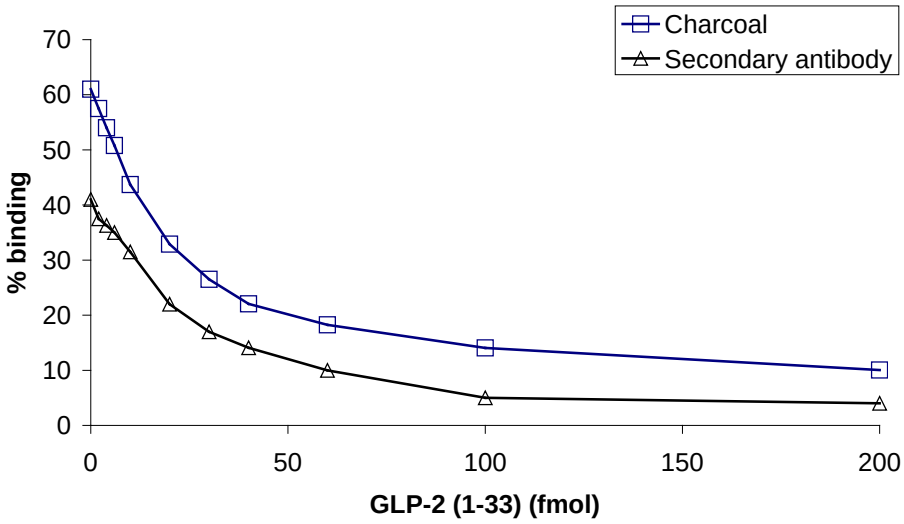


Figure 2-11: **Variation of separation method** (adsorption to charcoal versus immuno-precipitation with secondary antibody). Both methods achieve acceptable standard curves and assay sensitivity.

2.3.3 Optimization of sampling conditions

a) Protease inhibitor

Addition of PMSF to the collection tubes does not increase the measurements of GLP-2 IR in plasma samples. For most time points, no significant difference between measurements using Trasylol alone or in combination with PMSF was demonstrated. At 240 min, mean tGLP-2-IR was unexpectedly reduced in the Trasylol + PMSF samples (Table 2-2; Figure 2-12). This reduction was not significant in analysis of variance (ANOVA 0.31).

Timepoint (min)	Trasylol GLP-2 IR (pmol/l) mean (SEM)	Trasylol + PMSF GLP-2 IR (pmol/l) mean (SEM)	p
0	135 (18)	134 (14)	0.93
60	209 (37)	184 (33)	0.11
120	218 (43)	221 (42)	0.78
180	216 (34)	186 (26)	0.12
240	205 (29)	154 (25)	0.001

Table 2-2: Mean tGLP-2 IR at selected time-points. Lithium heparin tubes were prepared with Trasylol alone or Trasylol + PMSF. (n = 5 per time point), p (paired student T-test)

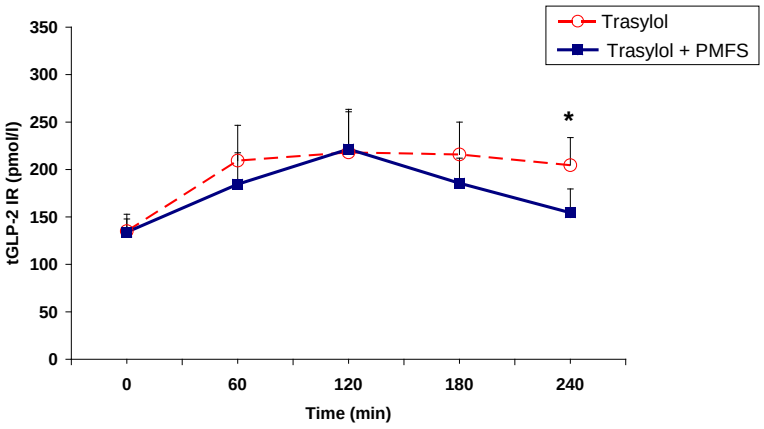


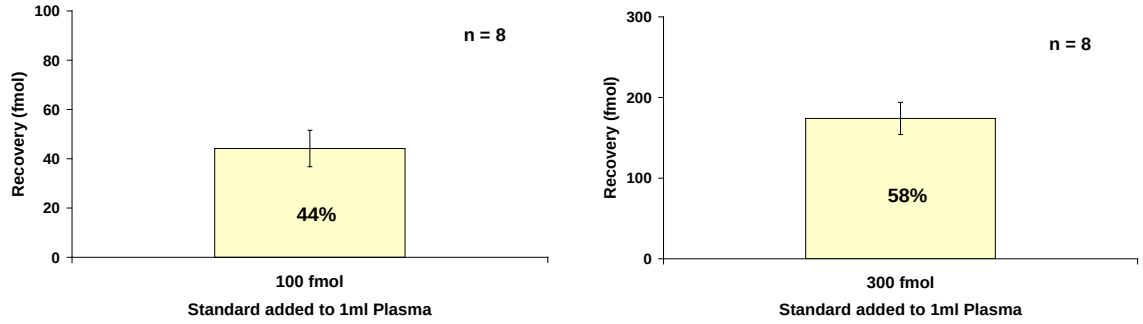
Figure 2-12: Mean tGLP-2-IR at selected pre- and post-prandial time-points. Lithium heparin tubes pre-prepared with Trasylol (broken line) or Trasylol + PMSF (cont. line). (n=5 per time point); * p = 0.001 (paired, student t-test); ANOVA: p=0.3.

b) Peptide extraction

Peptide recovery following SepPak extraction in plasma samples, spiked with synthetic GLP-2 standard, was 44% (on addition of 100fmol GLP-2, n=8) and 58% (on addition of 300fmol GLP-2, n=8) (Figure 2-13).

A significant increase of GLP-2-IR versus the fasting level could be demonstrated (t-test; $p \leq 0.05$) at all postprandial time points, equally in both non-extracted and SepPak concentrated samples (Figure 2-14). When values for concentrated samples were adjusted for approximately 50% reduced recovery, no significant difference could be shown between the samples (t-test; $p>0.05$ for all time points). Notably however, there was a substantial reduction in the GLP-2-IR SEM following solid-phase extraction; 27 vs. 13pmol/l; average SEM for all time points measured (paired t-test, $p<0.001$).

There was a trend towards a greater relative GLP-2-IR increase in SepPak extracted samples compared to unextracted samples (Figure 2-15), although this difference did not reach statistical significance for any time point (ANOVA; $p = 0.13$).



A **B**

Figure 2-13: Peptide recovery of after solid phase extraction with SepPak C18 cartridges, after spiking plasma with

A: 100fmol of synthetic GLP-2^{1-33} standard; n=8.
B: 300fmol of synthetic GLP-2^{1-33} standard; n=8.

Values are mean +/- SD

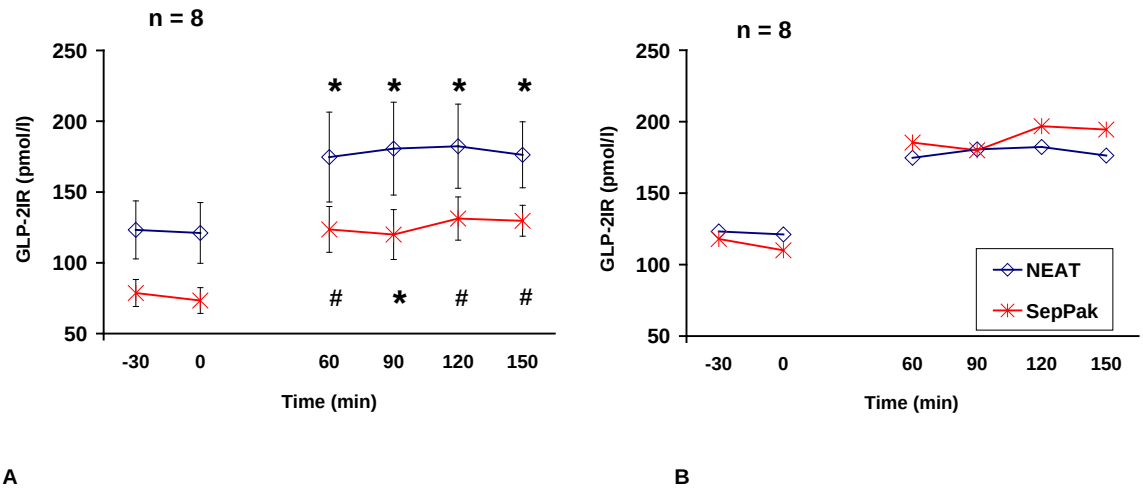


Figure 2-14: **Pre- and postprandial level of GLP-2 IR.**

A: Plasma samples were analysed unprepared and following peptide concentration in SepPak cartridges. (Mean+/-SEM) * p<0.05; # p<0.01 versus baseline (student T test)

B: Equivalent to diagram A, values for SepPak concentrated samples now adjusted for a 50% peptide loss of recovery (mean, error bars not included). No significant difference between the samples can be shown by paired student T test.

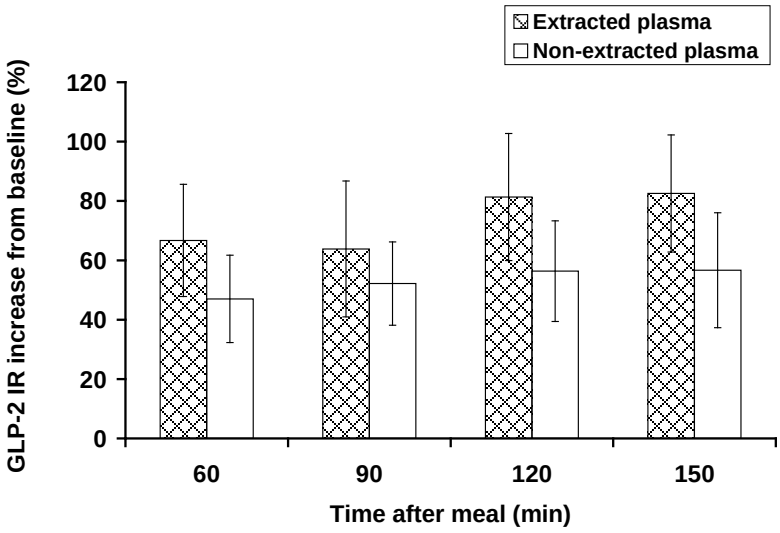


Figure 2-15: **Relative postprandial increase of tGLP-2-IR from baseline (%)**

measured at selected postprandial time points, following peptide concentration in SepPak cartridges **(hatched bars)** or in non-extracted plasma **(empty bars)**. MEAN +/- SEM. There is a trend towards a greater increase in extracted samples; however the difference is not significant for any time-point. ANOVA p=0.13

c) Freeze-thaw cycle

Up to 10 repeat cycles of defrosting and refreezing did not lead to significant alterations of the measured GLP-2-IR concentration in a random post-prandial plasma sample (single factor ANOVA p=0.68). The variation in the mean GLP-2-IR between all cycles was within the sample intra-assay variability.

Freeze-thaw cycles	Samples n	GLP-2IR Mean (pmol/l)	SD	SEM	CV
1-10	72	167.8	4.2	1.7	2.5
1	12	162.5	20.5	5.9	12.6
2	12	170.7	22.7	6.6	13.3
3	12	169.3	16.5	4.8	9.7
4	12	164.1	10.7	3.1	6.5
5	12	173.5	24.1	7.0	13.9
10	12	166.6	9.4	2.7	5.6

Table 2-3: **GLP-2-IR after cycles of plasma freezing and defrosting**

12 tubes were analysed for each freeze thaw cycle to determine intra-assay variability.

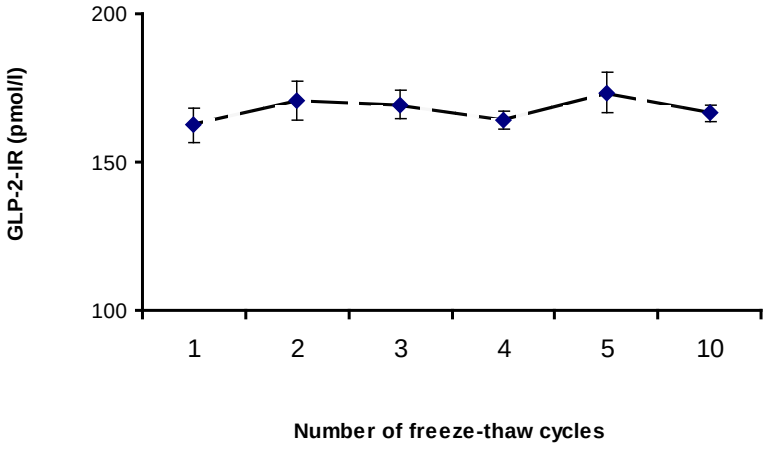


Figure 2-16: **GLP-2-IR after cycles of plasma freezing and defrosting**

12 tubes were analysed for each freeze-thaw cycle to determine intra-assay variability. Single factor Anova: p= 0.68

d) Haemolysis

The stepwise addition of haemolysed plasma to a random plasma sample led to a significant, dose dependent, increase of GLP-2-IR ($p<0.001$; Figure 2-17). Severe haemolysis lead to GLP-2-IR concentrations outside the steep part of the binding curve, resulting in increased variability of results (Table 2-3). Resultant GLP-2-IR concentrations fall outside the physiological range.

Binding curves, obtained by adding standard amounts of synthetic GLP-2 (1-33) to different concentrations of haemolysed plasma show that haemolysis significantly alters antibody binding to the peptide standard ($p<0.05$). A small degree of haemolysis (5% of total plasma volume) does not alter the standard curve. There is however considerable change with stronger haemolysis. This especially affects the steep part of the binding curve, resulting in a flat binding curve when 60% of the total plasma volume is haemolysed and thus causing overestimation of the GLP-2 concentration (Figure 2-18)

Factor	Heamolysis (%)	n	Mean (pmol/l)	SD	CV	p	vs. factor
1	0	10	151.7	26.9	17.7	<0.05	2, 3, 4
2	10	10	372.7	63.6	17.1	<0.05	1, 3, 4
3	50	10	1318.2	296.7	22.5	<0.05	1, 2
4	100	9	1142.7	288.5	25.3	<0.05	1, 2

Table 2-4: GLP-2-IR (mean+/-SD) in a random plasma sample is significantly altered with the stepwise increase of haemolysed plasma content.

In grossly haemolysed samples the coefficient of variation (CV) is increased. (p = Student-Newman-Keuls test (post hoc ANOVA analysis for pair-wise comparisons).

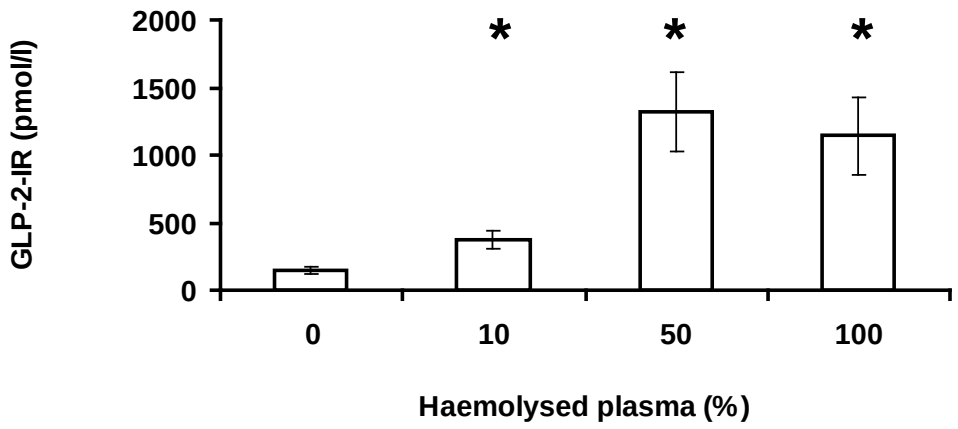
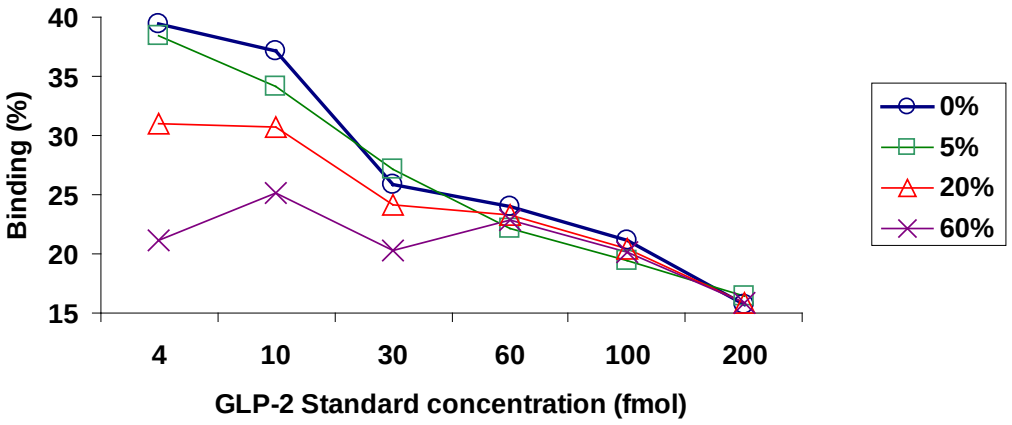


Figure 2-17: GLP-2-IR (mean +/-SD) of random plasma sample with stepwise addition of haemolysed plasma

* P<0.001 (vs. non-haemolysed plasma, student t-test), ANOVA p< 0.001



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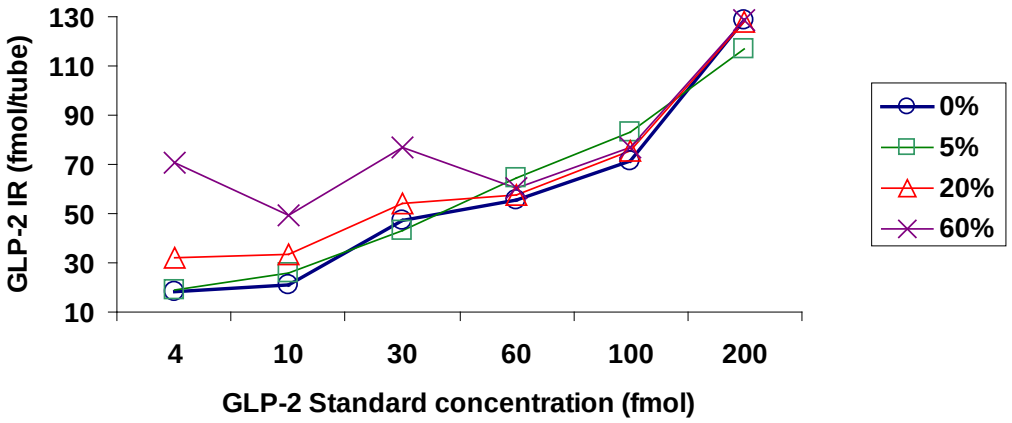


B

Figure 2-18: **A) FT-17 binding curves of GLP-2 (1-33) standard**, obtained in different concentrations of haemolysed plasma.

Plasma haemolysis needs to significant variation between the standard curves (ANOVA: $p<0.05$). Low level haemolysis (5% of total plasma volume) causes no significant change.

B) Estimation of GLP-2-IR.
The legend indicates the percentage of haemolysed plasma in the total plasma volume.

2.3.4 Quality control

a) Sensitivity

Assay sensitivity was determined as the smallest standard value, 2 SD below the mean of all zero standard values (95% CI). Assay sensitivity of 10 sequential RIAs is shown in Table 2-5. The mean sensitivity in these assays was 2.6 ± 0.9 fmol/tube, which equates to 26 ± 9 pmol/l for a 100 mcl sample.

Assay	Zero binding mean (%)	n	SD %	Sensitivity fmol/tube
1	54.2	45	2.3	2
2	54.3	29	1.2	2
3	59.8	26	2.1	2
4	56.7	20	1.3	2
5	60.4	18	2.0	2
6	51.5	50	2.6	2
7	57.2	16	2.0	2
8	53.4	38	2.4	4
9	52.6	22	2.1	4
10	55.6	18	1.9	4
Mean			**0.9**	**2.6**

Table 2-5: **Assay sensitivity (fmol/tube)**

Assay sensitivity was determined as the smallest standard value, 2 SD below the mean of all zero binding values (95% CI) shown for 10 sequential assays.

b) Reproducibility

The intra- and inter-assay coefficients of variation as calculated from repeat estimations of quality control samples are shown in Table 2-6. This was assessed in a random sample of fresh frozen plasma as well as in pooled, lyophilized plasma which had been spiked with GLP-2 (1-33) standard (such that 100 µl of plasma would read off the GLP-2 standard curve in the range of “high”, “medium” or “low”). The maximum intra-assay variation was 17.8%. The maximum inter-assay variation was 22%.

	Intra-assay variation					**Inter-assay variation**				
Plasma	**Frozen**	**Lyophilized**				**Frozen**	**Lyophilized**			
		Zero	Low	Medium	High		Zero	Low	Medium	High
Mean (fmol/tube)	11.3	13.9	16.4	34.4	72.9	13.1	11.6	14.5	28.9	62.6
SD	1	1.5	2.9	4.6	12.8	2.5	2.6	2.5	4.4	12.0
n	10	8	8	8	8	5	5	5	5	5
***CV (%)**	**8.8**	**11.1**	**17.8**	**13.5**	**17.6**	**19.1**	**22.0**	**17.3**	**15.4**	**19.1**

Table 2-6: **Reproducibility of RIA FT-17**

Intra- and inter-assay coefficients of variation were calculated from repeat estimations of quality control samples.

***CV= SD/mean*100**

The pre-and postprandial GLP-2-IR concentration of 8 healthy subjects was analysed at 10 time points over 270 minutes. Two RIAs (No 944 and No 950) were performed on all samples (n=80 samples per assay, analysed in duplicates).

Figure 2-19 shows a high correlation between the two RIA series (r = 0.91, r^2= 0.82; p<0.001). Sub-analysis of different time points in Table 2-7 demonstrated a highly significant positive correlation between the two sets of measurements at all time points. This result is comparable with the intra-assay correlation between the duplicates in each assay: r=0.91 (RIA 944) and r=0.94 (RIA 950).

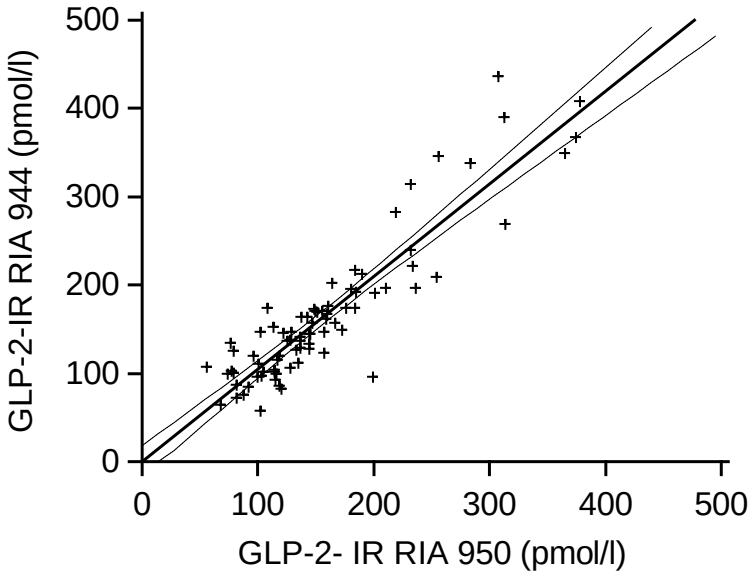


Figure 2-19: **Correlation of 2 consecutive RIAs**

Pre-and –postprandial GLP-2-IR concentration in 8 subjects over 270minutes, estimated in two sequential RIAs (“950” and “944”) (n=80 per assay).

A significant correlation can be demonstrated
(Pearson coefficient r=0.91, r^2 = 0.82, p<0.001).
Regression line and 95 % CI (dotted lines)

time	r	p
-30	0.91	0.002
0	0.97	<0.001
30	0.97	<0.001
60	0.98	<0.001
90	0.95	<0.001
120	0.93	<0.001
150	0.99	<0.001
180	0.98	<0.001
210	0.96	<0.001
240	0.98	<0.001

Table 2-7: **Correlation analysis of 2 consecutive RIAs**

Pre-and –postprandial GLP-2-IR concentration in 8 subjects over 270minutes, estimated in two sequential RIAs (“950” and “944”).

r = Pearson coefficient of variation.

c) Sample carryover

The average change of GLP-2-IR in low concentration plasma samples, analysed before and after high concentration plasma samples (spiked with 30fmol GLP-2 (1-33) standard), was <4%. This is well within the limits of intra-assay variation; indicating that there is no appreciable carryover effect when analysing adjacent high and low concentration samples.

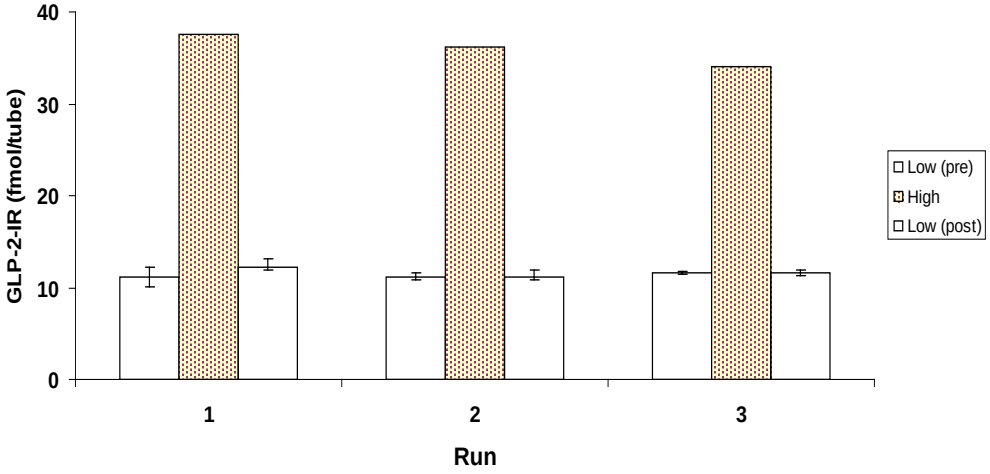


Figure 2-20: Sample carryover

Estimation of GLP-2-IR (mean ± SD) in low concentration plasma (2 fasting samples in duplicates; empty columns) before and after high concentration plasma (4 samples spiked with 30fmol GLP-2(1-33) standard; filled columns). 3 runs performed.

	GLP-2 (fmol/tube)		n	SD	Change %
	pre	post	pre/post	pre/post	
Run 1	11.2	12.2	2/2	1.0/0.9	8.82
Run 2	11.2	11.1	2/2	0.4/0.7	0.36
Run 3	11.6	11.6	2/2	0.2/0.3	0.41
Mean Change	**3.19%**				

Table 2-8: Sample carryover

% change of GLP-2-IR in low concentration plasma (2 fasting samples in duplicates) before and after high concentration plasma (4 samples spiked with 30fmol GLP-2 (1-33) standard). 3 runs performed.

2.3.5 Postprandial GLP-2-IR profile in healthy humans

Figure 2-22 depicts the concentration of GLP-2-IR in 8 healthy volunteers in the fasted state (-30 min; 0 min) and following (30-240 min) ingestion of a 920 kcal standardized meal.

Analysis of the two **fasting time points**, at -30 and 0, shows a small difference between the two times that was not statistically significant (p=0.80). As a result of this finding, the average value of the two fasting time points was taken for each subject (termed fasting or baseline value), and used in the subsequent analysis.

The mean fasting GLP-2-IR levels in 8 healthy individuals was 122.2pmol/l. There was considerable variation among subjects (Figure 2-21). It appeared that one individual had a particularly high fasting level (shown as an outlier). However, this subject had an appropriate postprandial increase of GLP-2-IR (65% increase from baseline at 90 minutes) and was therefore included in the analysis.

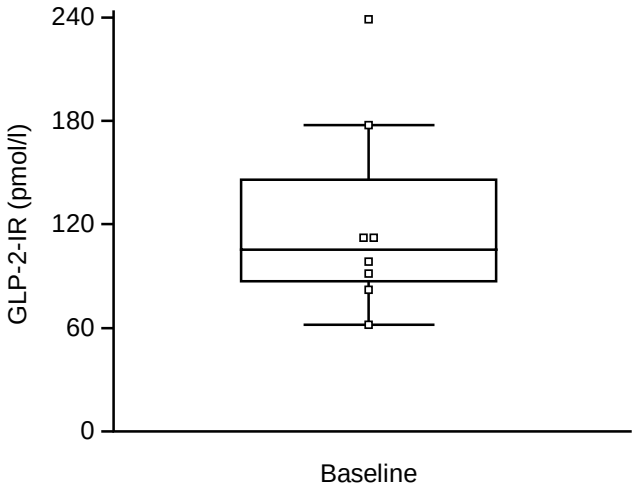


Figure 2-21: **Fasting GLP-2 IR** (average of time points -30 and 0), 8 healthy subjects.

Central box: 25 to 75 percentile; middle line: median; vertical line extends from the minimum to the maximum value, excluding an outlier (upper quartile plus 1.5 times the interquartile range).

A significant rise in mean **postprandial** GLP-2-IR was recorded at 30 minutes and levels remained significantly elevated at all postprandial time points until the end of the study period at 240 minutes (Figure 2-22, Table 2-9). The largest differences from the fasting times were observed from time 60 to time 180. When examining individual postprandial profiles, postprandial peaks were reached at different time-points: 90min (n=2); 120min (n=1); 150min (n=1); 180min (n=2); 240min (n=2).

Even though an increase from baseline was recorded in every subject, there were considerable differences in relative and absolute postprandial increase of GLP-2-IR between individual subjects. The area under the curve (AUC) was calculated for each subject and the increase of AUC from baseline determined.

Figure 2-23 shows the wide distribution of results. There was no correlation between the baseline GLP-2-IR and the magnitude postprandial AUC increase; Pearson correlation coefficient r=0.27, p=0.51 (Figure 2-23).

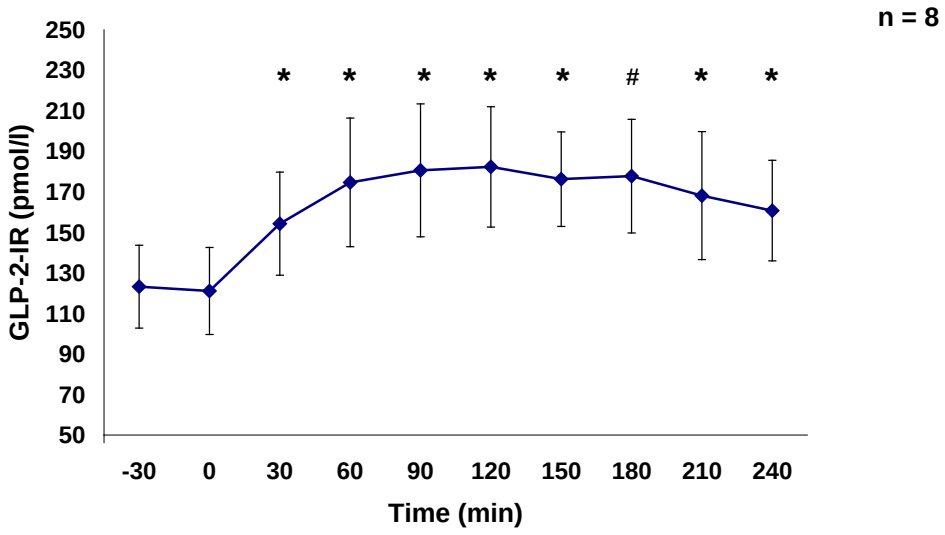


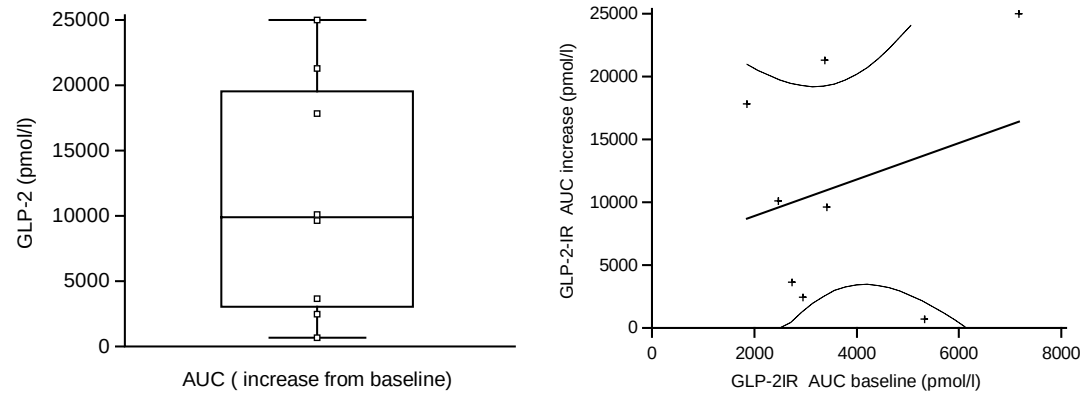
Figure 2-22: **Meal related GLP-2 IR (MEAN +/- SEM)**

8 healthy subjects were investigated before and after a 920cal standard meal. There is a significant increase in all post prandial time points versus the baseline GLP-2-IR concentration (average of time -30 and 0) * p <0.05 # p <0.01

Inrease from baseline			
Time	GLP-2-IR (pmol/l)	95% CI	P-value
30	32	4-60	0.03
60	53	11-93	0.02
90	58	16-100	0.01
120	60	20-101	0.01
150	54	17-91	0.01
180	56	21-90	0.007
210	46	8-84	0.02
240	38	6-76	0.03

Table 2-9: **Mean postprandial increase of GLP-2-IR over time.**

Difference between the average fasting values and each subsequent time point is calculated. Statistical analysis is performed using the paired t-test.



A **B**

Figure 2-23: **A)** Box-and-whisker plot of increase in area under the curve (AUC) from baseline AUC (-30 to 0) in 8 healthy subjects. (Central box: 25 to 75 percentile; middle line: median; vertical line: minimum to maximum value, excluding an outlier (upper quartile plus 1.5 times the interquartile range). It is shown that there is considerable variation between the subjects.

B) There is no significant correlation between the baseline level of GLP-2-IR and postprandial increase of the area under the curve (r=0.27, p=0.51). Shown are the linear regression line and the 95% confidence interval for the regression line (dotted lines).

2.3.6 Correlation to antiserum 92160

Plasma samples from 14 healthy subjects were collected in the fasted state and 60 and 120 minutes following a standard meal containing 866 calories. GLP-2-IR was determined in separate (first thaw) aliquots with RIA using antiserum FT-17 at our laboratory and antiserum 92160 at the University of Copenhagen.
(One subject was excluded from analysis as samples were incomplete).

Significant correlation could be shown between the two assays for all time points combined ($r=0.77$; $p<0.0001$; Figure 2-24).

On separate analysis of the time points however it became apparent that there is no correlation between the RIAs in the fasting levels ($r=0.18$; $p=0.5$). Correlation was high however between the postprandial GLP-2-IR readings (r was >0.8 for time points 60 and 120 as well as for the absolute increase of GLP-2 IR from fasting at 60 and 120 minutes ($p<0.001$) (Figure 2-25). The Pearson correlation coefficient (r) and coefficient of determination (r^2) for all time points are detailed in Table 2-10.

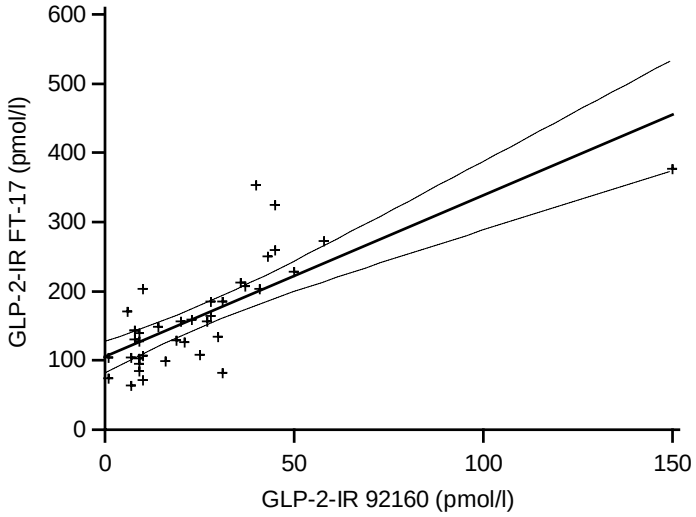


Figure 2-24: Correlation of GLP-2 IR using antiserum FT-17 and 92160

Scatter diagram of GLP-2-IR concentration measured during meal stimulation in 13 subjects with RIA using antiserum FT-17 and RIA using antiserum 92160 at time points 0, 60 and 120 (n=39).

There is significant correlation between the measurement methods; r^2=0.6.

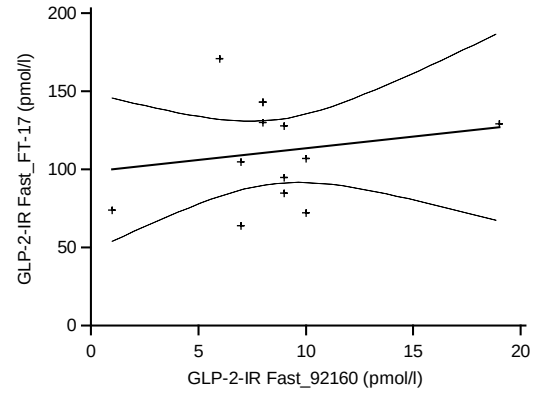
Shown are the linear regression line and the 95% confidence interval for the regression line (dotted lines).

Correlation RIA 92160 / RIA FT-17					
Timepoints		**n**	**r**	**r^2**	**p**
	0	13	0.30	0.09	0.55
	60	13	0.82	0.68	<0.001
	120	13	0.81	0.66	<0.001
PP	60+120	26	0.74	0.54	<0.0001
All	(0+60+120)	39	0.77	0.6	<0.0001
Increase		**n**	**r**	**r^2**	**p**
	60-0	13	0.87	0.75	<0.001
	120-0	13	0.85	0.72	<0.001
All	(60-0)+(120-0)	26	0.80	0.63	<0.0001

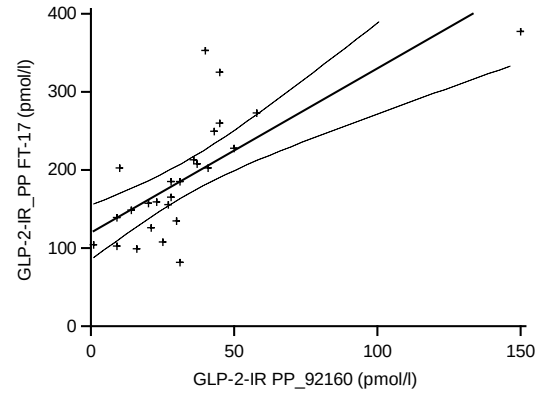
Table 2-10: Correlation of GLP-2 IR using antiserum FT-17 and 92160

Correlation of GLP-2-IR concentrations measured during meal stimulation in 13 subjects with RIA using antiserum FT-17 and 92160.

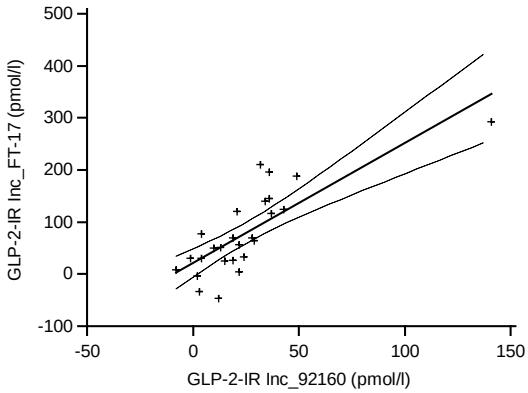
(PP=postprandial; significances given for correlation coefficient r)



A



B



C

Figure 2-25: Correlation of GLP-2 IR using antiserum FT-17 and 92160

Scatter diagram of GLP-2-IR concentration measured during meal stimulation in 13 subjects with RIA using antiserum FT-17 and RIA using antiserum 92160.

A) Fasting levels (0min; n=13; r= 0.3; P=0.55).

B) Postprandial levels (60min+120min; n=26; r=0.74; p<0.0001).

C) Increase of GLP-2-IR vs. baseline (60min+120min; n=26; r=0.8; p<0.0001).

Shown are the linear regression lines and the 95% confidence interval for the regression line (dotted lines).

2.3.7 Correlation to ELISA

A satisfactory GLP-2 standard curve was obtained with the commercial ELISA kit (Figure 2-26).

GLP-2 concentration was determined in plasma samples from 14 healthy subjects, collected in the fasted state and 60 and 120 minutes following a standard meal. All samples were analysed in duplicate. The coefficient of variation between duplicates was 9.2 ± 6.6%; (mean ± SD). Results were correlated with those obtained from the RIA FT-17 and RIA 92160.

Overall, there was significant correlation between postprandial GLP-2 immuno-reactivity measured with radioimmunoassay using **antiserum FT-17 and ELISA**. (r=0.8, p<0.001) (Figure 2-27). There was no positive correlation between the fasting levels (r=0.41, p=0.17). Correlation was significant between the postprandial GLP-2-IR readings as well as for the absolute increase of GLP-2 IR from fasting at 60 and 120 minutes (Table 2-11, Figure 2-28).

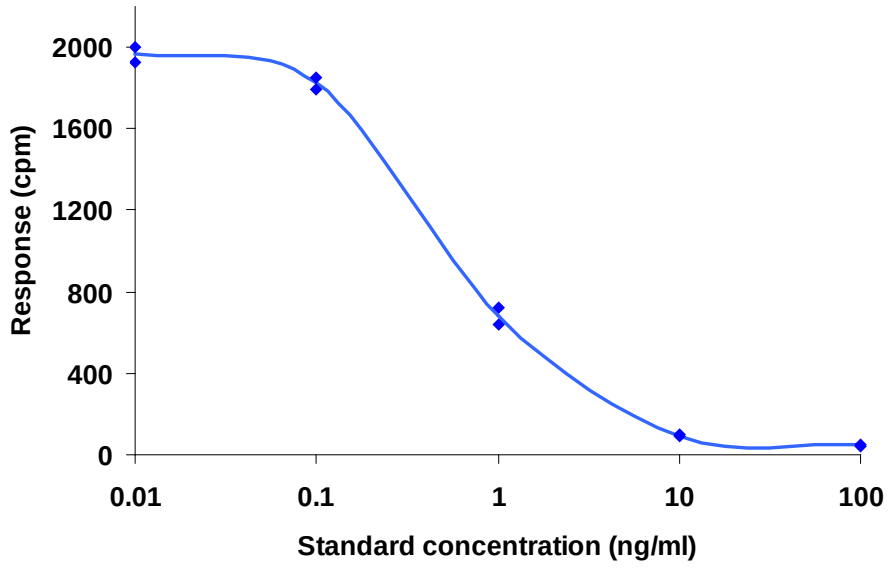


Figure 2-26: **GLP-2 ELISA standard curve**
cpm=count per minute

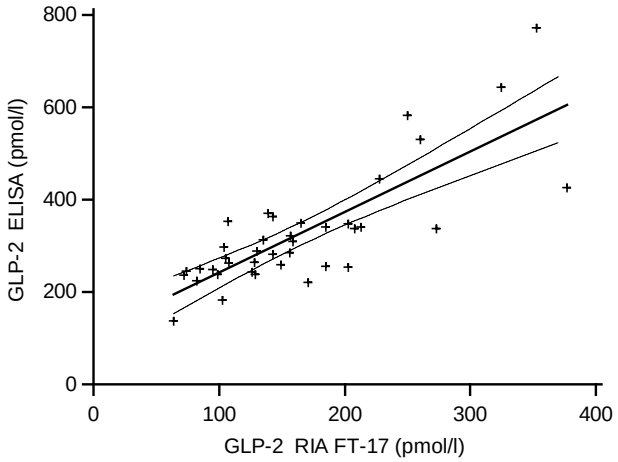


Figure 2-27: Correlation of GLP-2 IR using antiserum FT-17 and ELISA

Scatter diagram of GLP-2-IR concentration measured during meal stimulation in 13 subjects with RIA using antiserum FT-17 and ELISA at all time points (0, 60 and 120; n=39).

There is significant correlation between the measurement methods; r=0.8, r^2=0.6 (p<0.001).

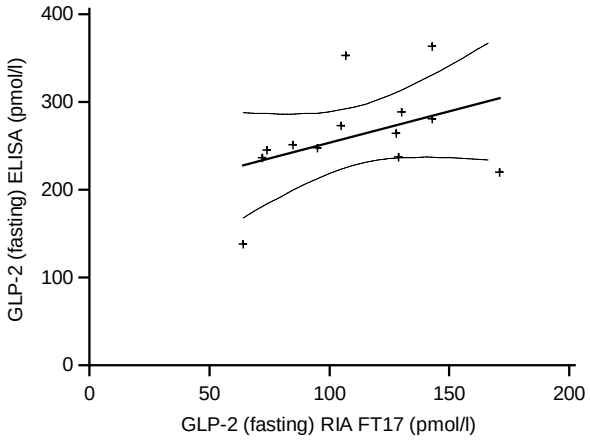
Shown are the linear regression line and the 95% confidence interval for the regression line (dotted lines).

Correlation RIA FT-17 / ELISA					
Timepoints		**n**	**r**	**r^2**	**p**
	0	13	0.41	0.16	0.17
	60	13	0.76	0.58	0.002
	120	13	0.85	0.72	0.0002
PP	60+120	26	0.80	0.64	<0.0001
All	(0+60+120)	39	0.80	0.64	<0.0001
Increase		**n**	**r**	**r^2**	**p**
	60-0	13	0.63	0.40	0.02
	120-0	13	0.72	0.52	0.006
All	(60-0)+(120-0)	26	0.66	0.44	0.0003

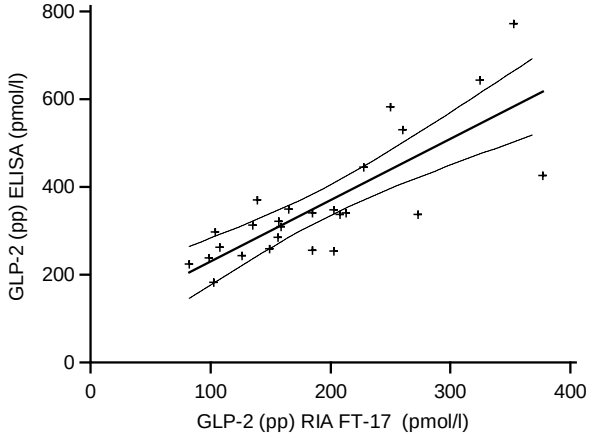
Table 2-11: Correlation of GLP-2 IR using antiserum FT-17 and ELISA

Correlation coefficient r and coefficient of determination r^2 of GLP-2 IR concentrations at separate time points.

(PP=postprandial; significances given for correlation coefficient r)



A



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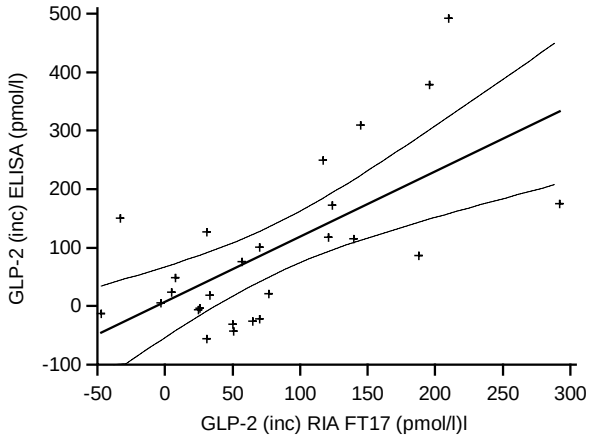


C

Figure 2-28: Correlation of GLP-2 IR using antiserum FT-17 and ELISA

A) Fasting levels (0min; n=13; r= 0.41; P=0.17).
B) Postprandial levels (60+120min; n=26; r=0.80; p<0.0001).
C) Increase of GLP-2-IR vs. baseline (60+120min; n=26; r=0.66; p=0.0003)

Shown are the linear regression lines and the 95% confidence interval for the regression line (dotted lines).

Correlation of the commercial ELISA with RIA (92160) was weak when all time points were combined (r=0.44; p=0.005). No significant correlation could be shown when analysing the fasting and postprandial levels alone or the postprandial increase of GLP-2 concentration (Table 2-12).

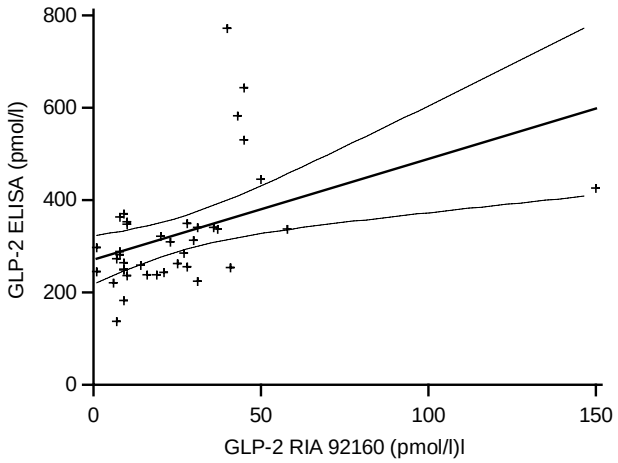


Figure 2-29: Correlation of GLP-2 IR using antiserum 92160 and ELISA

GLP-2-IR concentration measured during meal stimulation in 13 subjects with RIA using antiserum 92160 and ELISA at all time points 0, 60 and 120 (n=39). There is a weak correlation between the measurement methods; r^2=0.19. Shown are the linear regression line and the 95% confidence interval for the regression line (dotted lines).

Correlation RIA 92160 / ELISA					
Timepoints		**n**	**r**	**r^2**	**p**
	0	13	0.06	0.004	0.84
	60	13	0.49	0.24	0.09
	120	13	0.48	0.23	0.09
PP	60+120	26	0.34	0.12	0.08
All	(0+60+120)	39	0.44	0.19	**0.005**
Increase		**n**	**r**	**r^2**	**p**
	60-0	13	0.48	0.23	0.09
	120-0	13	0.48	0.23	0.09
All	(60-0)+(120-0)	26	0.36	0.13	0.007

Table 2-12: Correlation of GLP-2 IR using antiserum 92160 and ELISA

Correlation coefficient r and coefficient of determination r^2 of GLP-2 IR concentrations at separate time points.

(PP=postprandial; significances given for correlation coefficient r)

2.4 Discussion

GLP-2 secretion from intestinal L-cells has been acknowledged as a key process in the regulation of intestinal growth and adaptation. The ability to determine GLP-2 concentration accurately has not only furthered the understanding of intestinal physiology but has also paved the way towards exploration of therapeutic GLP-2 supplementation in a variety of intestinal conditions.

As detailed in section 1.1, intestinal GLP-2 is characterised by low plasma concentrations, a short half life and sequence homologies with its precursors, degradation products as well as other gut hormones. Measurement of this peptide therefore requires a method with a high degree of sensitivity and specificity. The radioimmunoassay, a standard technique in the quantification of gut hormones, can deliver these qualities. Availability of a specific GLP-2 antiserum is limited. In addition, commercially available RIA- and ELISA kits often do not provide sufficient information about the specificity of the antiserum used.

FT-17 is a polyclonal antiserum, raised in rabbits against synthetic human GLP-2 (1-33) coupled with BSA by carbodiiamide. It had been previously established that the antiserum has a high specificity for the detection of GLP-2 and does not cross-react with most related gut hormones. A negligible cross-reactivity with GLP-1 has been documented (Appendix 1). It was the aim of this study to characterise antiserum FT-17 specificity further and to optimise sampling and assay conditions and thus ensure maximal sensitivity before utilizing the assay for research into intestinal adaptation.

Cross-reactivity with the main GLP-2 degradation product, GLP-2 (3-33), has not been established previously. The chromatographic elution profile of synthetic GLP-2 (3-33) revealed GLP-2-IR at the point of GLP-2 (1-33) standard elution, confirming cross-reactivity. Quantitative RIA estimated 4% cross-reactivity with GLP-2 (3-33). It was postulated that FT-17 might also

cross-react with major proglucagon fragment (MPGF), a large proglucagon derived peptide (PG 72-158). MPGF is secreted as a by-product of glucagon synthesis in the pancreas and contains the entire GLP-2 (and GLP-1) sequence. Direct demonstration of cross-reactivity with MPGF is hampered by the fact that, due to its large size, a synthetic standard of this peptide is difficult to generate. As a surrogate marker, pancreatic and intestinal tissue extracts were examined. Pancreatic extract is known to contain large amounts of MPGF but only minimal GLP-2, whereas intestinal extracts predominantly contain GLP-2 (Orskov et al. 1987; Hartmann *et al.* 2000). In agreement with these studies, GLP-2-IR elution profile of rodent **intestinal tissue extract**, shown in this work, document an isolated peak at the elution point of GLP-2 (1-33) standard. The GLP-2-IR elution profile of **pancreatic extract** on the other hand, suggests cross-reaction of the antiserum with other substances of larger and smaller molecular weight. There were two major peaks (at K_{av} 0.15 and at 0.56-0.63) with no obvious peak at the elution point of GLP-2 (K_{av} 0.48). This indicates that FT-17 cross-reacts with other pancreatic proglucagon-derived peptides. It is possible that the elution fractions around K_{av} 0.15 contain MPGF. It is of note however that cytochrome C (MW 12 384 daltons) elutes at a later K_{av} (0.26). The estimated molecular weight of MPGF has been reported as 10 000 daltons (Patzelt et al. 1984). MPGF should therefore elute after cytochrome C, unless earlier elution is caused by reduced size due to protein folding. It is feasible that the antiserum cross-reacts with other large molecular weight constituents of the pancreatic extract. To elucidate this matter further, either synthetic MPGF would need to be tested on the column or the eluent of pancreatic extract should be analysed with a specific MPGF antiserum. The nature of the molecules eluting at K_{av} 0.56-63 also requires further exploration. Pancreatic extract contains glucagon which has been shown to elute after GLP-2 (Hartmann *et al.* 2000). Previous data (Appendix 1, Prof. Ghatei) however have shown no cross-reactivity of FT-17 with glucagon at concentrations between 1-100 pmol/tube. If cross-reactivity with glucagon exists at higher concentrations it is unlikely to be relevant when measuring GLP-2 concentration in plasma under physiological conditions.

Plasma profiles obtained by gel chromatography were investigated by Orskov et al. (Orskov *et al.* 1987). They concluded that: “the predominating glucagon-like peptide in the fasting state is a large peptide which is at least GLP-1 immuno-reactive”. After a meal however, a large proportion of GLP-1-immuno-reactivity eluted at the position of synthetic GLP-1, suggesting that the peptide released in response to a mixed meal corresponds to the intestinal GLP-1. Due to lack of a sensitive assay at the time of publication, this was not replicated for GLP-2. However it is now known that GLP-1 and GLP-2 are co-secreted in response to an appropriate nutritional stimulus (section 1.1.4). Chromatographic elution profiles of fasting and postprandial plasma (Figure 2-8), obtained for this study, are in agreement with Orskov’s findings. In the **fasting** sample, the GLP-2-IR mainly consisted of larger molecular weight material with only a very small peak corresponding to the elution of GLP-2 (1-33) standard. The peak of larger molecular weight material elutes after cytochrome C and is likely to contain MPGF. In the **postprandial** plasma, overall GLP-2-IR was increased. There was now a discernable peak, corresponding to the elution point of GLP-2 standard, comprising 25% of total GLP-2-IR.

Documentation of a clear response to a physiological stimulus is an important sign of a valid RIA assay. Measurement of plasma samples with antiserum FT-17 demonstrated a significant increase in GLP-2-IR in response to a high caloric, lipid rich, semi-liquid test-meal in healthy volunteers.

In the literature, **fasting** GLP-2 (1-33) levels in adults, measured using N-terminal specific antibody 92160, are reported in the region of 10-30 pmol/l (Hartmann *et al.* 2000; Jeppesen *et al.* 2000; Sorensen et al. 2003; Schmidt *et al.* 2005). In comparison, fasting levels of **total** GLP-2 immunoreactivity, measured with side viewing antibodies, are reported between 100 - 230 pmol/l (Brubaker *et al.* 1997; Xiao *et al.* 1999). This is in keeping with fasting GLP-2-IR concentrations measured here and supports the conclusion that the utilized GLP-2 antibody is a “side viewing antibody”, measuring total GLP-2-IR.

There was considerable variation in the amount of fasting GLP-2-IR levels between the subjects and the fasting GLP-2-IR was not correlated to the individual post-prandial GLP-2 response. One subject showed a particularly high fasting level, but still had an appropriate postprandial GLP-2 increase. All subjects were asked to undergo a minimum 9 hour fasting period. It is possible that there were differences in the timing of meals prior to the fasting period and/or differences in gastric emptying and small bowel transit. However, given the fact that GLP-2 (1-33) only accounts for minimal proportion of total GLP-2-IR in fasting plasma, it is more likely that the variation in fasting total GP-2IR levels is caused by variation in other circulating proglucagon derived peptides. This conclusion is also strengthened by the finding of a poor correlation between measurement of fasting total GLP-2-IR with antiserum FT-17 and fasting GLP-2 (1-33) with antiserum 92160. It implies that a comparison of fasting GLP-2-IR levels between subjects, measured with antiserum FT-17, can't reliably detect differences in GLP-2 (1-33). It is theoretically possible to separate the fasting samples with Sephadex Chromatography and quantify changes in the GLP-2-IR peak that elutes at the point of GLP-2 standard. This approach is very laborious and unlikely to be utilized in routine analysis. In addition, given the small concentration of GLP-2 (1-33) in fasting plasma and the peptide loss due to the procedure, a large amount of sample would be required.

The utilized test meal had high lipid content and caused significant **postprandial** increase of GLP-2-IR at all measured time points (30-240 min post meal). A rise of GLP-2 IR following a lipid meal (35% whipping cream) has been demonstrated by (Hartmann *et al.* 2000). In their study, fat induced a biphasic tGLP-2-IR release with peaks at 45 and 90 minutes. In the study presented here, the greatest increase occurred between 60 and 180 minutes. The maximum postprandial tGLP-2-IR increase of 69.7% is comparable with results published by Hartman et al., who report a 69% and 96% increase at 45 and 90 minutes respectively.

We have shown a strong correlation between the measurements of postprandial total GLP-2-IR, (measured with antiserum FT-17) and GLP-2 (1-

33) (measured with antiserum 92160). A strong correlation could also be shown for the estimation of tGLP-2-IR/GLP-2(1-33) increase from baseline. This indicates that the post-prandial increase of tGLP-2-IR is a good measure of GLP-2 (1-33) secretion.

Total GLP-2-IR might also be altered by non-specific interaction of the GLP-2 antibody with other plasma components. It is shown in section 2.3.1 that peptide extraction of postprandial plasma through Sep Pak cartridges can lead to a reduction of large molecular weight material and therefore to a relative increase of GLP-2 (1-33) within the total GLP-2-IR. This method of peptide extraction however is very time and labour-intensive and may introduce additional sources of error. Even though extraction was performed according to protocol, peptide recovery was lower (44% and 58%) compared to other reports: 84 ±17%. (Xiao et al. 2000). Different reagents were used to acidify the sample prior to passage trough the cartridge (trifluoroacetic acid vs. hydrochloric acid) and for peptide elution (isopropanolol vs. methanol), which might account for some of the difference. Other, methods of GLP-2 extraction have been described, e.g. the use of 96% ethanol (Hartmann *et al.* 2000) which is not as labour intensive. However reported peptide recovery was only 68%.

When examining the GLP-2-IR response to nutrient stimulation, the expected post-prandial rise of GLP-2-IR could be demonstrated in unextracted plasma as well as after peptide extraction. There was a trend towards a greater relative increase of GLP-2-IR in extracted plasma samples, although no significant difference could be shown. A greater postprandial increase could indicate that the specificity of the assay to detect postprandial increase of GLP-2 is enhanced through removal of interferences that are not nutrient dependent and that therefore any changes in tGLP-2-IR are a more accurate reflection of postprandial increase in active GLP-2(1-33). A confounding factor however is the finding that in control samples (using different concentrations of synthetic GLP-2 standard) higher GLP-2 levels before extraction have led to a higher peptide yield.

The mean sensitivity of the GLP-2-RIAs performed was 2.6 ± 0.9 fmol/tube. This is equal to published results of side viewing antiserum UTTH7 where sensitivity was quoted as 10pg/tube which equates to 2.6 fmol/tube (Xiao *et al.* 1999). The intra-assay variation of freshly frozen quality control plasma samples was 8.8 %, the inter-assay variation 19.2%. It is generally recognized that the inter-assay variation between RIAs can be very high and comparison between assays is therefore not usually undertaken. An intra-assay variation of <10% appears acceptable, although better performance of other assays has been reported (3.2% (Xiao *et al.* 1999)). It is unclear why the lyophilized control samples showed more inconsistency with a maximum intra-assay variation of 17.8%. It is possible that the generation of these samples (dilution of standard, separation into aliquots, freeze-drying and reconstitution) has introduced additional error. A high level of correlation was found (r=0.91) when 2 consecutive complete assays of the same samples were compared.

Assay conditions depend upon the characteristics of a given antibody and antigen. Antibody-antigen interactions might be influenced by a variety of factors like type and pH of the buffer solution, incubation time and temperature. It has previously been established that for antibody FT-17, the use of phosphate buffer (pH 7.4) and an incubation time of 3 days achieve optimum binding conditions. Two additional factors that might alter assay sensitivity, buffer albumin content and separation method were investigated. The present results indicate that variation of albumin in the buffer solution (ranging from 0.3-3%) had very little impact on assay performance. In subsequent assays, buffer BSA content was therefore maintained at 0.3%, as previously used. Similar analytical sensitivity was achieved when separation of antibody-antigen complexes from free antigen through adsorption to charcoal was compared to immuno-precipitation with a secondary antibody. As charcoal separation is readily available, rapid, and inexpensive, it was utilized for all subsequent assays.

Usage of a specific serine protease inhibitor (PMSF) in addition to Trasylol, used in the described manner, did not increase the diagnostic yield when

measuring GLP-2-IR. Assuming that a certain amount of GLP-2 undergoes degradation during sample processing and measurement, the most likely explanation for an apparent lack of protection through PMSF would be that both full length peptide as well as peptide degradation products are equally detected by the antibody used. It is unclear why measurements of tGLP-2-IR are lower at 240 minutes but overall it would seem that this finding is likely to be random. In the current experimental set up, enzyme activity should be minimal. A non-specific enzyme inhibitor (Trasylol) continued to be used routinely in all collection tubes. Samples are transported on ice and spun down immediately in a refrigerated centrifuge, stored at -70 °C and, after thawing, processed on cold trays or in the cold room at 4°C. It is likely that these precautions alone are adequate measures of minimizing peptide degradation.

The impact of haemolysis on the binding characteristics of FT-17 was examined by diluting severely haemolysed plasma with non haemolysed plasma obtained at the same venepuncture. Mild haemolysis (5% of total volume) had no significant impact on the binding curve of GLP-2 standard. Severe haemolysis however, leads to over-estimation of GLP-2 concentration and increased variability of results. Visibly haemolysed samples should probably be excluded from analysis.

In order to protect samples from multiple freeze-thaw cycles and thus enhance their longevity, it is usual practice to separate plasma into several aliquots prior to storage. However, if the total sample volume is limited, samples may undergo repeat freeze-thawing. It is shown here that repeat freeze-thawing of a postprandial plasma sample does not lead to significant changes of GLP-2-IR. As detailed above, GLP-2 (1-33) only accounts for a certain proportion of total GLP-2-IR. To gain more specific insight into the effect of repeat freeze-thawing on GLP-2 (1-33), assessment of the increase of GLP-2-IR from fasting levels might be a more sensitive marker of GLP-2 (1-33) in the samples. In the meantime, the safest policy is to continue

freezing samples in small aliquots and to use first defrost samples if possible.

Carry over was assessed, because during the GLP-2 RIA, a single glass pipette is used for the entire assay to separate the bound and unbound fractions. This practice does not appear to lead to significant error if high and low concentration samples are measured in succession.

GLP-2-ELISA has been shown in the literature to be a very suitable alternative to RIA in the measurement of GLP-2. Previous published results by (Martin *et al.* 2005), using an ELISA kit that is not commercially available (anti-ALE-0303 antiserum, raised against the first 18 residues of native human GLP-2, NPS pharmaceuticals) have shown a strong correlation to RIA using antiserum 92160 (r^2 = 0.95%; confidence interval of 0.944 to 0.985; n = 36). The results of a different ELISA kit (Phoenix Pharmaceuticals, Inc) presented here appear less favourable with no correlation to RIA (92160). Due to financial constraints, only a single ELISA kit could be tested. Samples were analysed in duplicate with an acceptable level of variation (9.2%). However a repeat correlation with antiserum 91260 should be attempted before reaching final conclusions about the utility of this ELISA kit. Only limited information was available with regards to antibody specificity with no data indicating cross-reactivity with GLP-2 (3-33) or MPGF. This emphasises the need to validate any analytical method against the current gold standard in order to interpret results accurately.

Summary: It has been shown that a radioimmunoassay using antiserum FT-17 provides a sensitive and specific method for the measurement of total GLP-2 immuno-reactivity in plasma samples and tissue extracts. FT-17 is not specific for the detection of biologically active GLP-2 (1-33), but belongs to the category of “side viewing antibodies” that cross-react with GLP-2 precursors and degradation products. It has to be taken into consideration that the concentration of active GLP-2 (1-33) in the fasted state is low in relation to the total GLP-2 immuno-reactivity. The concentration of fasting total GLP-2 immuno-reactivity is subject to considerable variation and

correlates only poorly to GLP-2 (1-33) concentration. When using antiserum FT-17, estimation of biologically-active GLP-2 (1-33) secretion is therefore probably most precise when the dynamic GLP-2 response to a physiological stimulus is measured.

Chapter 3

GLP-2 secretion as part of the L-cell response in short bowel syndrome

3.1 Introduction and aim

Enteroendocrine L-cells are part of the diffuse endocrine system of the gut, originating from endodermal stem cells (Hocker et al. 1998). The majority of L-cells are located in the distal small bowel, colon and rectum, where they are scattered amongst endothelial cells in the crypts of Lieberkühn (Eissele *et al.* 1992). Recently it was established that L-cells are also present in the human duodenum (Theodorakis *et al.* 2006).

Peptide tyrosine tyrosine (PYY) and proglucagon derived peptides (PGDP) are the main products of L-cell secretion. Proglucagon undergoes posttranslational processing into glucagon-like peptides-1 and -2 (GLP-1 and GLP-2) as well as Glicentin and Oxyntomodulin. The latter two both contain the entire pancreatic glucagon sequence and were previously identified as enteroglucagon.

Over the last two decades much progress has been made towards delineating the diverse biological activity of these peptides and it has become evident that they possess therapeutic potential for a variety of clinical conditions. As an incretin hormone, GLP-1 enhances insulin secretion, whilst inhibiting glucagon release and has been shown to improve glycaemic control in animal models of type-2 diabetes as well as in human clinical studies (Todd et al. 2007). Reduction in food intake has been demonstrated following systemic administration of PYY (Batterham et al. 2003), GLP-1 (Verdich et al. 2001) and Oxyntomodulin (Wynne et al. 2005) making these peptides possible targets for the treatment of obesity. Enhancement of L-cell secretion also appears advantageous in a variety of intestinal conditions. PYY and GLP-1 are potent inhibitors of gastro-intestinal motility and secretion (Savage et al. 1987; Wettergren et al. 1993) and thus might be developed into novel anti-diarrhoeal drugs. GLP-2 has received much attention for its specific effects on intestinal mucosal proliferation and repair (Drucker *et al.* 1996; Bulut et al. 2004). Early clinical trials have suggested positive treatment effects in patients with short bowel syndrome

(Jeppesen *et al.* 2001; Jeppesen *et al.* 2005) and Crohn's disease (Buchman et al. 2006).

The morphology of L-cells as open type cells with long projections toward the intestinal lumen suggests that these cells are sensing luminal contents (Theodorakis *et al.* 2006). In keeping with this, ingestion of nutrients, particularly of carbohydrates and lipids, constitutes a strong, secretory stimulus for PYY and PGDPs. It remains largely unexplored how the release of these peptides is coordinated, to what extend their production and secretion is mediated by the same stimuli or can be driven by separate signals and how changes of intestinal anatomy or biochemical environment impact on this balance.

In animals subjected to massive small bowel resection, the remnant intestine undergoes a patterned response known as intestinal adaptation. Well-documented indices of this adaptive response include structural (increases in intestinal crypt depth and villus height) and functional (increases in digestive and absorptive capacity per unit intestinal length) changes. The significance of intestinal adaptation is that its net effect is to increase the absorptive efficiency and capacity of the remnant intestine in animals subjected to intestinal resection. However, there is a paucity of evidence documenting the significance of this phenomenon in humans (Tavakkolizadeh et al. 2002).

Animal models of massive small intestinal resection have demonstrated an adaptive increase of both PGDPs and PYY if the L-cell reservoir in the distal bowel is preserved (Fuller et al. 1993; Bilchik et al. 1995; Adrian et al. 1996). Fuller and colleagues have shown a parallel increase of PYY and proglucagon mRNA in the ileal mucosa following massive small bowel resection in rats, suggesting that the increased synthetic activity is an L-cell specific response (Fuller *et al.* 1993). In a similar model however, Bilchik and colleagues demonstrated differences between concentrations of PYY and proglucagon mRNA in different parts of the intestine and came to the conclusion that the response to intestinal resection might be gene specific (Bilchik *et al.* 1995).

In clinical practice, patients with short bowel syndrome following extensive or repeated intestinal resections, divide into two common groups: those with a jejunostomy and those with jejunum in continuity with the colon. The two patient groups greatly differ in their requirements of nutritional support. Whereas both groups have problems with nutrient absorption, jejunostomy patients have additional problems of maintaining their fluid and electrolyte balance. It is thought that differences in gut hormone secretion may contribute to variations in gastric emptying rate and gastrointestinal secretions between short bowel patients with and without a colon (Nightingale et al. 1996).

Different aspects of L-cell secretion in this patient population have previously been studied. Nightingale and colleagues have shown low fasting and postprandial PYY values in short bowel patients without a colon and high values in short bowel patients with a retained colon (Nightingale et al. 1996). Jeppesen and colleagues have found impaired postprandial GLP-2 secretion in patients without a colon (Jeppesen *et al.* 1999) and elevated fasting and postprandial plasma levels of GLP-1 and GLP-2 in patients with a retained colon (Jeppesen *et al.* 2000). Although these results suggest parallel changes of PYY and PGDP secretion, it needs to be considered that these results were derived from different patient populations. If the mucosal response to injury leads to a general reduction or enhancement of L-cell mass or function, a positive correlation between PYY and PGDP secretion could be assumed. If however, as suggested by Bilchik's study, the response to intestinal resection is gene specific, the secretory response between PGDPs and PYY might deviate.

In order to investigate changes in L-cell secretory response following extensive intestinal resection in humans, we measured fasting and postprandial plasma levels of PYY, GLP-1 and GLP-2 in patients with short bowel syndrome and healthy controls.

3.2 Methods

3.2.1 Subjects

The study was performed according to the principles of the Declaration of Helsinki and approved by the Harrow research ethics committee (REC reference 04/Q0405/83). All participants gave written consent to take part in the study after receiving oral and written information. All samples were collected at St Mark's hospital between 2005 and 2006. All of the work, including experimental design, subject recruitment, sample collection, sample analysis and data interpretation was carried out by the author. Three subject groups were investigated.

HC: Healthy controls. These subjects, mainly hospital staff, had no previous history of intestinal surgery or other significant co-morbidity. This group included 14 subjects (8 male, 6 female) with a median age of 40 years (range 31-54 years) and a median BMI of 26 (range 24-29).

SBS-1: Short bowel patients with **no terminal ileum or colon in continuity**. This group included 16 subjects (4 male, 12 female) with a median age of 52 years (45-58) and a median BMI of 22 (19-25). Underlying diagnosis leading development of SBS was: Crohn's disease (n=8); mesenteric infarct (n=5); volvulus (n=2); colorectal cancer (n=1). Five patients had a jejunostomy and no remaining colon. Three patients had a jejunostomy and an unconnected rectal stump. A further 8 patients had either the whole or part or their colon preserved but this was not in continuity. The median small bowel length was 100 cm (68-100). Eight patients (subgroup SBS-1-adapted) were clinically stable patients who had undergone their last small bowel resection more than two years prior to participation in the study. It can be assumed that maximum intestinal adaptation has taken place in this group (Goodlad et al. 2001). The other 8 patients (SBS-1-non-adapted) were acutely hospitalized patients who had undergone their last small bowel resection less than one year prior to the study (usually at the same admission). All but one patient

(subject 05) was in need of parenteral nutritional support. Patient details are listed in Table 3-1.

SBS-2: Short bowel patients **with terminal ileum and/or colon in continuity**. This group included 11 patients (4 male, 7 female) with a median age of 50 years (44-62) and a median BMI of 21 (21-23). Underlying diagnosis leading development of SBS was: mesenteric infarct (n=10); volvulus (n=1). These patients had either all (n=4) or part of the colon (n=7) preserved and this was in continuity with the small bowel. Median small bowel length was 40 (18-60) cm with 80 % (65-100) of colon in continuity. This group consisted of 5 stable (SBS-2-adapted) and 6 acute (SBS-2-non-adapted) patients as described above. Patient details are listed in Table 3-1.

The small bowel length was measured at the time of the last surgical resection in 21 cases and assessed radiologically with a barium follow through in 5 patients. The small bowel length could not be quantified in one subject (SBS-1-11). The operation notes stated that "proximal jejunum was brought out as jejunostomy". Colonic length was estimated as sum of: caecum + ascending colon = 25%; transverse colon = 25%; descending colon = 20%, sigmoid colon = 20% and rectum = 10%. Subjects were continued on their regular medications which included standard treatment for SBS: antidiarrhoeal medications (loperamide and codeine), antisecretory drugs (proton pump inhibitors). Only one patient (SBS-1-10) received twice daily octreotide injections. The morning dose was withheld until after the collection of blood. One subject was included in both groups, before and after restoration of continuity between jejunum and colon (SBS-1-10 = SBS-2-27).

The basic metabolic rate (BMR) of all subjects was calculated using the Harris Benedict equation (Harris et al. 1918): BMR (Men) = 66.4730 + (13.7516 × Weight) + (5.0033 × Height)− (6.7550 × A ge); BMR (Women) = 655.0955 + (9.5634 × Weight) + (1.8496 × Height) − (4.6756 × Age)

SBS-1: no colon in continuity

ID	Age/Sex	BMI	D	Small bowel (cm)	Colon (%)	Stoma	Out of stream	Last resection (months)	PN
01	36M	23.2	CD	100	0	jejunostomy	——	180	yes
02	47F	21.5	CD	60	0	jejunostomy	——	180	yes
03	54F	18.6	CD	40	0	jejunostomy	——	48	yes
04	54F	21.8	CD	80	0	jejunostomy	——	156	yes
05	58M	26.0	CD	100	10	jejunostomy	rectal stump	72	no
06	58F	18.7	CD	110	10	jejunostomy	rectal stump	36	yes
07	59F	19.8	CD	80	10	jejunostomy	rectal stump	216	yes
08	59F	26.4	MI	75	75	jejunostomy	colon	60	yes
09	52F	18.2	Ca	100	0	jejunostomy	——	3	yes
10*	24M	19.6	V	110	95	jejunostomy	colon	2	yes
11	42F	37.6	MI		100	jejunostomy	jejunum, ileum, colon	2	yes
12	33F	24.6	MI	25	65	jejunostomy	colon	3	yes
13	52F	30.0	MI	100	75	jejunostomy	colon	3	yes
14	52F	18.1	CD	0	75	duodenostomy	colon	1	yes
15	46M	24.4	V	120	100	jejunostomy	ileum, colon	9	yes
16	70F	19.1	MI	100	80	jejunostomy	ileum, colon	2	yes

SBS-2: colon in continuity

ID	Age/Sex	BMI	D	Small bowel (cm)	Colon (%)	Stoma	Anastomosis	Last resection (months)	PN
17	59F	22.2	MI	0	55	none	duodeno-colonic	72	yes
18	40F	23.5	MI	29	35	colostomy	jejuno-colonic	48	yes
19	62F	21.2	MI	0	100	none	duodeno-caecal	276	yes
20	47F	23.4	MI	20	100	none	duodeno-ileal	72	yes
21	62F	20.9	MI	50	80	none	jejuno-colonic	144	yes
22	68F	17.9	MI	45	100	none	jejuno-caecal	2	yes
23	63M	21.0	MI	120	65	none	jejuno-colonic	6	yes
24	48M	27.9	MI	70	100	none	gastro-ileal	4	yes
25	41M	22.9	MI	15	75	colostomy	jejuno-colonic	8	yes
26	50F	21.2	MI	40	65	colostomy	jejuno-colonic	1	yes
27*	24M	18.3	V	110	95	none	jejuno-caecal	1	yes

Table 3-1: **Patient details**

D: Diagnosis, CD: Crohn's disease, MI: Mesenteric infarct, V: Volvulus
PN: Parenteral nutrition

***** subject SBS-1-10 = SBS-2-27 (before and after restoration of bowel continuity)

(Small bowel length of 0cm indicates: duodenum only)

3.2.2 Test meal

A calorie-dense, lipid-rich, standardized test meal was chosen in order to induce adequate and reproducible postprandial GLP-2 secretion. Palatability and ease of consumption were other important considerations. These criteria were met by using a combination of liquid nutritional supplements. One serving of Calshake® (a gift from Fresenius Kabi), made up with 250ml whole milk, was enriched with 60ml of the long chain triglyceride emulsion Calogen® (a gift from Nutricia Clinical). To enhance palatability, the drink was served in a choice of flavours and mixed with ice. Table 3-2 details the macronutrient composition of the meal. Subjects were asked to consume the meal completely within 20 minutes. 10 (out of 16) patients from the SBS-1 and 9 (out of 11) patients from the SBS-2 group completed the test meal. Reasons for not taking or finishing the standard meal included lactose intolerance, nausea, fullness and concerns over fluid restriction. In patients who had not completed the full meal, only fasting blood samples were analysed.

Standard meal	Per serving
Energy (kcal)	866
Protein (g)	11.6
Carbohydrate (g)	68.4
Fat (g)	60.8
Fibre (g)	0

Table 3-2: **Macronutrient composition of standard meal** (as per manufacturers' information).

3.2.3 Sampling

Venous blood was collected after an overnight fast. Patients received their normal parenteral nutrition over night. In hospitalized patients blood was collected in the morning (between 8 and 10am). As most stable outpatients have a long journey time to the hospital, sample collection occurred during the nutrition clinic in the afternoon (between 1 and 3pm). These patients were allowed unsweetened tea or coffee without milk in the morning and then water until the test. Samples were collected in 10ml lithium-heparin tubes containing 0.2ml Trasylol and immediately spun down in a refrigerated centrifuge at 4°C for 10 min at 1600g. Plasma was collected and stored at -70°C until analysis. In patients who consumed the standard meal two further blood samples were drawn 60 min and 120 minutes after the meal was ingested.

3.2.4 Radioimmunoassay

Total GLP-2-IR was measured using antiserum FT-17 and **GLP-2 (1-33)** concentration was determined by Prof. Jens Holst, using antiserum 92160. Both assays are described in chapter 2.

Plasma glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) were measured in duplicate by established specific and sensitive in-house assays (Adrian et al. 1985; Kreymann *et al.* 1987).

GLP-1 (7-36) was measured using an antibody, produced in rabbits against GLP-1 coupled by carbodi-imide to BSA. The antibody cross reacted 100% with all amidated forms of GLP-1 but did not cross react with glycine extended forms [GLP-1 (1-37) and GLP-1 (7-37)] or any other known pancreatic or gastrointestinal peptide. 125I-GLP-1 (7-37 amide) was prepared by the iodogen method (Wood et al., 1981) and purified by HPLC. The specific activity of the 125I GLP-1 label was 48 Bq/fmol. The assay was performed in a total volume of 0.7 ml of 0.06 M sodium barbitone buffer (pH

8.0) containing 0.3 % BSA. The assay was incubated for three days at 4ºC before separation of the free and antibody bound label by charcoal absorption. The limit of detection was 7.5p mol/l, with an intra-assay variation of 5.4%. All samples were assayed in duplicate and in one assay to avoid inter-assay variation.

PYY-like immunoreactivity measured the biologically active components, both the full length (PYY1-36) and the fragment (PYY3-36). The antiserum (Y21) was produced in a rabbit against synthetic porcine PYY (Bachem, UK) coupled to bovine serum albumin with glutaraldehyde and used at a final dilution of 1:50 000. This antibody cross-reacts fully with the biologically active circulating forms of human PYY, but not with pancreatic polypeptide, neuropeptide Y, or any other known gastrointestinal hormone. 125I-labeled PYY was prepared by the iodogen method and purified by high pressure liquid chromatography. The specific activity of the 125I-labelled PYY was 54 Bq per fmol. The assay was performed in a total volume of 700 µL of 0.06 M phosphate buffer, pH 7.26, containing 0.3 % bovine serum albumin. The samples were incubated for 3 days at 4 ºC before separation of free and antibody-bound label by sheep anti-rabbit antibody. 200 µL of unextracted plasma was assayed. The assay has been reported to detect changes of 2pmol/L, with an intra-assay coefficient of variation (CV) of 5.8 % and an inter-assay CV of 9.8 %.

3.2.5 Statistics

Calculations were performed using Excel® and MedCalc® statistical programmes. Non-parametric statistical tests were applied in view of the sample size and the inequality of sample numbers between groups. Results are expressed as median (range). Wilcoxon test for paired samples was applied to assess the postprandial response (time point 0 vs. 60 and 120) within each group. Kruskal-Wallis test was used for comparison between all subject groups. Mann-Whitney-U test for independent samples was used for post hoc analysis. Spearman’s coefficient of rank correlation was applied to

assess the correlation between intestinal length and hormone concentrations. A value of $p<0.05$ was considered significant.

3.3 Results

3.3.1 Demographic characteristics

The study groups included 14 healthy control subjects (8 male, 6 female), 16 SBS-1 subjects: short bowel patients with no colon in continuity (4 male, 12 female) and 11 SBS-2 subjects short bowel patients with all or part of the colon preserved and in continuity with the small bowel (4 male, 7 female). Full patient details are described in the methods section (3.3).

There were no significant differences in age or height between the study groups. Body weight, body mass index (BMI) and basic metabolic rate (BMR) were lower in both SBS groups compared with the healthy controls. The residual small bowel was shorter in the SBS-2 subjects who had part of their colon in continuity. There was no difference in the overall time from surgery between the SBS-1 and SBS-2 group (Table 3-3).

Group characteristics

		HC		SBS-1		SBS-2		p
Number	**all**	**14**		**16**		**11**		
	a	NA		8		6		
	n-a	NA		8		5		
Sex	**all**	**8/6**		**4/12**		**4/7**		0.19
(F/M)	a	NA		2/6		4/2		
	n-a	NA		2/6		0/5		
Age	**all**	**40**	**(31-47)**	**52**	**(45-58)**	**50**	**(44-62)**	0.2
(years)	a	NA		49	(40-52	49	(43-60)	
	n-a	NA		56	(52-58)	59	(47-62)	
	p (a vs. n-a)			0.08		0.9		
Weight	**all**	**81**	**(69-89)**	**57 ***	**(51-74)**	**61 ***	**(55-66)**	0.01
(kg)	a	NA		63	(50-78)	66	(62-71)	
	n-a	NA		55	(52-72)	56	(54-59)	
	p (a vs. n-a)			0.7		0.08		
Height	**all**	**173**	**(168-180)**	**164**	**(161-174)**	**165**	**(157-177)**	0.2
(cm)	a	NA		166	(162-176)	177	(172-180)	
	n-a	NA		163	(160-169)	158	(156-163)	
	p (a vs. n-a)			0.6		0.03		
BMI	**all**	**26**	**(24-29)**	**22 ***	**(19-25)**	**22 ***	**(21-23)**	0.02
(kg/m^2)	a	NA		22	(19-26)	21	(18-23)	
	n-a	NA		22	(20-24)	22	(21-23)	
	p (a vs. n-a)			0.9		0.5		
BMR	**all**	**1627**	**(1548-1870)**	**1291***	**(1193-1631)**	**1322***	**(1191-1570)**	0.01
(cal/day)	a	NA		1415	(1191-1716)	1577	(1361-1709)	
	n-a	NA		1128	(1200-1424)	1197	(1184-1287)	
	p (a vs. n-a)			0.6		0.08		
Time since surgery	**all**	**NA**		**23**	**(3-93)**	**8**	**(3-72)**	0.9
(months)	a	NA		3	(2-3)	3	(1-6)	0.9
	n-a	NA		114	(57-180)	72	(72-276)	0.9
	p (a vs. n-a)				<0.0001	0.004		
Small bowel	**all**	**NA**		**100**	**(68-100)**	**40**	**(18-60)**	0.05
(cm)	a	NA		100	(63-105)	58	(41-100)	0.7
	n-a	NA		80	(71-100)	10	(0-29)	0.005
	p (a vs. n-a)			0.3		0.05		
Colon in continuity	**all**	**NA**		**NA**		**80**	**(65-100)**	NA
(%)	a	NA		NA		85	(68-99)	
	n-a	NA		NA		80	(55-100)	
	p (a vs. n-a)					0.7		

Table 3-3: **Demographic characteristics of subjects.**

Results are given as median (interquartile range).

The study groups were compared using the Kruskal-Wallis test (Chi-square test for sex of subjects). A p-value < 0.05 was considered significant.

Mann Whitney U test was applied for post hoc analysis * = p<0.05 and for comparison of adapted (a) vs. non-adapted (n-a) subjects within the groups.

3.3.2 Hormone concentration

Measurement of **fasting hormone concentration** in **SBS-1** patients (without terminal ileum and/or colon in continuity) revealed reduced plasma concentrations of GLP-1 ($p<0.001$), and PYY ($p<0.001$) compared with healthy controls. There was a trend towards reduction for GLP-2 (1-33) ($p=0.07$). This could not be demonstrated for tGLP-2-IR measured with antiserum FT-17.

In **SBS-2** patients (with preserved terminal ileum and/or colon), the median fasting concentrations of GLP-1 and GLP-2 (1-33) were also reduced compared with controls but this difference was not significant. In contrast, the median fasting plasma PYY concentration was 3-fold higher in this patient group ($p< 0.001$) (Table 3-4 and Figure 3-1).

Fasting

	HC		SBS-1		SBS-2	
n	**14**		**16**		**11**	
GLP-1 (pmol/l)	51	(43-56)	22	(17-30)	25	(23-56)
p vs. HC			**0.0003**		0.1	
GLP-2 (1-33) (pmol/l)	9	(7-10)	5	(1-9)	6	(5-14)
p vs. HC			0.07		0.4	
tGLP-2 IR (pmol/l)	118	(85-143)	111	(79-148)	147	(90-185)
p vs. HC			0.9		0.2	
PYY (pmol/l)	20	(12-30)	6	(5-12)	69	(49-95)
p vs. HC			**0.0004**		**0.0001**	

Table 3-4: **Fasting hormone concentrations;** median (range).
p vs. HC (Mann-Whitney-U test)

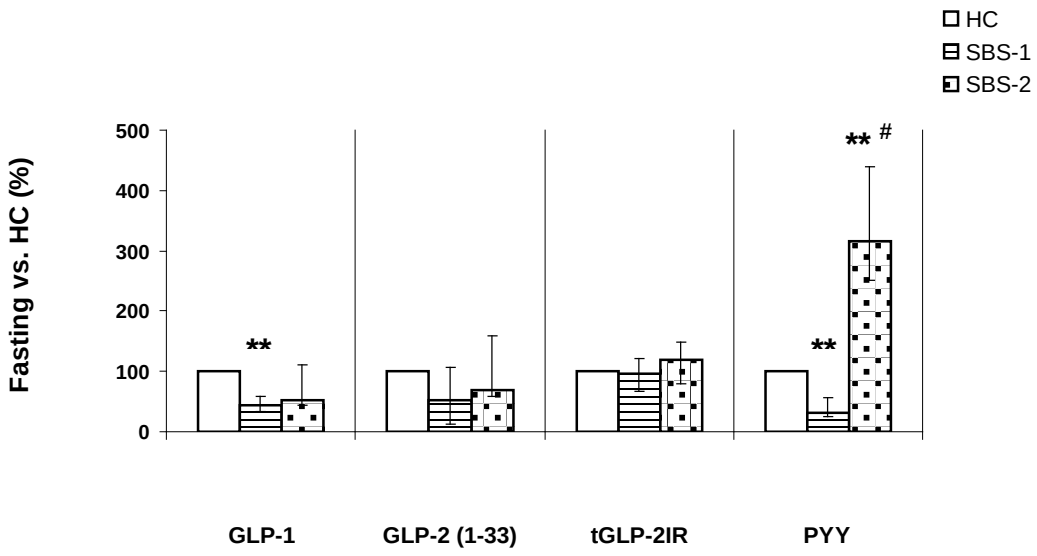


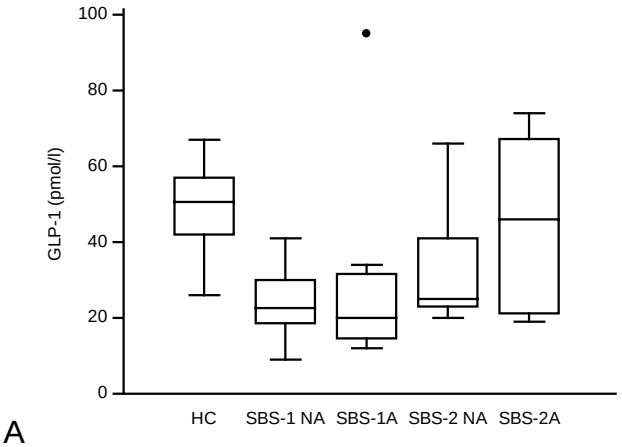
Figure 3-1: **Fasting hormone concentrations; change versus HC** (set as 100%)
**p<0.001 vs. HC; # p<0.00001 vs. SBS-1 (Mann-Whitney-U test)

Comparison between non-adapted (last resection <12 months) and adapted (last resection > 24 months) patients in the SBS-1 group shows a significant reduction in fasting GLP-1 and PYY in both subgroups. Fasting GLP-2 was only reduced in the acute subgroup.

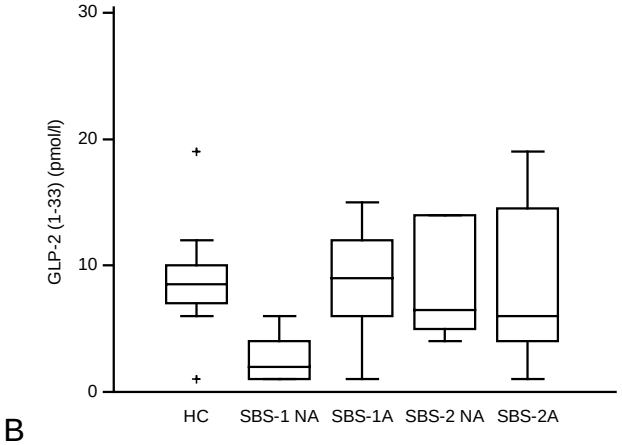
In the SBS-2 group GLP-1 is significantly reduced in the non-adapted subgroup. There is no change in fasting GLP-2 in either subgroup. Fasting PYY values are elevated in non-adapted and adapted SBS-2 patients (Figure 3-2).

Fasting	HC	SBS-1		SBS-2	
		non-adapted	adapted	non-adapted	adapted
n	14	8	8	6	5
Last resection (months)	NA	3 (2-3)	114 (57-180)	3 (1-6)	72 (72-276)
Jejunum (cm) p (non-adapted vs. adapted)	NA	100 (63-105) 0.3	80 (71-100)	58 (41-100) **0.05**	10 (0-29)
Colon in continuity (%) p (non-adapted vs. adapted)	NA	NA	NA	85 (68-99) 0.7	80 (55-100)
GLP-1 (pmol/l) p (vs HC) p (non-adapted vs. adapted)	51 (43-56)	22 (19-29) **0.0008** 0.8	20 (15-30) **0.001**	25 (24-37) **0.03** 0.7	46 (22-65) 0.7
GLP-2 (pmol/l) p (vs HC) p (non-adapted vs. adapted)	9 (7-10)	2 (1-4) **0.0007** **0.007**	9 (7-12) 0.7	7 (5-13) 0.5 0.9	6 (5-13) 0.6
PYY (pmol/l) p (vs HC) p (non-adapted vs. adapted)	20 (13-30)	6 (5-13) **0.006** 0.8	7 (5-10) **0.002**	69 (55-69) **0.0005** 0.5	59 (48-103) **0.006**

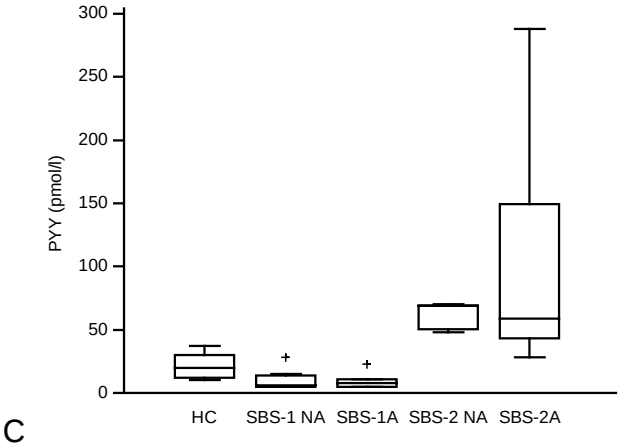
Table 3-5: **Fasting hormone concentration in adapted and non-adapted subgroups.**
p (Mann-Whitney-U test)



A



B



C

Figure 3-2: **Fasting hormone concentration in non-adapted (NA) and adapted (A) subgroups:**

A) GLP-1 **B) GLP-2 (1-33)** **C) PYY**

(Values shown: median, 25 to 75 percentile, minimum to maximum, outliers)

SBS-1 NA	= No colon in continuity,	resection < 12 months ago	(n=8)
SBS-1 A	= No colon in continuity,	resection > 24 months ago	(n=8)
SBS-2 NA	= Colon in continuity,	resection < 12 months ago	(n=6)
SBS-2 A	= Colon in continuity,	resection > 24 months ago	(n=5)
HC	=	Healthy controls	(n=14)

Postprandial hormone concentrations were determined 60 and 120 minutes following a standardized nutrient challenge. The highest hormone level of the two time-points (Max) was compared with the fasting concentration. A significant postprandial rise of GLP-1, GLP-2 ((1-33) and tGLP-2-IR) and PYY concentration from baseline was demonstrated in all subject groups (Table 3-6 and Figure 3-3).

		HC		SBS-1		SBS-2	
n		**14**		**10**		**9**	
GLP-1 (pmol/l)	Fasting	**50.56**	(43.3-56.1)	**28.1**	(23.4-33.5)	**41.4**	(23.5-65.2)
	Max	**69.7**	(66.3-89.2)	**42.4**	(33.3-64.5)	**97**	(70.7-126.4)
	p	0.0001		0.01		0.012	
GLP-2 (1-33) (pmol/l)	Fasting	**8.5**	(7.3-9.8)	**3.5**	(1.0-7.5)	**6**	(5.0 -13)
	Max	**30.5**	(23.7-44.0)	**11.5**	(9.3-14.5)	**35**	(23-52)
	p	0.0002		0.008		0.004	
tGLP-2 IR (pmol/l)	Fasting	**117.5**	(85-143)	**93.4**	(71-166.1)	**146.6**	(101.8-193.9)
	Max	**203**	(156.7-260.5)	**169.9**	(117-232.4)	**371.6**	(237.4-376.1)
	p	0.0006		0.002		0.004	
PYY (pmol/l)	Fasting	**19.5**	(12.8-29.9)	**10.1**	(7.4-14.2)	**70.2**	(58.5-239.5)
	Max	**39.6**	(29-57.7)	**23.8**	(14.8-36.1)	**200.9**	(159.2-396.6)
	p	0.0001		0.002		0.004	

Table 3-6: **Hormone concentration in fasted state versus maximum postprandial level**
(Max = concentration at 60 or 120 minutes post meal)
Values given are: median (interquartile range). (p: Wilcoxon test for paired samples)

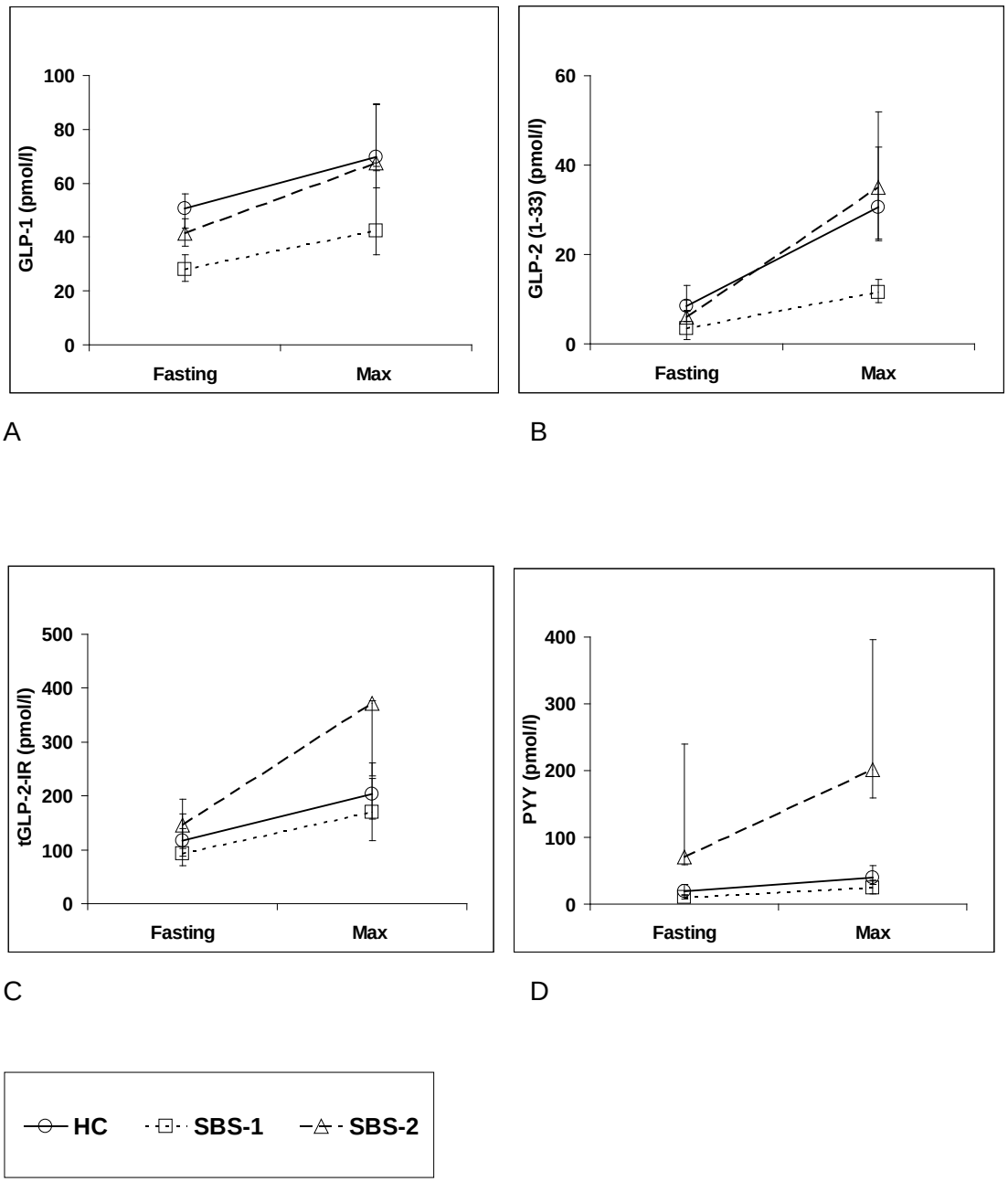


Figure 3-3: **Hormone concentration in fasted state versus maximum postprandial level**

(Max = concentration at 60 or 120 minutes post meal)

Values given are: median (interquartile range).

The difference between fasting and maximum hormone concentration was significant for all groups and hormones analysed (see Table 3-5).

HC = Healthy controls
SBS-1 = No colon in continuity
SBS-2 = Colon in continuity

The **magnitude of the postprandial secretory response** for each hormone was assessed by calculating the maximum increase of hormone concentration from baseline (at 60 or 120 minutes).

SBS-1 patients showed a reduced response of GLP-2 (1-33) compared to HC. No statistically significant changes could be demonstrated for tGLP-2-IR, GLP-1 or PYY.

In **SBS-2** patients, there was no significant difference in postprandial GLP-1 or GLP-2 secretory response. However, postprandial PYY secretion in SBS-2 patients showed a very strong increase (8.5-fold higher compared to HC) (Table 3-7; Figure 3-4).

Maximal postprandial increase from fasting

	HC		SBS -1		SBS-2	
n	**14**		**10**		**9**	
GLP-1 (pmol/l)	21	(12-36)	12	(6-31)	30	(22-43)
p vs. HC			0.2		0.6	
GLP-2 (1-33) (pmol/l)	23	(12-36)	10	(3-12)	27	(15-38)
p vs. HC			**0.02**		0.2	
tGLP-2 IR (pmol/l)	70	(32-144)	31	(26-106)	168	(91-225)
p vs. HC			0.3		0.1	
PYY (pmol/l)	18	(13-24)	12	(8-24)	150	(91-163)
p vs. HC			0.5		**0.0001**	

Table 3-7: **Maximum postprandial increase in hormone concentrations** (at 60 or 120 min)

Values: median (range);
p vs. HC: (Mann-Whitney-U test)

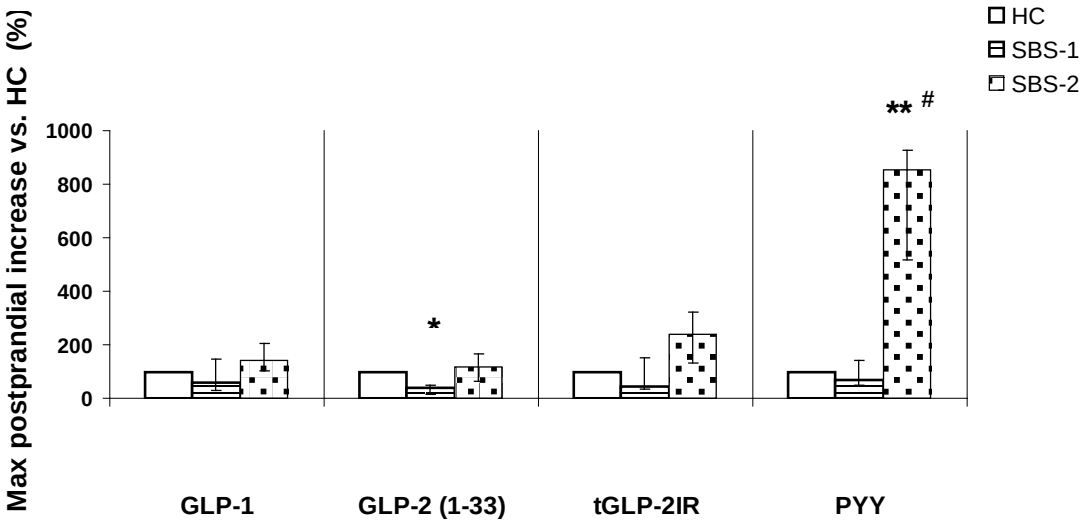


Figure 3-4: **Maximum increase in hormone concentrations** (at 60 or 120min)

Change versus healthy controls: HC (set as 100%)

*p<0.05 vs. HC; **p<0.001 vs. HC, # p<0.0002 vs. SBS-1 (Mann-Whitney-U test)

Comparison between non-adapted (last resection <12 months) and adapted (last resection > 24 months) patients in the **SBS-1** group shows a reduction of the postprandial GLP-1 and GLP-2 response vs. healthy controls in the non-adapted group but no difference in the adapted group. The postprandial PYY increase is similar to that of healthy controls in both subgroups.

In the **SBS-2** group the GLP-1 and GLP-2 response is similar to the controls in non-adapted and adapted patients. The strongly increased postprandial PYY response can be shown in both subgroups. (Table 3.8 and Figure 3-5).

Max increase	HC	SBS-1		SBS-2	
		non-adapted	adapted	non-adapted	adapted
n	14	5	5	5	4
Last resection (months)	NA	3 (2-3)	72 (60-156)	4 (2-6)	108 (66-177)
Jejunum (cm) p (non-adapted vs. adapted)	NA	100 (25-100) 1	80 (80-100)	45 (40-70) 0.1	25 (15-34)
Colon in cont. (%) p (non-adapted vs. adapted)	NA	0	0	75 (65-100) 0.6	90 (69-100)
GLP-1 (pmol/l) p (vs HC) p (non-adapted vs. adapted)	21 (12-36)	11 (2-12) **0.02** **0.03**	35 (22-36) 0.4	26 (22-30) 1 0.4	40 (29-46) 0.4
GLP-2 (pmol/l) p (vs HC) p (non-adapted vs. adapted)	23 (12-36)	4 (3-9) **0.02** 0.22	11 (10-14) 0.19	19 (15-38) 0.7 0.9	31 (24-39) 0.4
PYY (pmol/l) p (vs HC) p (non-adapted vs. adapted)	18 (13-24)	9 (6-16) 0.9 0.2	19 (9-28) 0.3	150 (91-157) **0.006** 0.9	152 (115-167) **0.001**

Table 3-8: **Maximum increase in hormone concentrations** (at 60 or 120min) in non-adapted and adapted subgroups.

p (Mann Whitney U test).

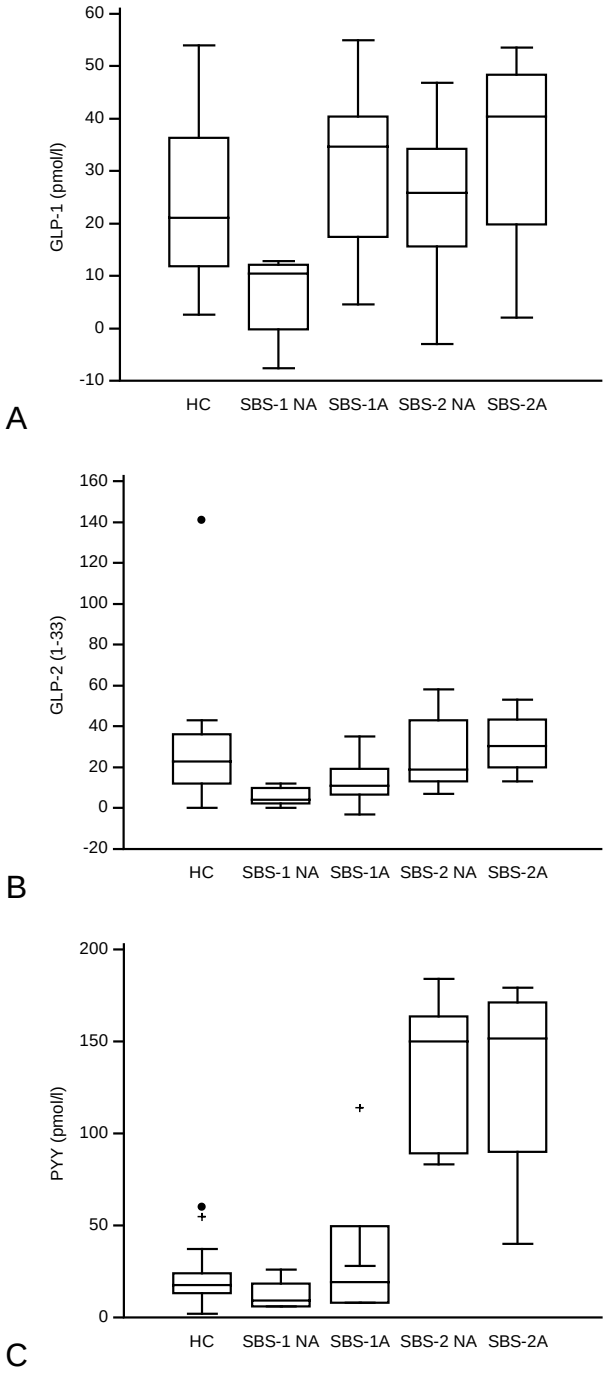


Figure 3-5: **Maximum postprandial secretory response** (at 60 or 120 minutes) in non-adapted (NA) and adapted (A) subgroups:

A) GLP-1 B) GLP-2 (1-33) C) PYY

(Values shown: median, 25 to 75 percentile, minimum to maximum, outliers)

SBS-1 NA	= No colon in continuity,	resection < 12 months ago	(n=5)
SBS-1 A	= No colon in continuity,	resection > 24 months ago	(n=5)
SBS-2 NA	= Colon in continuity,	resection < 12 months ago	(n=5)
SBS-2 A	= Colon in continuity,	resection > 24 months ago	(n=4)
HC	=	Healthy controls	(n=14)

Analysis of **fasting** L-cell hormone concentration in a **single subject** with SBS secondary to mesenteric volvulus demonstrates reduced GLP-1, GLP-2 and PYY levels (compared to median concentrations in HC) 2 months following resection and formation of jejunostomy (110 cm small bowel). One month after restoration of colonic continuity (95% colon preserved), fasting GLP-1 and GLP-2 concentration remain low but a strong increase in fasting PYY concentration can be seen (Figure 3-6).

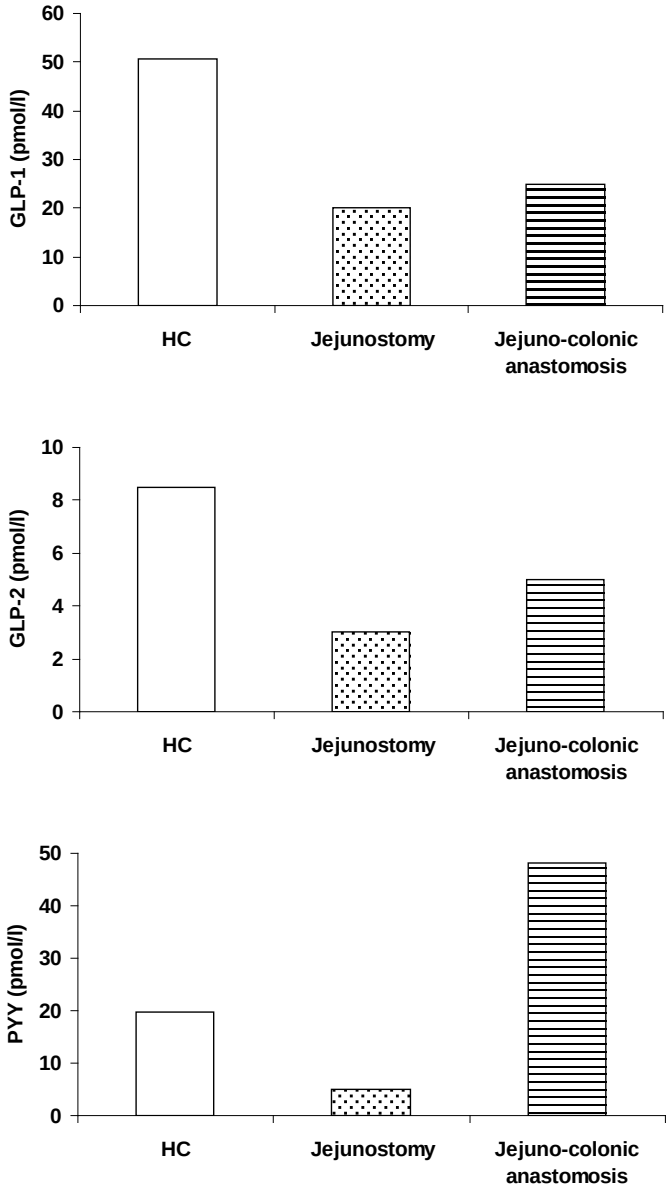


Figure 3-6: **Fasting hormone concentrations in a single subject with SBS**
2 months after jejunostomy formation (110cm small bowel) (dotted bar)
1 month following restoration of colonic continuity (striped bar)
Compared to median hormone levels in healthy controls (HC; empty column)

The **correlation between remaining intestinal lengths and hormone plasma concentrations** (fasting and maximum increase) was calculated using the Spearman's coefficient of rank correlation (rho). There was a suggestion of a positive correlation between length of remaining small bowel and maximum postprandial PYY increase (rho=0.69, p=0.03) in the SBS-1 group. No other significant correlations could be demonstrated.

Correlation to intestinal length

		n	**GLP-1 rho**	p	**GLP-2 rho**	p	**PYY rho**	p
SBS-1								
Fasting	SB length	15	**0.25**	0.4	**-0.11**	0.6	**0.02**	0.9
Max Increase	SB length	10	**0.12**	0.7	**0.48**	0.1	**0.69**	***0.03***
SBS-2								
Fasting	SB length	11	**0.47**	0.1	**-0.04**	0.9	**0.37**	0.2
	Colon length	11	**-0.07**	0.8	**-0.06**	0.8	**0.07**	0.8
Max Increase	SB length	9	**-0.3**	0.3	**0.48**	0.2	**0.23**	0.5
	Colon length	9	**0.5**	0.1	**0.46**	0.2	**0.19**	0.6

Table 3-9: **Correlation between hormone concentration and intestinal length.**

The remaining intestinal lengths (SB=small bowel and Colon) was correlated with GLP-1, GLP-2 and PYY plasma concentration (fasting and maximum postprandial increase).

Spearman's correlation coefficient (rho); n= number of subjects.

3.4 Discussion

The enteroendocrine L-cell is the source of intestinal PYY and proglucagon derived peptide (PGDP) production. These hormones have important regulatory functions within the gastrointestinal tract as well as exert a range of extra-intestinal effects. Changes in PYY and PGDP secretion from L-cells following massive intestinal resection have been demonstrated in animal models and humans (Fuller *et al.* 1993; Bilchik *et al.* 1995; Adrian *et al.* 1996; Nightingale *et al.* 1996; Jeppesen *et al.* 1999; Jeppesen *et al.* 2000). The question, whether this adaptive response is L-cell specific or whether the secretion of different L-cell products may be regulated independently has not yet been answered.

Patients with short bowel syndrome (SBS) due to extensive intestinal resection appear to achieve better functional adaptation if their colon has been retained. It has been suggested that this might partly be caused by differences in gut hormone secretion (Nightingale *et al.* 1996).

The results of this study demonstrate that **SBS patients, who have no terminal ileum or colon in continuity (SBS-1 group)**, have deficient secretion of PYY as well as GLP-1 and GLP-2. The fasting plasma concentration of GLP-1 and PYY was significantly reduced compared to healthy controls and there was a trend towards reduction for GLP-2 (1-33). No difference was shown for tGLP-2-IR. The latter finding is not unexpected, given that the amount of glucagon-like peptides in fasting samples is thought to be very small (Orskov *et al.* 1987) and we have found no correlation between fasting levels of GLP-2 (1-33) and tGLP-2-IR (section 2.3.6). Reductions in fasting GLP-1 and PYY were shown in non-adapted and adapted SBS-1 patients.

The SBS-1 patients in this study showed a preserved postprandial secretory response with significant increases of GLP-1, GLP-2 and PYY. However, the maximum postprandial increase of GLP-2 (1-33) was attenuated when

compared with healthy controls. Reductions in median postprandial response for GLP-1 and PYY did not reach statistical significance.

Nightingale and colleagues showed that jejunostomy patients (median jejunal length 90 cm (55-125)) had low fasting PYY values and a reduced secretory response following a mixed 640 kcal meal (Nightingale *et al.* 1996). A study from Morgan et al. describes a preserved but attenuated GLP-1 response following a 625 kcal meal in 6 jejunostomy patients (residual jejunal length 50-160 cm) (Morgan et al. 1993). Jeppesen and colleagues also describe impaired postprandial GLP-2 secretion in jejunostomy patients but in contrast to our findings showed no significant difference in fasting GLP-2 levels between jejunostomy patients and healthy controls (Jeppesen *et al.* 1999). It is conceivable that this is due to the fact that their patient population (n=7) had slightly longer jejunal remnants (111 cm (range 30-150cm) vs. 100 (68-100). The majority of L-cells are located in the distal ileum and colon. However, smaller quantities can also be found in the proximal jejunum and duodenum (Eissele *et al.* 1992; Theodorakis *et al.* 2006). Thus, a positive correlation between remaining jejunal length and L-cell secretions could be postulated. However, we were unable to demonstrate a correlation between fasting peptide levels and length of jejunal remnant in jejunostomy patients. It is very possible that the subject number and the variations of residual intestinal length were insufficient to demonstrate such correlation.

In Jeppesen's study, the jejunostomy patients had only a small postprandial GLP-2 response following a liquid 450 kcal and a separate mixed 936 kcal meal (meal A and B respectively) with no significant increase from baseline at any time point (10-180min). The test meal used in our study contained 866 kcal, which is comparable to meal B, however was of different consistency (liquid) and nutrient composition. This might cause differences in gut distension and speed of gastric empting and nutrient delivery to sensor cells, thus resulting in a different L-cell response.

Intestinal adaptation occurs over a period of time. Initial changes to mucosal DNA synthesis and cell production can occur rapidly. In animal models, increase in PYY and proglucagon mRNA has been demonstrated within hours from resection (Bilchik *et al.* 1995). The majority of intestinal adaptation takes place within the first 12-24 months (Goodlad et al. 2001). Certain intestinal functions, for example the intestinal calcium absorption, may continue to increase for more than 2 years after resection (Gouttebel et al. 1989). It is of note that the subgroup of non-adapted jejunostomy patients in our study had a significantly reduced postprandial GLP-1 and GLP-2 response compared to healthy controls (in keeping with Jeppesen's findings); whereas no difference was demonstrated in the adapted subgroup (both groups had comparable amounts of jejunal remnant). This could suggest that GLP secretion from L-cells may continue to adapt beyond 12 months. However this hypothesis would need to be tested by monitoring subjects longitudinally over several years.

Overall, the data from this work and the available literature suggests that L-cell secretion of PYY as well as PGDP is impaired in SBS patients with a jejunostomy. The length of jejunal remnant might be related to fasting PGDP levels, although a positive correlation could not be shown here. Prolonged time for adaptation (>12 months) might increase the level of postprandial PGDP secretion.

It is generally acknowledged that the preservation of colon during extensive intestinal resection is beneficial in the functional adaptation of the intestinal remnant and reduces the need for parenteral nutrition (Nightingale et al. 1992). The colon itself has a large capacity for electrolyte and water absorption. In addition, colonic fermentation of carbohydrates to short chain fatty acids that can be easily absorbed from the colon, contributes towards salvage of energy (Nordgaard et al. 1996; Jeppesen et al. 1999). Lastly, activation of the colonic brake mechanism in SBS patients with a preserved colon is thought to prohibit the increased rate of gastric emptying, found in jejunostomy patients (Nightingale et al. 1993). The colonic bake is thought to be mediated through L-cell hormone secretion, in particular PYY, which is

known to slow gastric emptying (Savage *et al.* 1987). Increased fasting and postprandial levels of PYY in SBS patients with a preserved colon have been demonstrated (Nightingale *et al.* 1996). Jeppesen and colleagues found a two-fold increase of GLP-1 and a more than threefold increase of GLP-2 level in short bowel patients with a preserved colon, suggesting that increased secretion of these PGDPs might also contribute to the positive effect of a preserved colon in short bowel syndrome (Jeppesen *et al.* 2000).

In our study, **SBS patients with preserved colon in continuity (SBS-2 group)** also demonstrated a marked increase in fasting and postprandial PYY concentration. Surprisingly, and in contrast to Jeppesen's findings however, fasting GLP-1 and GLP-2 levels were not increased. In the non-adapted subgroup, the fasting concentration of GLP-1 was even reduced. Equally, the maximum postprandial PGDP response did not differ significantly from that of healthy subjects. The patient cohort in Jeppesen's study consisted of 7 subjects with an average small bowel length of 89cm (range 45-140). The mean colonic remnant was 84% (57-100). Only 2 patients received parenteral support. In our cohort the median remnant small bowel was shorter (average 41cm, median 40cm; range 18-60 cm). All patients were dependent on parenteral nutritional support indicating a lower level of functional adaptation.

In contrast to Jeppesen's hypothesis that L-cells in the human colon are capable of significant GLP-1 and GLP-2 secretion when the ileum has been removed, our results suggest that a preserved colon alone might not suffice for an adaptive increase of PGDP secretion. It is interesting to speculate that, whereas the increase of PYY secretion occurs in the colon, the residual jejunum is primarily responsible for the adaptive hyperenteroglucagonemia as has been proposed previously (Bilchik *et al.* 1995). However, given the deficient peptide secretion in subjects after colectomy, the colon appears to play a pivotal role in signalling mechanisms for PGDP increase.

The finding of differential L-cell secretion appears to be corroborated by the observation of fasting L-cell hormone concentrations in a single jejunostomy

patient. One month after restoration of colonic continuity, there is only a small increase of fasting GLP-1 and GLP-2 concentration and a disproportionally strong elevation in fasting PYY (Figure 3-6). Low plasma glucagon-like peptide -2 levels despite the presence of residual colon are also described in a study of premature infants with severe short bowel syndrome (Sigalet et al. 2004).

The differential secretory response of L-cells surprises, as PYY and PGDPs are co-localized in these cells. In rabbit colon, where L-cells constitute 33% of enteroendocrine cell mass, PYY and enteroglucagon co-localization was documented in all L-cells examined (Nilsson et al. 1991). The labelling density was generally higher for PYY but there appeared to be variations in the PYY/enteroglucagon labelling ratio between cells. Co-localization was seen in 83% of secretory granules, 15% contained PYY and 2% enteroglucagon only. It is therefore not surprising that parallel stimulation of PYY and PGDP production release can usually be seen postprandially (Feltrin et al. 2006). Nonetheless a differential response to certain nutrients and stimuli has also been described. In isolated perfused rabbit colon, selective release of either PYY or enteroglucagon occurs upon stimulation with bile salts or fatty acids respectively. In rats, a differential secretory response was seen following intra-colonic oleic acid, short chain fatty acids and L-Name. Similar results have been reported in humans, where infusions of oleic acid into the ileal pouch of patients with total colectomy did not modify enteroglucagon levels but induced a dose-dependent increase of PYY (Pironi et al. 1993).

It is also noteworthy that other hormones appear to be co-localised in L-cells. Hoersch et al. describe complex, zone related coexistence patterns of peptides and other transmitters found in individual cell populations in the mucosa of the human anal canal. In the colonic mucosa they not only describe co-existence of GLP-1 and PYY but also GLP-1 and serotonin (5-HT) (Horsch et al. 1994). In human duodenal L-cells, Theodorakis et al. have identified co-localisation of GLP-1 and gastric inhibitory polypeptide (GIP). They reach the conclusion that the concept of distinct cell populations

dedicated to the synthesis and release of separate peptides should be reviewed under the perspective of a multipotent cell population, producing peptides according to stimuli in the metabolic or biochemical milieu (Theodorakis *et al.* 2006).

Cell turnover in the intestinal mucosa is very high. The life span of enteroendocrine cells is estimated at 3-4 days in mice (Schonhoff et al. 2004). It has been shown that normal enteroendocrine cells are a terminally differentiated, non-proliferating population and that therefore population expansion precedes differentiation (Barrett et al. 1995). Endocrine-committed, intermediate cells are possibly the proliferating elements responsive to tissue-specific physiological stimuli (Rindi et al. 2004). In response to intestinal resection, L-cells show an increase of rough endoplasmic reticulum indicating a hyper-functional state (Buchan et al. 1985). Whether different populations of L-cells respond to varying physiological stimuli, whether adaptation of L-cells occurs early on during entero-endocrine differentiation resulting in a changing L-cell-phenotype or whether the profile of secretory granules in a mature L-cell can adapt to a changing biochemical environment requires further research.

In addition to its anti-motility and anti-secretory properties, PYY is known to have inhibitory effects on appetite and food intake (Vincent et al. 2008). Adaptive hyperphagia is a known phenomenon in adult short bowel patients. Given the marked increase in fasting and postprandial PYY concentration in SBS patients with colonic continuity, it is interesting to note that in a study of 90 SBS patients, the presence or absence of the colon did not appear to be related to oral energy intake (Crenn et al. 2004).

Both, PYY and PGDPs have therapeutic potential in SBS. PYY, as inhibitor of gastro-intestinal motility and secretion (Savage *et al.* 1987), might lead to a more favourable fluid balance, especially in jejunostomy patients. This has not yet been assessed in clinical trials. The long acting GLP-2 analogue teduglutide has been highly effective in improving fluid absorption in SBS patients with and without preserved colon (Jeppesen *et al.* 2001; Jeppesen

et al. 2005). A subsequent larger multicentre double blinded placebo controlled trial has recently shown that this can lead to meaningful reductions in parenteral nutrition requirements for patients with short bowel syndrome (O'Keefe et al. 2008).

Our findings could have implications for the development of therapeutic strategies in short bowel syndrome. Jeppesen et al. have demonstrated elevated endogenous GLP-2 levels in most (but not all) of their SBS patients with a retained colon. They have shown that this patient group still derived significant benefit from exogenous teduglutide administration (Jeppesen *et al.* 2005). As a possible mechanism they speculate that supraphysiological doses of teduglutide produce high concentrations locally, which suppress gastric acid secretion and induce small bowel growth. Our work suggests that in addition, a subgroup of SBS patients who have a retained colon but only a very short remaining small bowel remnant might be GLP-2 deficient and therefore benefit from replacement therapy.

As an alternative to exogenous hormone administration, stimulation of endogenous peptide secretion might constitute a worthwhile therapeutic concept. Our results suggest that blind/non-specific stimulation of L-cells might not lead to a uniform secretory response. Instead secretions might vary in different individuals depending on their intestinal anatomy. It is conceivable that other factors like underlying pathology and mucosal health might also be relevant. Further research into the mechanisms of L-cell differentiation and stimulation is needed.

The results of this study in context with the other cited publications illustrate that SBS patients are a very heterogeneous subject group. All studies, published so far describe a relatively small number of subjects. This is also a weakness of the presented study. We have postulated that differences in gut hormone secretion might be related to the length of remaining small intestine, although a correlation could not be shown in this patient population. It would be useful in future studies to achieve a sufficient subject number in order to allow a subgroup analysis of different small bowel length.

Other potential confounding factors are time of surgery and underlying pathology. Recruitment of SBS patients can be problematic as SBS is a rare condition and patients are usually followed up in specialist hospitals. This means long journeys to the study centre and therefore potential subjects may not be able to commit sufficient time for meal stimulated test. Admission of subjects might be necessary but is often inconvenient for the patient and very costly.

The small bowel length of most, but not all patients was determined from surgical notes. Although it is now considered good surgical practice to record the remaining intestinal lengths following resection, this has not always been the case in the past when often the length of resected intestine alone was documented. Although in these patients the small bowel length was estimated radiologically there might have been room for error. Given the important implications of the intestinal anatomy on functional adaptation and post resection recovery, accurate documentation of postsurgical anatomy is of great importance.

SBS patients in this study were not malnourished and had a body mass index within the normal range. However, there was a miss-match with the healthy control group, which contained a higher proportion of overweight and obese subjects. It is of note that attenuated secretion of PYY has been shown in morbidly obese subjects (mean BMI 40.3) (Le Roux et al. 2006). However the magnitude of PYY increase in SBS-2 patients compared to controls, shown in this study and others, is unlikely to be explained through the difference in weight alone. Nonetheless, body weight might need to be considered as a confounding factor when assessing PYY secretion. Secretion of GLP-1 and GLP-2 does not appear to differ between normal weight and obese subjects (see Chapter 4).

The choice of test meal was determined by various factors. In order to elicit a good postprandial response it needed to provide a sufficient caloric load. Other important considerations were the ease of preparation, standardization and reproducibility, ease of consumption and palatability. A liquid meal of

nutritional supplements was therefore chosen. Gastric emptying of liquids can be rapid in SBS patients. In 2 patients with very short jejunal remnant the test meal rapidly emptied into the stoma bag. To allow better comparability with other studies, it might be useful to investigate the response to a mixed solid meal. However some SBS patients have problems consuming such meal (Nightingale *et al.* 1996).

Summary: In keeping with previous studies, we have shown deficient L-cell secretion of PYY and PGDP (GLP-1 and GLP-2) in SBS patients with jejunostomy. Patients with retained colon had an increase of PYY- but not of PGDP plasma concentration. This finding contrasts previously published results which describe elevated PGDP fasting levels in this patient group. We propose that despite their co-localisation in enteroendocrine L-cells, the secretion of PYY and PGDP is regulated differentially and may vary depending on the intestinal anatomy.

Chapter 4

Meal stimulated glucagon-like peptide secretion in normal weight and obese subjects

4.1 Introduction and aim

The burden of obesity related illnesses threatens to overwhelm healthcare systems worldwide. It is hoped that unveiling the physiological pathways of appetite control may lead to new strategies for fighting this epidemic. A variety of gut hormones have been shown to influence appetite, nutrient intake and satiety. It is now thought that changes in hormonal release pattern may contribute towards the pathophysiology of obesity.

The proglucagon derived peptides, glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) are gut hormones, co-secreted from mucosal enteroendocrine L- cells. They are partly co-localized with glucose-dependent insulinotropic polypeptide (GIP) in the proximal small bowel and with peptide tyrosine tyrosine (PYY) in the distal small bowel and colon (Mortensen *et al.* 2003). Nutrients, in particular fats and carbohydrates, are the primary physiologic stimulus for glucagon-like peptide (GLP) and PYY secretion (Adrian *et al.* 1985; Hartmann *et al.* 2000).

PYY is a major mediator of the ileal and colonic brake which slows gastro-intestinal transit and secretion, allowing for increased nutrient absorption. Identification of its role in appetite regulation has been a key advance in obesity research. PYY shows a blunted postprandial secretion profile in the obese, indicating that the release of PYY from the intestinal tract may be inhibited. Moreover, exogenous PYY administration reduces food intake in rodents and humans (Batterham *et al.* 2003; Le Roux *et al.* 2006).

As an incretin hormone, GLP-1 enhances insulin secretion, whilst inhibiting glucagon release. GLP-1 is also part of the ileal brake mechanism. Intravenous infusions of GLP-1 reduces energy intake in lean and overweight subjects in a dose dependent manner. It is thought that the reduced gastric emptying rate may contribute to increased satiety (Verdich *et al.* 2001). An attenuation of the postprandial GLP-1 response in obese humans has been reported (Verdich et al. 2001). Other studies have

however shown an increased GLP-1 response to oral nutrients in the obese (Fukase et al. 1993) or no difference of GLP-1 secretion between normal weight and obese subjects (Feinle *et al.* 2002).

GLP-2 has recently received much attention for its specific effects on intestinal mucosal proliferation, perfusion and nutrient absorption. GLP-2 has also been shown to delay gastric emptying but is less potent than GLP-1 (Nagell *et al.* 2004). Although intracranial injection of GLP-2 appears to confer anorexic effects in rodents (Lovshin et al. 2001), peripheral administration of GLP-2 in humans has shown no impact on hunger sensation (Nagell *et al.* 2004). There is a paucity of literature investigating GLP-2 release in the obese. One study detected no difference between lean and obese subjects in the fasted state (Mersebach et al. 2003).

The aim of this study was to determine the GLP-1 and GLP-2 response to a series of standard meals in normal weight and obese subjects. We specifically wanted to determine whether, given the reduced postprandial PYY response in obese subjects, a corresponding reduction of glucagon-like peptide secretion would be found, suggesting global reduction of L-cell response in this patient group.

4.2 Methods

The study was performed according to the principles of the Declaration of Helsinki and approved by the Local Research and Ethics Committee at Hammersmith Hospital (REC number 03/6499). All participants gave written consent to take part in the study after receiving oral and written information. This study was performed in collaboration with Dr. Carel Le-Roux who developed the experiment and coordinated subject recruitment and sample collection. Sample analysis with radioimmunoassay and data interpretation was performed by the author.

4.2.1 Subjects

Nineteen normal weight (N) and 19 obese (O) subjects were studied. The obese subjects were recruited from the Lifestyle or Diabetes clinics at Hammersmith Hospital and the lean subjects from internal advertising on the hospital site. The body weight of all subjects had been stable for 3 months. Mean body mass index was 21.7 ± 0.4 kg/m^2 in the normal weight group and 40.3 ± 1.1 kg/m^2 in the obese group. Both groups consisted of 13 females and 6 males. Mean age was 28.6 ± 1.6 years in the normal-weight group and 29.0 ± 2.0 years in the obese group. Insulin resistance was determined using the homeostasis model assessment insulin resistance index [HOMA-IR index; calculated as: fasting glucose (mM) × fasting insulin (mU/L) / 22.5; values $\geq$ 3.8 were considered diagnostic of insulin resistance (Ascaso et al. 2001)]. Nine out of 19 obese subjects were insulin resistant (HOMA-IR > 3.8). None of the normal weight subjects was insulin resistant.

The following exclusion criteria applied: chronic medical or psychiatric illness, substance abuse, pregnancy, more than two alcoholic drinks per day, and aerobic exercise for more than 30 minutes three times per week.

4.2.2 Study protocol

The trial was performed in a double blind, randomized manner. All subjects fasted from 21:00 on the evening prior each study day. For twenty-four hours prior to each meal subjects refrained from taking strenuous exercise or drinking alcohol. All subjects were asked to attend hospital on three separate occasions. Lean and obese subjects were randomly allocated to two groups (Group-1: 10 lean and 10 obese subjects; Group-2: 9 lean and 9 obese subjects). Depending on their study group, subjects received (in random order) either a 500 ml (Group 1: 250, 500 and 1000 kcal) or a 900 ml (Group 2: 1000, 2000 and 3000 kcal) liquid standard meal. Venous blood was collected through an indwelling forearm cannula in the fasted state followed by sampling in 30 min intervals for 3-h after every meal. Blood samples were collected into lithium heparin bottles containing aprotinin (50000 units), centrifuged and plasma immediately stored at -20^{o}C until analysis. The subjects were sedentary during the entire study. Conditions for each subject remained constant on each occasion as the temperature was controlled and the subjects were allocated the same chair or bed. The subjects were encouraged to relax and allowed to read or watch videos.

4.2.3 Test meal

The postprandial secretion of glucagon-like peptides is related to the macronutrient composition and calorie content of a meal (Chapter 1.1.4). Palatability and ease of consumption are other important considerations when choosing a standardized test meal. In this experiment, ice cream with variable lipid content was utilized. This allowed investigation of the secretory response to different caloric challenges, whilst maintaining comparable meal composition. The macronutrient content of each meal is detailed in Table 4.1. The ice cream was prepared to the highest food safety standards and consumed at 4 °C in a semi liquid state. All subjects were required to drink the entire volume. Each different calorie meal looked and tasted similar and

the subjects were blinded to the calorie content. On each occasion the meal was given in the same containers, and at the same temperature.

	Carbohydrate (g)	Fat (g)	Protein (g)	Calories (kcal)
500mL meal				
250 calories	32.3	10.2	16	258
500 calories	52	26.5	18	518
1000 calories	63.3	75.3	17.1	1000
900mL meal				
1000 calories	99	52.9	32.6	1002
2000 calories	107.5	161.7	29.5	2004
3000 calories	94.5	274.95	24.75	2952

Table 4-1: Macronutrient content of the standard meals

Meals consisted of liquid drinks with similar consistency.
CHD: Carbohydrate.

4.2.4 Radioimmunoassay

Total GLP-2-IR was measured using antiserum FT-17 as described in chapter 2. Detection limit was 25pmol/l; intra-assay CV 8.3%. Antibody FT-17 was validated by correlating it with the current gold-standard, antibody 92160 (Hartmann *et al.* 2000). The coefficient of determination R^2 was 0.77 ($p<0.01$) for the detection of postprandial increase of GLP-2.

GLP-1 (7-36) was measured using an established specific and sensitive in-house assay (Kreymann et al. 1987). The GLP-1 antibody was raised in rabbits against GLP-1 coupled by carbodi-imide to BSA. It cross reacts 100%

with all amidated forms of GLP-1 but does not cross react with glycine extended forms [GLP-1 (1-37) and GLP-1 (7-37)] or any other known pancreatic or gastrointestinal peptide. 125I-GLP-1 (7-37 amide) was prepared by the iodogen method (Wood et al., 1981) and purified by HPLC. The specific activity of the 125I GLP-1 label was 48 Bq/fmol. The assay was performed in a total volume of 0.7 ml of 0.06 M sodium barbitone buffer (pH 8) containing 0.3 % BSA. The assay was incubated for three days at 4ºC before separation of the free and antibody bound label by charcoal absorption. The limit of detection was 7.5p mol/l, with an intra-assay variation of 5.4%. All samples were assayed in duplicate and in a single assay.

4.2.5 Statistics

Data analysis of the data was performed in Excel and MedCalc ® software. Normality of data distribution was tested by using the Kolmogorov-Smirnov test. Data are expressed as mean values ± SEM. For comparison of the postprandial secretory response to the 3 meals within a subject group, we used one way ANOVA and the Student-Newman-Keuls test for post hoc pair wise comparisons. Differences in postprandial response between lean and obese were compared with the use of two tailed Student's t tests. The values for the area under the curve (AUC) were calculated using the trapezoid rule.

4.3 Results

There was no difference in the **fasting** hormone concentration levels between normal weight controls (NWC) and obese subjects for either GLP-1 or tGLP-2-IR (Table 4-2, Figure 4-1).

The **meal stimulation** curves for all groups are shown in Figures 4-2 to 4-4. The 500ml-250kcal meal did not elicit a significant increase of GLP-1 or tGLP-2-IR from baseline at any time point.

To investigate the postprandial secretory response, the increase of postprandial plasma concentration from baseline for each postprandial time-point as well as the change of area under the curve from baseline (Δ AUC 30-180 minutes) was determined.

It was firstly evaluated, how the subject groups responded to the increasing calorie load (Table 4-3). In the 500ml groups, Δ AUC (GLP-1 and tGLP-2-IR) was significantly greater following the 1000kcal meal compared to the 250 and 500kcal meal in NWC subjects. There was a similar trend in the obese subjects but this difference was not significant. In the 900ml groups, there was no difference in the GLP-1 response between the 1000, 2000 and 3000 kcal meal in the NWC group, whereas in the obese subjects the greatest response was achieved at 3000kcal. Δ AUC tGLP-2-IR was greatest in the 2000kcal group in NWC subjects, whereas there was no significant difference between the 3 meals in the obese subject group.

Meal response curves, comparing the postprandial increase of GLP-1 and tGLP-2-IR plasma concentration from baseline between NWC and obese for each time-point, are illustrated in Figure 4-6 (500ml groups) and 4-7 (900ml groups). In the 500ml meal groups a significant difference between NWC and the obese is only shown in a single time-point (GLP-1, 90min, 250 kcal). This is unlikely to have physiological relevance. In the 900ml meal groups, GLP-1 concentration appears higher in the NWC group at 2000kcal (120min

and 150min); whereas the GLP-1 concentration at these time points appears lower at 3000kcal.

Accordingly the only significant differences of Δ AUC (Figure 4-8A) are shown in these 2 groups: Δ AUC (GLP-1, 30-180 minutes) in the 2000 kcal meal, showed a higher level in the NWC, whereas in the 3000 calorie meal, Δ AUC (GLP-1) was significantly higher in the obese. This was not accompanied by corresponding differences in tGLP-2-IR concentration. Investigating the early postprandial response only (Δ AUC, 30-60 minutes), no difference between NWC and obese groups was shown in any meal (Figure 4-8B).

Le Roux et al. have shown a consistent reduction of PYY 90min-peak secretory response in the same subject group (Le Roux *et al.* 2006). This time point was therefore analysed separately and comparative peak hormone concentrations for all meals are illustrated in Figure 4-9. It is apparent that the trend of reduced L-cell hormone secretion in the obese, shown for PYY, can't be demonstrated for GLP-1 or tGLP-2-IR. There were also no significant differences in the 90min peak hormone concentrations of any meal if only obese subjects with insulin resistance (HOMA-IR > 3.8; n=5 in 500ml group and n=4 in 900ml group) were compared with NWC.

The correlation between GLP-1 and tGLP-2-IR was calculated for all samples and is detailed in Figure 4-10. There was no correlation between the fasting samples. This is due to the fact that, as detailed in chapter 2, tGLP-2-IR correlates only poorly to the concentration of active glucagon-like peptide secretion. A significant positive correlation was demonstrated for all postprandial time points as well as for the maximum increase from baseline.

Fasting Levels of GLP-1 and tGLP-2-IR

	Time	NWC	Obese	p
GLP-1 (pmol/l)	**-30** **0**	**21.4** **20.8**	**20.6** **18.9**	**0.5** **0.1**
tGLP-2IR (pmol/l)	**-30** **0**	**195.9** **185.1**	**217** **209**	**0.1** **0.07**

Table 4-2: **Fasting hormone concentrations (mean)**

p (unpaired student t-test, normal weight controls (NWC) vs. obese subjects)

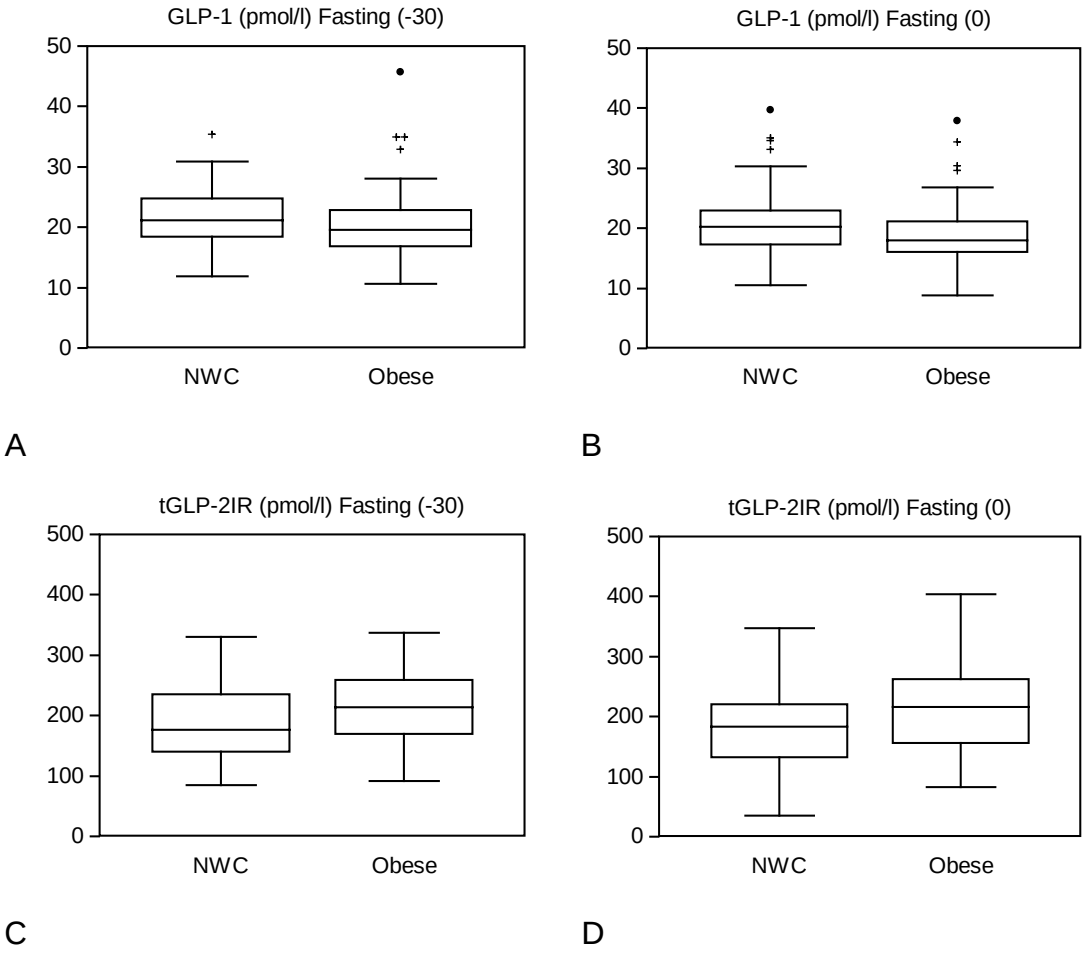


Figure 4-1: **Fasting hormone concentrations at time -30 and 0**

Median values, 25 to 75th percentile, minimum to the maximum value and outside values are shown.

Meal stimulated plasma concentration of GLP-1:

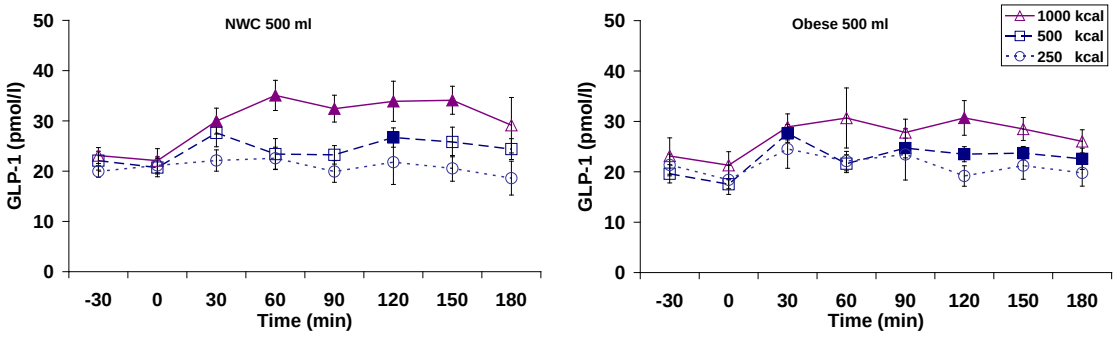


Figure 4-2: **GLP-1 meal response curves (500ml)**

Fasting (time -30-0) and postprandial (time 30-180) plasma concentration of GLP-1 in obese subjects (n=9) and normal weight controls (NWC, N=10) consuming a 500ml test-meal with increasing caloric content (circle: 250kcal, square 500kcal, triangle 1000kcal)

Values are mean (SED)
Filled points = t-test, p<0.05 vs. fasting (time 0)

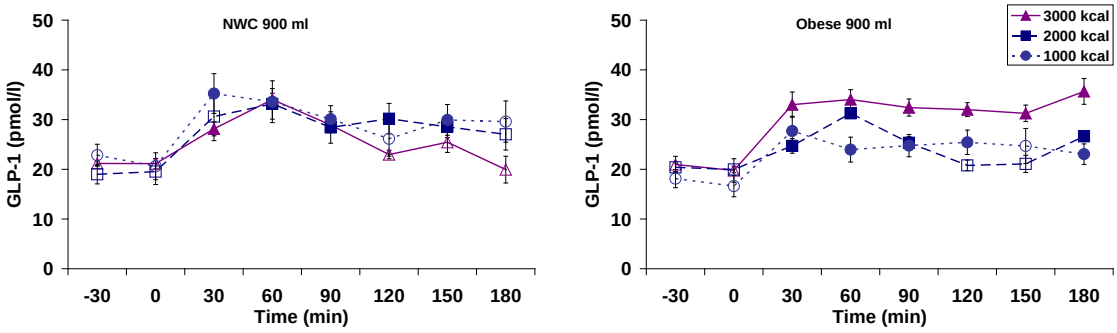


Figure 4-3: **GLP-1 meal response curves (900ml)**

Fasting (time -30-0) and postprandial (time 30-180) plasma concentration of GLP-1 in obese subjects (n=10) and normal weight controls (NWC, n=9) consuming a 900ml test-meal with increasing caloric content (circle: 1000kcal, square 2000kcal, triangle 3000kcal)

Values are mean (SED)
Filled points = t-test, p<0.05 vs. fasting (time 0)

Meal stimulated plasma concentration of tGLP-2-IR

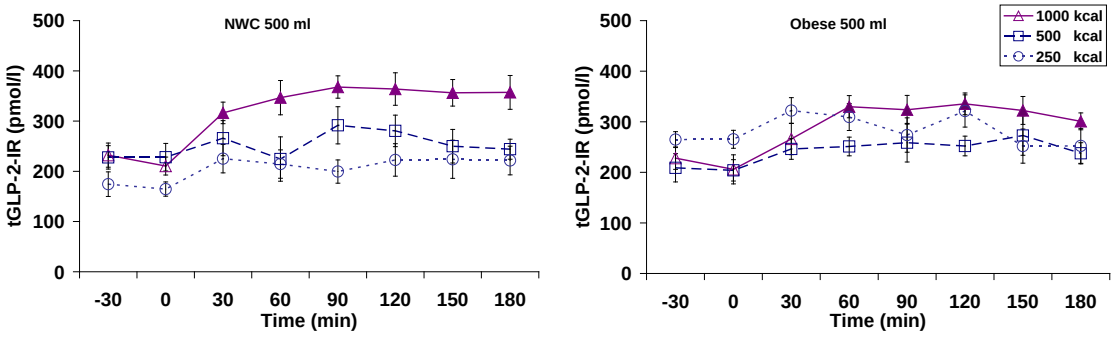


Figure 4-4: tGLP-2-IR meal response curves (500ml)

Fasting (time -30-0) and postprandial (time 30-180) plasma concentration of tGLP-2-IR in obese subjects (n=9) and normal weight controls (NWC, N=10) consuming a 500ml test-meal with increasing caloric content (circle: 250kcal, square 500kcal, triangle 1000kcal)

Values are mean (SED)
Filled points = t-test, p<0.05 vs. fasting (time 0)

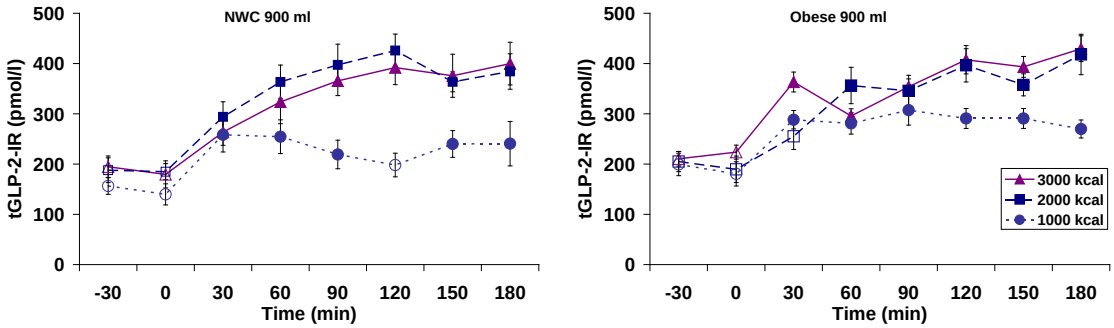


Figure 4-5: tGLP-2-IR meal response curves (900ml)

Fasting (time -30-0) and postprandial (time 30-180) plasma concentration of tGLP-2-IR in obese subjects (n=10) and normal weight controls (NWC, n=9) consuming a 900ml test-meal with increasing caloric content (circle: 1000kcal, square 2000kcal, triangle 3000kcal)

Values are mean (SED)
Filled points = t-test, p<0.05 vs. fasting (time 0)

Δ AUC (30-180min) in response to increasing caloric load within each subject group:

GLP-1(pmol/l)

ml	kcal	Group	n	Δ AUC 180
500	250	NWC	9	151 (285)[3]
	500	NWC	9	629 (256)[3]
	1000	NWC	9	1716 (323)[1,2]
			p	***0.003***
900	1000	NWC	8	1621 (240)
	2000	NWC	8	1768 (352)
	3000	NWC	8	981 (168)
			p	0.1
500	250	Obese	8	314 (268)
	500	Obese	8	858 (341)
	1000	Obese	8	1275 (200)
			p	0.07
900	1000	Obese	8	1264 (276)[3]
	2000	Obese	8	761 (143)[3]
	3000	Obese	8	2049 (251)[1,2]
			p	***0.003***

tGLP-2-IR (pmol/l)

ml	kcal	Group	n	Δ AUC 180
500	250	NWC	9	8100 (4900)[3]
	500	NWC	9	5710 (4020)[3]
	1000	NWC	9	20220 (2440)[1,2]
			p	***0.03***
900	1000	NWC	7	15590 (4600)[2]
	2000	NWC	7	30230 (3330)[1]
	3000	NWC	7	26670 (4010)
			p	***0.0048***
500	250	Obese	6	4770 (4170)
	500	Obese	6	3380 (3990)
	1000	Obese	6	14680 (3270)
			p	0.1
900	1000	Obese	9	16300 (2840)
	2000	Obese	9	24940 (3720)
	3000	Obese	9	25290 (3110)
			p	0.1

Table 4-3: **Δ AUC of postprandial response**

Δ AUC (over 180min) in response to increasing calorie load within each subject group.

Data are expressed as mean values ± SEM
p was determined with one way ANOVA.
If $p<0.05$; Student-Newman-Keuls test for post hoc pair wise comparisons was applied.

Only subjects for whom samples of all three meals were available were included.

Increase from baseline in obese subjects and normal weight controls (NWC); 500ml meal

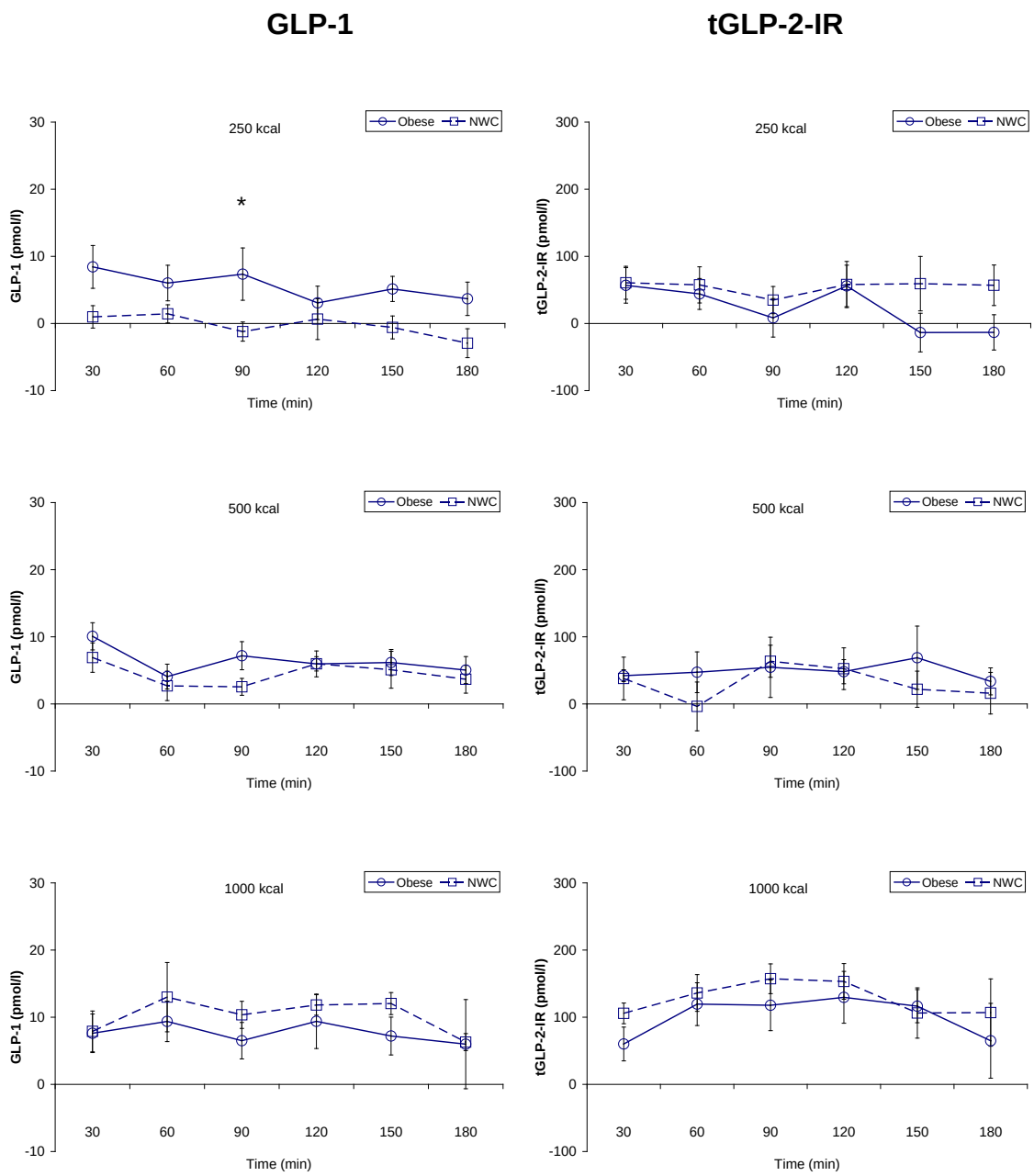


Figure 4-6: **Postprandial increase of plasma GLP-1 and tGLP-2-IR concentration (30-180min)**

Obese subjects and normal weight controls (NWC)
Consuming a 500ml meal of 250, 500 and 1000 kcal
Data are expressed as mean values ± SEM
*p<0.05 (Student T-test, obese vs. NWC)

Increase from baseline in obese subjects and normal weight controls (NWC); 900ml meal

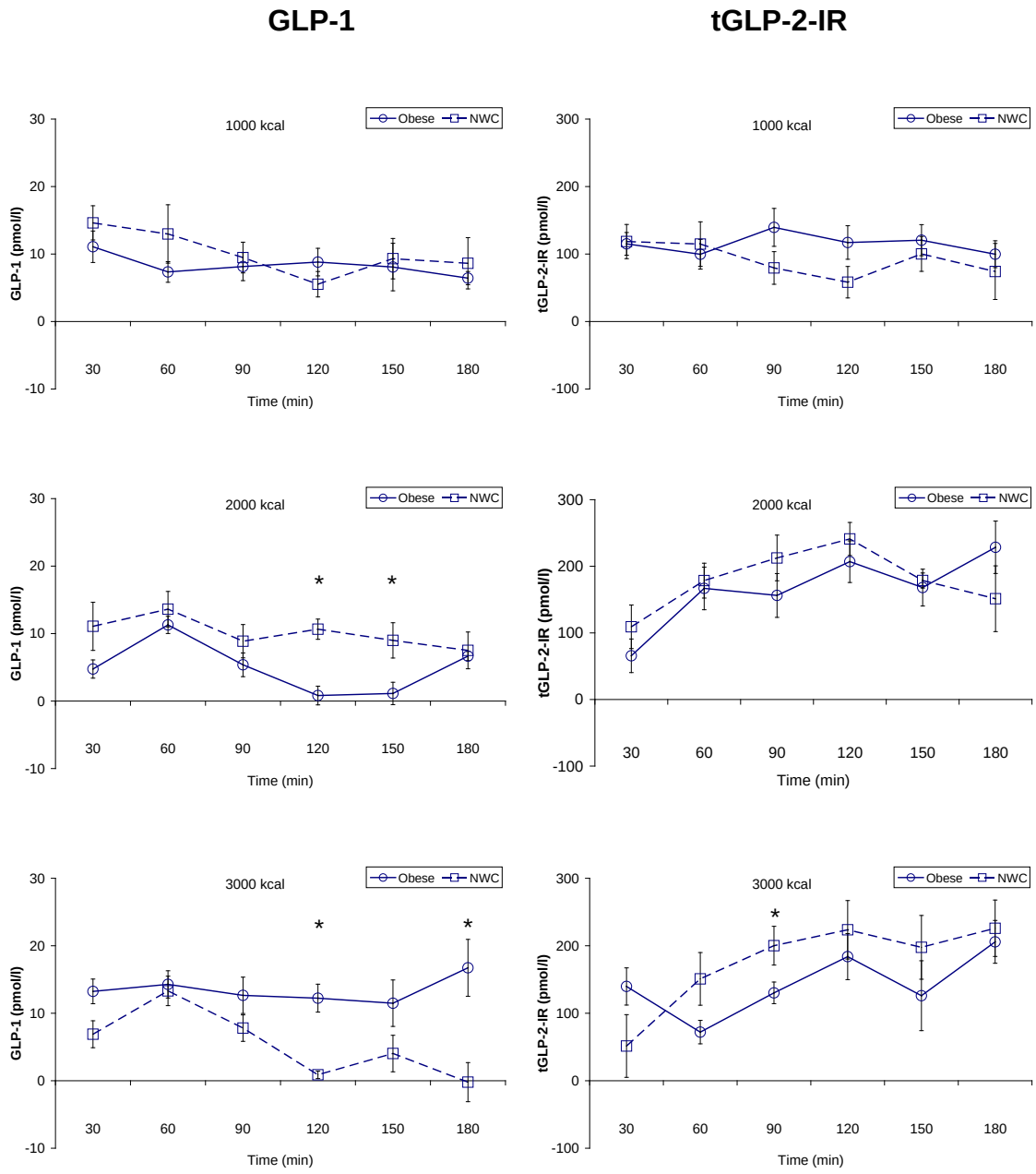
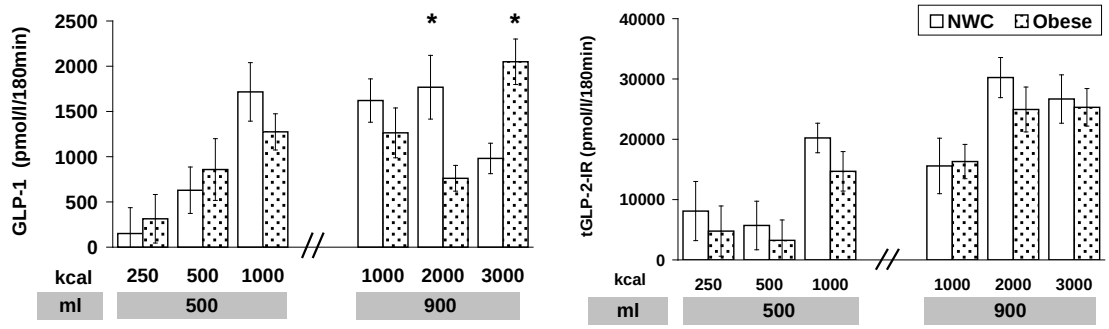


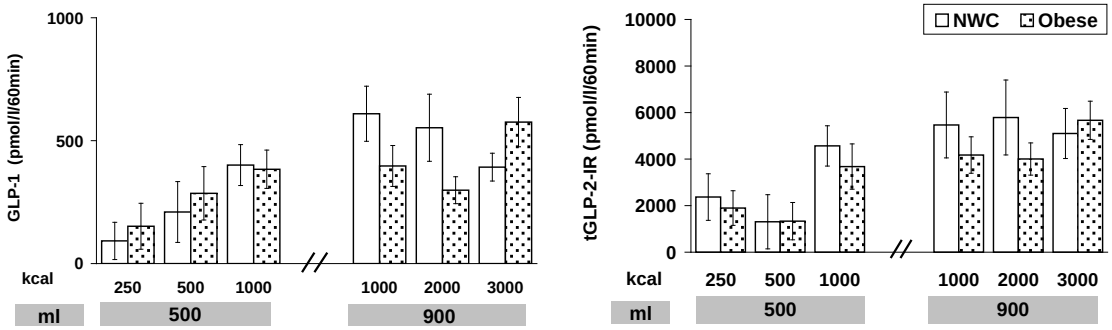
Figure 4-7: **Postprandial increase of plasma GLP-1 and tGLP-2-IR concentration (30-180min)**

Obese subjects and normal weight controls (NWC)
Consuming a 900ml meal of 1000, 2000 and 3000 kcal
Data are expressed as mean values ± SEM
*p<0.05 (Student T-test, obese vs. NWC)

Δ AUC in obese subjects and normal weight controls (NWC)



A: Δ AUC from fasting (time 0) **to 180 min**



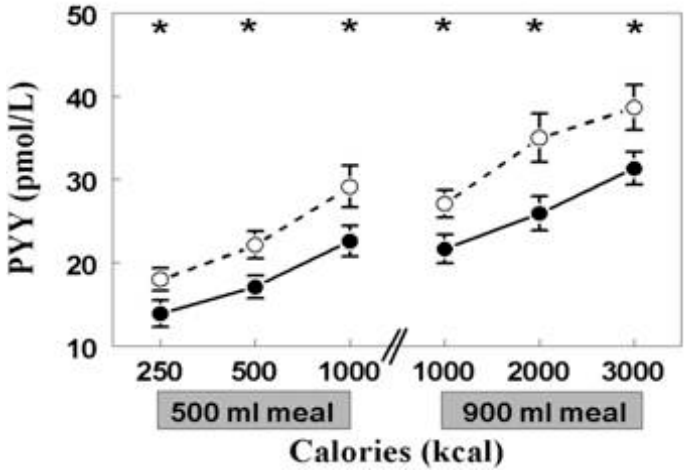
B: Δ AUC of early postprandial response (from fasting (time 0) **to 60 min**)

Figure 4-8: **Δ AUC (GLP-1 and tGLP-2-IR)**

Obese subjects (dotted bar) and normal weight controls (NWC, empty bar)

A: over 180 minutes
B: over 60 minutes (early postprandial response)

Data are expressed as mean values ± SEM
***p<0.05, unpaired student-t test**



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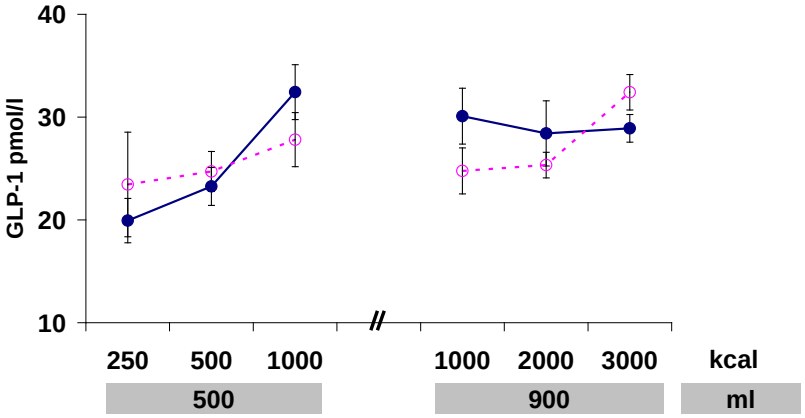

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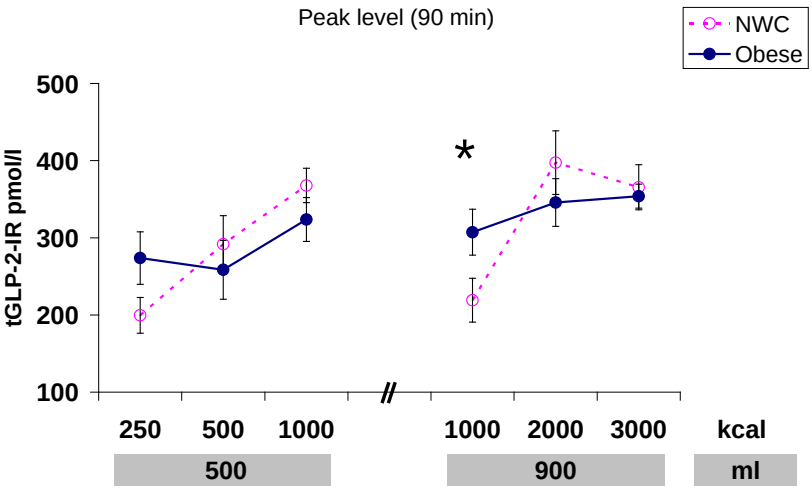

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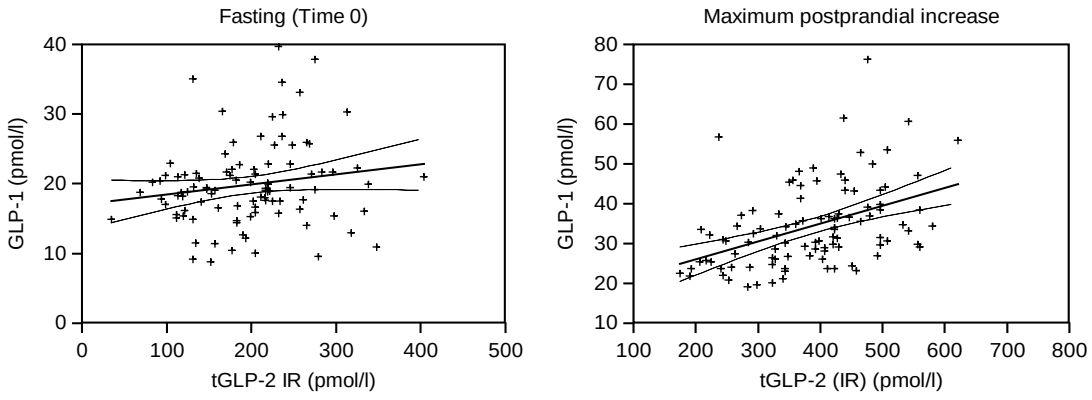
Figure 4-9: **Peak concentration (pmol/l) at the 90 minute time-point in obese (bullet) and normal-weight controls (NWC) (empty circle).**

A) Peak PYY level, *p<0.05 (unpaired t-test) (Le Roux et al. 2006).
B) GLP-1
C) tGLP-2-IR
* p=0.048 (unpaired t-test NWC vs. Obese)

Correlation between GLP-1 and tGLP-2-IR

Correlation of GLP-1 and tGLP-2-IR plasma concentration					
	Time	**n**	**r**	**r^2**	**p**
Fasting	**-30**	100	0.2069	0.04	**0.03**
	0	98	0.1684	0.03	**0.1**
Postprandial	**30**	98	0.3306	0.11	**0.0009**
	60	98	0.401	0.16	**<0.0001**
	90	100	0.4154	0.17	**<0.0001**
	120	99	0.3889	0.15	**0.0001**
	150	98	0.3605	0.13	**0.0003**
	180	91	0.4378	0.19	**<0.0001**
Max	**30-180**	100	0.4426	0.20	**<0.0001**

A



B **C**

Figure 4-10: **Correlation between GLP-1 and tGLP-2-IR**

A: All fasting and postprandial time-points
(Differences in n-numbers due to missing samples)

B: Fasting (linear regression line and 95% CI)

C: Point of maximum postprandial increase (linear regression line and 95% CI)

4.4 Discussion

Hormones, released from the gastrointestinal tract in response to nutrients, have received attention for their possible role in the initiation, termination and frequency of meals. It is thought that an imbalance of anorexigenic (e.g.: Cholecystokinin, PYY, Oxyntomodulin, GLP-1) and orexigenic (e.g.: ghrelin) mediators may result in disorders of feeding behaviour.

Attenuation of PYY, a L-cell product, has been reported in the obese (Le Roux et al. 2006). PYY reduces food intake in rodents and humans. It was postulated that lower postprandial PYY levels may result in an increase in food intake to achieve the same level of satiety as seen in normal-weight subjects and thus form part of the pathophysiological mechanism that helps to maintain the obese state.

Similar observations have been shown for glucagon-like peptides. Some studies have demonstrated attenuation of fasting levels and postprandial GLP-1 in the obese (Ranganath et al. 1996; Verdich *et al.* 2001; Adam et al. 2005). However, these findings have not been consistent. Importantly, no differences were found when nutrients were given intraduodenally, thus avoiding the influence of gastric emptying (Fukase *et al.* 1993; Feinle *et al.* 2002). In this study obese subjects and healthy normal weight controls (NWC) exhibited similar fasting levels of GLP-1 and tGLP-2-IR. There was also no meaningful difference in early and overall postprandial secretory response to a large range of caloric challenges.

A variety of factors might account for discrepancies between the studies. In a report of decreased GLP-1 secretion in obese diabetic patients, compared with matched obese healthy subjects, it was suggested that the presence and magnitude of insulin resistance might influence GLP-1 secretion in the obese (Vilsboll et al. 2003). Addressing this point, we analysed the data, including only insulin resistant obese subjects. No significant attenuation of the secretory profile in the obese was found and no trend was apparent.

However, the subject numbers were not large enough to exclude small changes confidently. It has also been suggested that the obese have slightly decreased sensitivity for GLP-1 release that could be overcome by providing a stronger nutritional challenge (Adam *et al.* 2005). This study did not detect any differences over a wide range of caloric stimulation. Nutrient composition might be another relevant factor. Our study used a meal stimulus with mixed nutrient composition. Thus, Ranganath's suggestion that L-cell responsiveness to carbohydrates might be particularly affected in the obese could not be tested (Ranganath *et al.* 1996). We aimed to investigate the secretory response to a standard nutritional stimulus. It remains to be explored in a future study whether adjusting each test meal for the individual's metabolic rate would provide additional information. Lastly, the antibodies used for radioimmunoassay were not specific for active forms of GLP-1 or GLP-2 and thus could not detect subtle changes in secretion.

The results of this study, suggesting similar fasting levels and postprandial responses of glucagon-like peptide concentrations in lean and obese subjects contrast the finding of attenuated PYY release in the obese subjects of the same study group (Le Roux *et al.* 2006). This is particularly interesting since glucagon-like peptides and PYY can be found in the same cells and are co-localised in secretory granules. However, this co-localization does not appear exclusive. It has been shown in rabbits that colonic L-cells can exhibit different populations of secretory granules (Nilsson *et al.* 1991). Moreover, differential secretion of PYY and proglucagon derived peptides in response to specific nutritional stimuli has been demonstrated in rodents (Anini 1999) and also in patients with short bowel syndrome (chapter 3). In this context, a possible explanation for our findings could be that the stimulation of proPYY and proglucagon synthesis is differentially regulated in the obese and thus may lead to altered L-cell peptide content compared to normal weight subjects. Alternatively, different secretion patterns following luminal stimulation could be caused by a predominant loss of sensitivity for PYY secretion in the obese. A previous report of disproportionate postprandial secretion of PYY and GLP-2 in pigs has suggested that GIP may have a differential effect on L-cell hormone secretion (van Goudoever et al. 2001).

The strength of this study lies in the fact that glucagon-like peptide concentrations could be compared with PYY measurements from the same subject groups. A significant drawback however is the small subject number within each group. In view of the high standard deviations for both GLP-1 and GLP-2 measurements, type II errors can't be excluded. The statistical power to detect a 20% reduction in the 90 minute postprandial GLP-1 peak was 56% on average (range 35-96% depending on meal and time-point). It would not be practically feasible to repeat the same experimental design with a greater subject number. In order to confirm the findings presented here, a future study should utilize a single meal with a good post-prandial response (e.g. using 1000kcal). Estimating a 90 minute peak of 30 pmol/l in NWC and a SD between subjects of 7 pmol/l; at least 17 (68) subjects need to be recruited into both study groups (NWC and obese) to allow 80% power of detecting a significant ($p<0.05$) reduction of 20% (10%) in the obese.

Summary: In contrast to an attenuated postprandial PYY response, no evidence of a reduction in glucagon-like peptide secretion could be found in obese subjects. It may be possible that the balance of glucagon-like peptides and PYY in secretory L-cells granules is altered in the obese or that the secretion of these peptides is regulated by differential signals. Limitations of this study in view of limited subject number must be taken into consideration.

Chapter 5

Glucagon-like peptide-2 response after Roux-en-Y gastric bypass

5.1 Introduction and aim

Bariatric surgery is not only the most effective means of achieving sustained weight loss in subjects with morbid obesity; it also consistently alleviates obesity-related complications (Sugerman et al. 2003; Buchwald et al. 2009). The Roux-en-Y gastric bypass (RYGB), a technique in which a small stomach pouch is joined to the proximal jejunum through a narrow gastro-jejunal anastomosis (Figure 5-1) achieves sustained loss of 35–40% of total body weight, and most of this effect is maintained for at least 15 years (Cummings et al. 2004). Although initially evolving from procedures designed to cause malabsorption, subjects undergoing RYGB report only transient gastrointestinal side effects and, despite the loss of intestinal length, macronutrient malabsorption is rarely clinically relevant. The degree of weight loss appears to be disproportionate to the degree of macronutrient malabsorption, suggesting that a change in energy intake is the principal mechanism of weight loss after RYGB (Cummings et al. 2004). Recently, the postsurgical alteration of gut hormone secretion, in particular changes in PYY and GLP-1 concentration, has been implicated in causing decreased appetite and calorie intake. The insulinotropic properties of GLP-1 may contribute to the early resolution of type 2 diabetes (Borg et al. 2006; le Roux et al. 2007).

Morphological adaptation of the intestine might also contribute to reducing post-operative malabsorption. An increase in small bowel villus height has been described in humans following bilio-pancreatic diversion, a similar bariatric procedure to RYGB (Stock-Damge et al. 1986). Intestinal thickening and enhanced proliferative activity can be shown in rodents following RYGB (le Roux, unpublished results). A change in intestinal growth factor concentration has not yet been demonstrated in this context. The intestinotrophic gut hormone glucagon-like peptide-2 (GLP-2) is a key mediator of post-resection intestinal adaptation (see chapter 1). GLP-2 levels correlate with the magnitude of gut resection, the severity of malabsorption,

and the degree of crypt cell proliferation (Martin *et al.* 2005). We aimed to determine changes in GLP-2 levels in obese patients after RYGB.

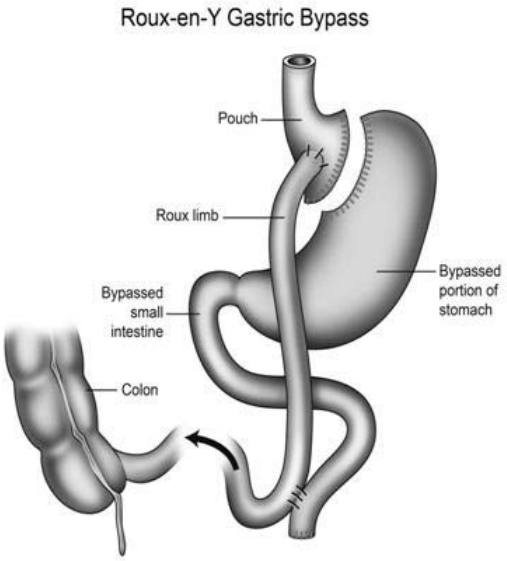


Figure 5-1: Principle of Roux-en-Y Gastric bypass.

The technique involves creating a stomach pouch out of a small portion of the stomach and attaching it directly to the small intestine through gastro-jejunal anastomosis. Thus, storage capacity of the stomach is reduced. Ingested food bypasses approximately 95% of the stomach, the entire duodenum, and a small portion of the proximal jejunum. (Figure from the Imperial Weight Centre at Imperial College Healthcare NHS Trust, with permission.)

5.2 Methods

The study was performed according to the principles of the Declaration of Helsinki and approved by the Local Research and Ethics Committee at King's College Hospital, London (LREC protocol number: 01-03-020) and Musgrove Park Hospital, Taunton (LREC protocol number: 05/Q2202/96). All participants gave written consent to take part in the study after receiving oral and written information.

This study was performed in collaboration with Dr. Carel Le-Roux who developed the experiment and coordinated subject recruitment and sample collection. The surgical procedures were performed by Mr Richard Welbourn. Sample analysis with radioimmunoassay and data interpretation was performed by the author.

5.2.1 Subjects

Obese patients (WHO grade III, body mass index (BMI) > 40 kg/m^2) attending an obesity clinic, who had elected to undergo RYGB, with stable weight for at least 3 months prior to recruitment, were invited to participate.

Laparoscopic Roux-en-Y gastric bypass was performed creating a small isolated lesser curve-based gastric pouch excluding the fundus. The length of the Roux limb was 100 cm for patients with a body mass index (BMI) below 50 kg/m^2 and 150 cm for patients with a BMI above 50 kg/m^2.

Diabetes was defined according to the World Health Organisation definition (– fasting plasma glucose ≥ 7.0mmol/l or 2–h plasma glucose after ingestion of 75g oral glucose load ≥ 11.1mmol/l).

Exclusion criteria included pregnancy, substance abuse and more than two alcoholic drinks per day.

Two subject groups were investigated pre- and postoperatively. Each patient acted as their own control in a prospective controlled study design:

In **study 1 (short term follow up),** 5 male and 11 female subjects; mean age 48 (±2.0) years; mean preoperative BMI 49 (±1.3) kg/m^2 were recruited. Plasma samples were collected according to protocol **before and 2, 4, 7 and 42 days after RYGB**.

In study 2 (long term follow up), 6 female obese patients, mean age 47 (±3.4) years; mean preoperative BMI 48 (±1.4) kg/m^2 were recruited. Plasma samples were collected according to protocol **before and 1, 3, 6, 12 and 24 month after RYGB**. The RYGB procedures were performed laparoscopically with a Roux limb of 112 cm.

5.2.2 Study Protocol

Patients attended after a 12-hour fast, an intravenous cannula was sited and venous samples were taken at baseline and at 30 min intervals thereafter up to 180 min following a standard semi-liquid mixed 420kcal meal. Plasma was collected, immediately separated and stored at -70ºC until analysis.

5.2.3 Radioimmunoassay

Total GLP-2-IR was measured using antiserum FT-17 as described in chapter 2. FT-17 cross-reacts 100% with human GLP-2, less than 0.1% with GLP-1 and does not cross-react with any other known gastrointestinal or pancreatic hormone. FT-17 detects intact GLP-2 (1-33), its primary degradation product GLP-2 (3-33) and major proglucagon fragment (MPGF). Detection limit was 25pmol/l; intra-assay CV 8.3%. We validated our antibody FT-17 by correlating it with the current gold-standard, antibody 92160 (Hartmann *et al.* 2000). The coefficient of determination R^2 was 0.77 ($p<0.01$) for the detection of postprandial increase of GLP-2.

5.2.4 Statistics

Data are expressed as mean ± SEM unless stated otherwise. tGLP-2-IR concentration is described as peak concentration, increase from fasting and by calculation of the area under the curve (AUC) using the trapezoidal rule. Fasting concentrations were compared with repeated measure ANOVA. Total tGLP-2-IR AUC (0-180 min) was determined for each individual response, and responses were analysed by repeated measures analysis of variance (ANOVA). Comparisons were made for the increase in AUC against the pre-operative response using repeated measures ANOVA, and where the F-statistic was significant, using paired student t-test.

5.3 Results

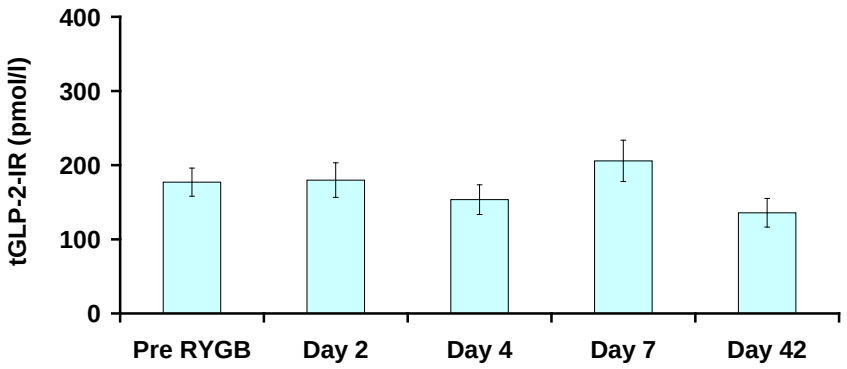
5.3.1 Study 1 (short term follow up)

Fasting levels of tGLP-2-IR remained constant during the post-operative period to day 42 (p=0.3); Figure 5-2 A.

During the preoperative meal, the 420 kcal **nutritional stimulus** did not cause a significant increase of the post-prandial tGLP-2-IR concentration from fasting at any time point (p=0.9, single factor ANOVA). The maximum peak occurred at 120minutes (209 ± 25pmol/l). The increase was equal to 23% of the fasting value. Following RYGB, postprandial tGLP-2-IR concentrations were significantly raised compared to fasting levels between 30 and 120 minutes in every test meal up to 42 days. The maximum post-prandial peak occurred at 60 minutes (day 2, 4, 7) or 30 minutes (day 42); with the highest value of 396±65pmol/l at day 42 (increase equal to 185% of the fasting value, Figure 5-2B). Postprandial profiles before and after RYGB (for day 2 and 42) are shown in Figure 5-2C.

The area under the curve (AUC 0-180min) was calculated for every meal-stimulated tGLP-2-IR profile. The tGLP-2-IR AUC in post-RYGB meals was significantly increased in every meal up to 42 days post surgery with a maximum AUC increase at day 7 (85% from pre-RYGB meal (Table 5-1 and Figure 5-3).

There was a significant disparity in the tGLP-2-IR response between diabetic (n=8) and non-diabetic subjects (n=8) at day 2 post RYGB. In contrast to the increased postprandial response in non-diabetics, diabetic subjects had a flat postprandial meal profile, similar to the pre-operative response. This difference gradually reduced over the trial period (Figure 5-4, Figure 5-5; Table 5-2).



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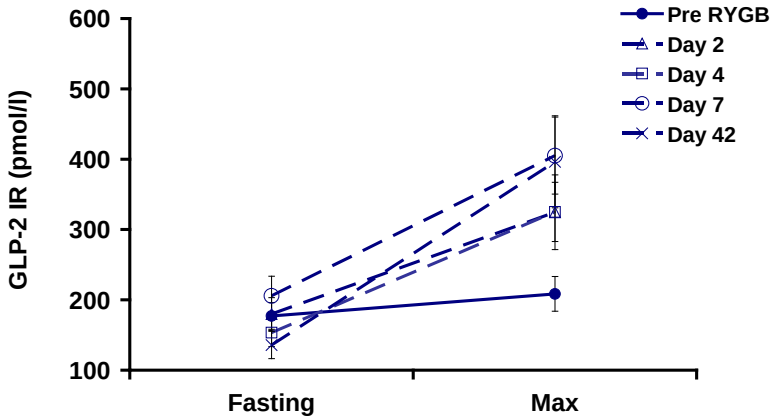

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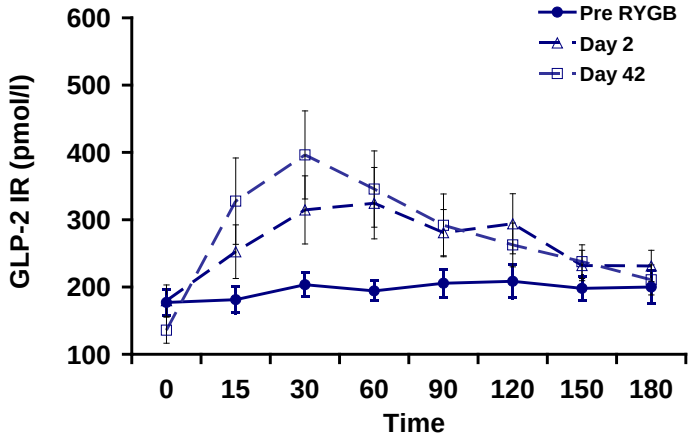


C

Figure 5-2: **Meal related tGLP-2-IR plasma concentration pre- and up to 42 days post RYGB (n=16).**

A) Fasting tGLP-2-IR.

B) Fasting and maximum tGLP-2-IR. Solid line; pre RYGB (p=0.2 fasting vs. max), broken lines post RYGB (p<0.05 fasting vs. max for all meals).

C) Meal profiles before and after RYGB (shown for day 2 and 42; day 4 and 7 have similar appearance to day 2 graph and were omitted for clarity).

All depicted values represent the mean (SEM).

	pre RYGB	post RYGB			
		Day 2	Day 4	Day 7	Day 42
AUC (pmol/l)	**35726** (3191)	**50227** (6493)	**50121** (5242)	**60599** (6312)	**51428** (6555)
Δ AUC (pmol/l)		**14623** (5463)	**14724** (4612)	**24573** (5280)	**18492** (5534)
Δ AUC (%)		**47** (17)	**52** (16)	**85** (23)	**73** (27)
p		*0.018*	*0.007*	*0.0004*	*0.032*

Table 5-1: **Total tGLP-2-IR AUC (0-180) and postoperative change (Δ AUC)**

n=16
Values are mean (SEM)
Total AUC ANOVA: p=0.04
p= paired student t–test (total AUC pre vs. post RYGB)

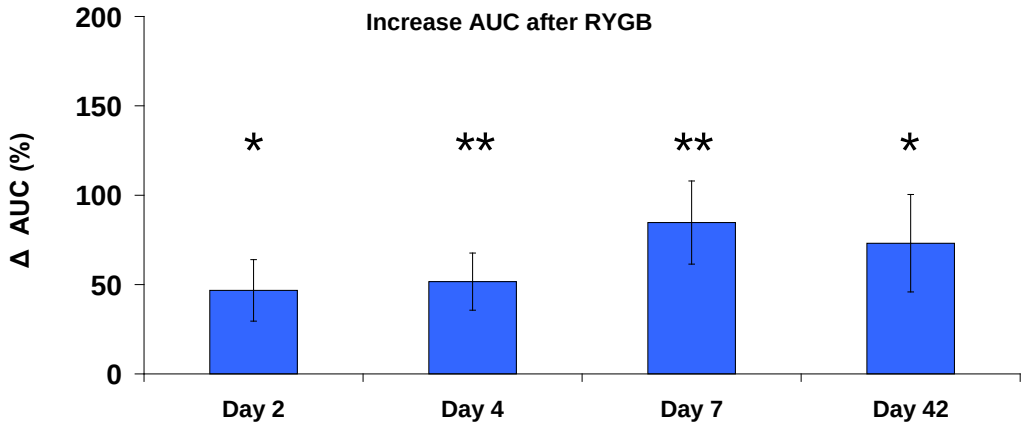


Figure 5-3: **Postoperative change of tGLP-2-IR AUC (0-180) (% change from pre-RYGB)**

n=16
Values represent mean (SEM)
**p<0.01, *p<0.05 (paired student t–test, total AUC pre vs. post RYGB)

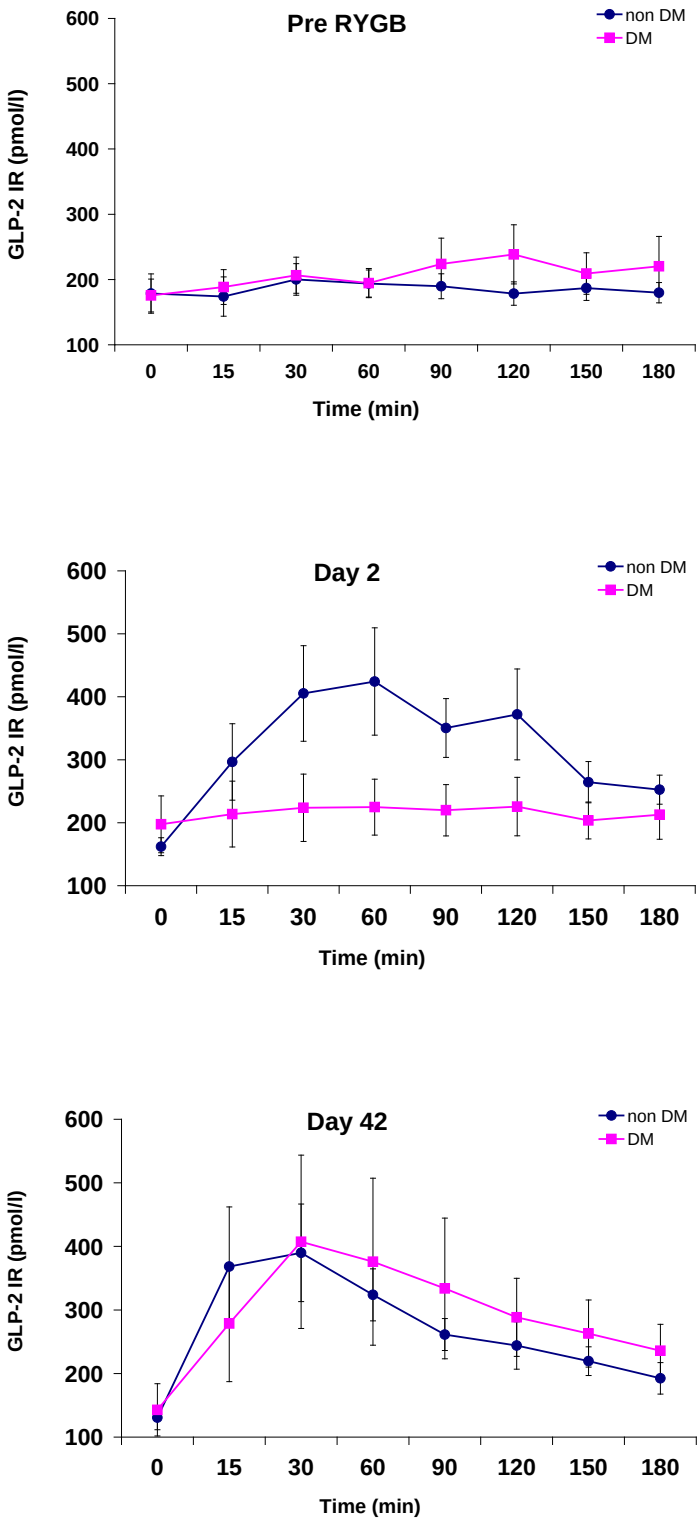


Figure 5-4: **tGLP-2-IR meal profiles pre-and post RYGB; diabetic and non-diabetic subjects**

n=8 for each group
Values represent the mean (SEM)

		pre RYGB	post RYGB			
			Day 2	Day 4	Day 7	Day 42
AUC	All	**35726** (3191)	**50227** (6493)	**50121** (5242)	**60599** (6312)	**51428** (6555)
(pmol/l/180min)	nonDM	**33621** (3202)	**62908** (9435)	**53971** (7039)	**66333** (6857)	**49296** (4936)
	DM	**37831** (5567)	**39132** (7323)	**45721** (8080)	**55582** (10316)	**54412** (15101)
nonDM / DM	*p*	*0.53*	*0.06*	*0.45*	*0.42*	*0.72*
Δ AUC	All		**14623** (5463)	**14724** (4612)	**24573** (5280)	**18492** (5534)
(pmol/l/180min)	nonDM		**29848** (8215)	**20351** (7063)	**32369** (6352)	**16312** (6655)
	DM		**1301** (2643)	**8293** (5190)	**17752** (7748)	**22307** (10866)
nonDM / DM	*p*		***0.004***	*0.17*	*0.15*	*0.97*
Δ AUC	All		**47** (17)	**52** (16)	**85** (23)	**73** (27)
(%)	nonDM		**96** (25)	**71** (26)	**110** (33)	**69** (37)
	DM		**4** (9)	**30** (15)	**63** (33)	**81** (44)
nonDM / DM	*p*		***0.003***	*0.21*	*0.33*	*0.85*

Table 5-2 **Total tGLP-2-IR AUC (0-180) and postoperative change (Δ AUC) in diabetic and non-diabetic subjects**

n=8 for each group
Values are mean (SEM)
p= unpaired student t-test, diabetic vs. non-diabetic subjects

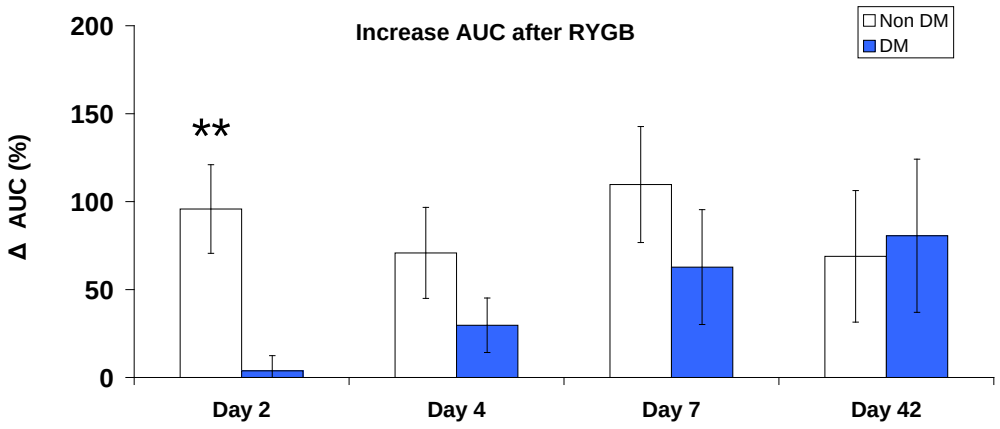


Figure 5-5: **Postoperative change of tGLP-2-IR AUC (0-180) (% increase from pre-RYGB) in diabetic (empty bars) and non-diabetic (filled bars) subjects.**

n=8 for each group
Values represent the mean (SEM)
**p<0.01 (unpaired student t-test, diabetic vs. non-diabetic subjects)

5.3.2 Study 2 (long term follow up)

This study recruited six female patients with a pre-operative BMI of 48.3 ± 1.4 kg/m^2. Post-RYGB mean **body weight and BMI** showed significant reductions compared to pre-surgery values at all time points from 1 month to 24 months. Mean weight loss at 24 months was 39.2 ± 3.6 kg (BMI of 33.9 kg/m^2; Figure 5-6).

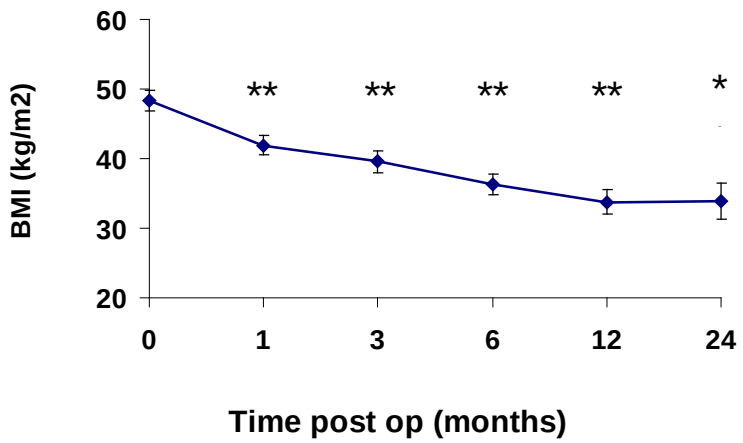


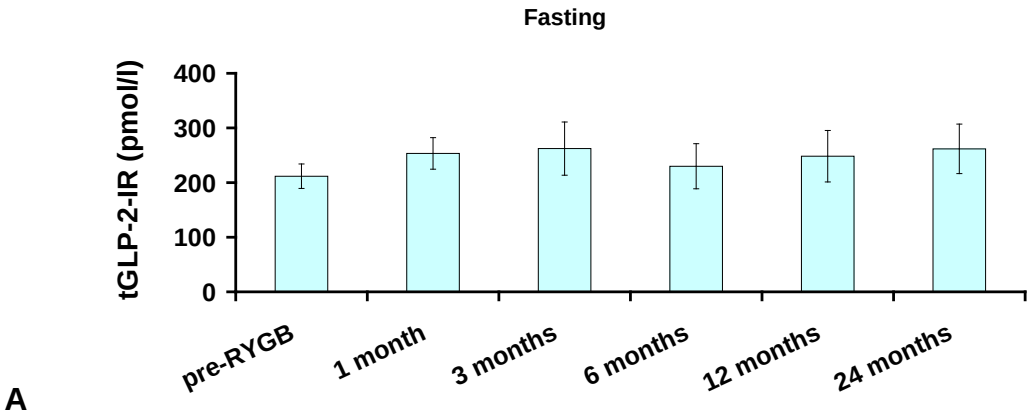
Figure 5-6: **Change of BMI**

6 female subjects following RYGB surgery.
Values represent the mean (SEM)
**p<0.01 (paired student t-test pre- vs. post RYGB).

Fasting levels of tGLP-2-IR remained constant throughout the pre- and post-operative period (p=0.9, single factor ANOVA; Figure 5-7a).

During the pre-RYGB meal, the 420 kcal **nutritional stimulus** did not cause a significant increase of the post-prandial tGLP-2-IR concentration from fasting at any time point (p=0.9, single factor ANOVA). The maximum peak occurred at 120 minutes (244±24pmol/l). The increase was equal to 15% of the fasting value. (Figure 5-7b, c). Following RYGB, postprandial tGLP-2-IR concentrations were raised compared to fasting levels at varying time-points between 30 and 120 minutes for up to 12 months. The maximum post-prandial peak occurred at 30 minutes (1, 3, 12, 24 months) or 90 minutes (6 months); with the highest value of 477±59pmol/l at 3 months (increase equal to 62% of the fasting value, Figure 5-7B). Postprandial profiles before and after RYGB (at 1, 6 and 24 months) are shown in Figure 5-7C.

The area under the curve (AUC 0-180min) was calculated for every meal-stimulated tGLP-2-IR profile. The tGLP-2-IR AUC in post-RYGB meals was significantly increased at 3 months and 6 months post surgery with a maximum AUC increase at 6 months (78% from pre-RYGB meal with a continued trend towards increase at 24 months (Table 5-3 and Figure 5-8).



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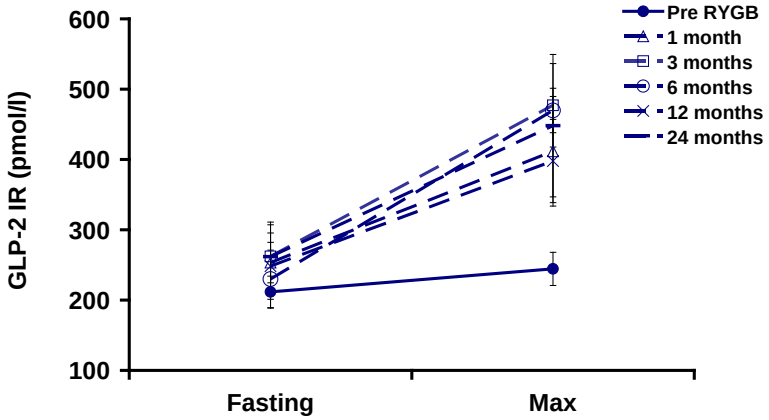

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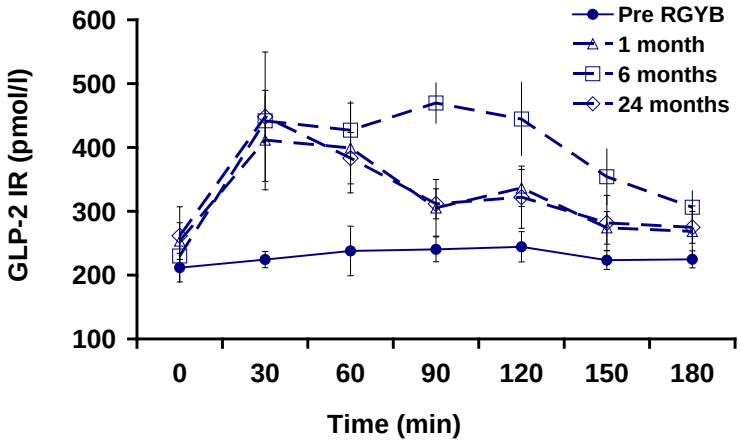


C

Figure 5-7: **Meal related tGLP-2-IR concentration pre- and post RYGB (n=6).**

A) Fasting tGLP-2-IR

B) Fasting and maximum tGLP-2-IR
Solid line; pre RYGB (p=0.2 fasting vs. max), broken lines post RYGB (p<0.05 fasting vs. max for all meals).

C) Postprandial profiles pre-and post RYGB
(shown for 1, 6 and 24 months; other time-points were omitted for clarity).

All depicted values represent the mean (SEM).

	pre RYGB	post RYGB (months)				
		1	3	6	12	24
AUC	**41656** (3110)	**59633** (7596)	**67403** (4976)	**72614** (5983)	**58744** (7652)	**60498** (5512)
Δ AUC		**17612** (7792)	**25747** (5712)	**30958** (6232)	**17089** (8205)	**15607** (5212)
Δ AUC (%)		**44** (18)	**66** (15)	**78** (16)	**45** (21)	**35** (13)
p		*0.087*	***0.006***	***0.004***	*0.092*	*0.058*

Table 5-3 **Total tGLP-2-IR AUC (0-180) and postoperative change (Δ AUC)**

n=6
Values represent mean (SEM)
Total AUC ANOVA: p=0.02
p= paired student t–test (total AUC pre vs. post RYGB)

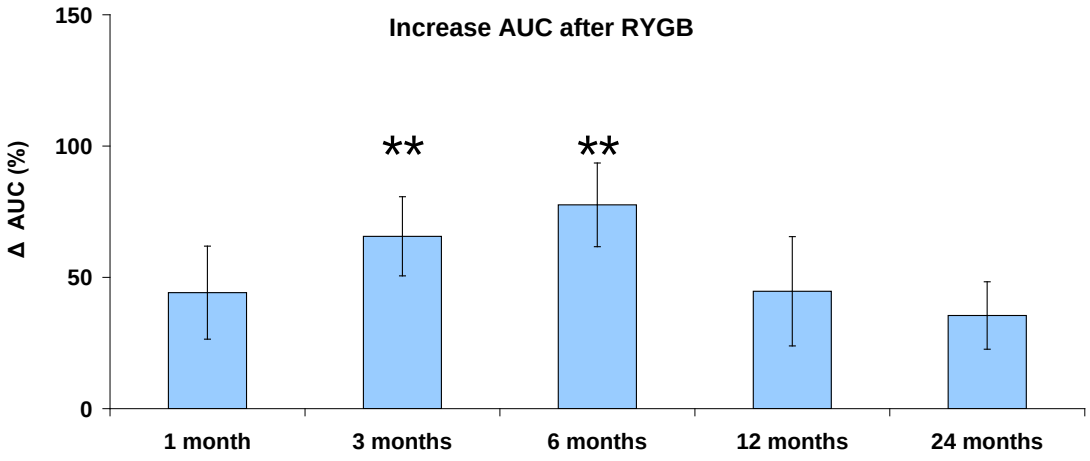


Figure 5-8 **Postoperative change of tGLP-2-IR AUC (0-180) (% change from pre-RYGB)**

n=6
**p<0.01 (paired student t–test; total AUC pre vs. post RYGB)
Values represent mean (SEM)

5.4 Discussion

The observation that bariatric surgical procedures, designed to bypass a significant portion of the intestinal tract, achieve lasting weight loss but only lead to modest and transient symptoms of malabsorption warrants further investigation. The Roux-en-Y gastric bypass (RYGB) procedure restricts the storage capacity of the stomach and allows undigested nutrients to bypass most of the stomach, duodenum and proximal jejunum. Nutrients and bilio-pancreatic secretions are united in the distal jejunum. Consequently, nutrients have access to a reduced absorptive surface area of the small intestine. Although deficiencies in vitamins and minerals have been recorded (Pournaras et al. 2009), macronutrient malnutrition is a rare complication (Skroubis et al. 2006). Instead it is thought that a reduction in energy intake, mediated by gastric restriction and post-surgical alteration of gut-hormone secretion, is the principal mechanism of weight loss after RYGB. Increased secretion of the anorectic intestinal hormones PYY and GLP-1 as well as suppression of the gastric “hunger hormone” ghrelin have been implicated in decreasing appetite and calorie intake (Cummings et al. 2004).

Morphological adaptation of the intestinal mucosa has been demonstrated in obese patients following bilio-pancreatic diversion, a similar bariatric procedure to RYGB, with an increase of villous height by 80% in the proximal and 58% in the distal ileum (Stock-Damge et al. 1986). Studies in a RYGB rodent model have shown increased cellular proliferation in the ileal crypts with increased mitoses and cellular uptake of the antibody to Ki67 (Carel Le Roux, unpublished data).

The results of this study demonstrate a significantly enhanced post-prandial release of the intestinotrophic hormone GLP-2 in obese patients after RYGB. This effect can be demonstrated as early as 2 days following surgery and is still evident 24 month post surgery. There is no demonstrable change in fasting tGLP-2-IR concentration at any time point following RYGB surgery. As detailed in chapter 2 however, changes in fasting GLP-2 (1-33)

concentration are difficult to exclude completely with the measurement method used.

Secretion of GLP-2 from enteroendocrine L-cells predominantly occurs in the distal jejunum and ileum. Nutrients are the chief secretory stimulus and appear to increase GLP-2 release through indirect neuro-hormonal mechanisms as well as direct L-cell contact (Section 1.1.4). However, bilio-pancreatic secretions might also act as effective GLP-2 secretagogues (Chapter 6).

It could be hypothesised that the accelerated and increased delivery of partially digested nutrients and/or digestive juices to the distal small bowel is largely responsible for the increase of postprandial GLP-2 secretion. This would be in keeping with findings of unchanged basal GLP-2 secretion. As described in Chapter 1, a small early peak of GLP-2 secretion can be observed under physiological conditions and is believed to be caused by early indirect L-cell stimulation through neuro-humoral signalling. A second, often more prominent peak occurs between 90-150 minutes (Balks *et al.* 1997; Xiao et al. 1999) which is thought to result from direct stimulation through nutrients reaching the distal intestine (Brubaker et al. 2003). In 8 healthy volunteers consuming a 920kcal meal, peak GLP-2 concentrations were recorded between 90 and 240 minutes (Section 2.3). In contrast, the main post-prandial GLP-2 peak following RYGB surgery occurred very early (often 30min) after ingestion.

The hypothesis that accelerated stimulation of the ileum and consequent release of L-cell products (GLP-1 and PYY) contribute to the effects of some bariatric surgery has been tested in rodent models of ileal transposition (IT). In this procedure, an isolated segment of ileum is transposed to the jejunum, resulting in an intestinal tract of normal length but an alteration in the normal distribution of endocrine cells along the gut. It was shown that rats with IT lost more weight and consumed less food than controls. Weight loss in the IT rats was not due to nutrient malabsorption but was thought to be caused by increased synthesis and release of the anorectic ileal hormones GLP-1 and

PYY (Strader et al. 2005). Another study of ileal-jejunal transposition demonstrated significant increases in mucosal mass, villus height, and crypt depth in the transposed ileum as well as in the remainder of the small intestine (Tsuchiya et al. 1995). As GLP-1 is co-secreted with the intestinotrophic GLP-2 it is likely that GLP-2 is involved in this transposition hypertrophy.

Physiologically, the increase of L-cell secretion in response to contact with a higher concentration of undigested nutrients could be interpreted as part of an adaptive response designed to decrease intake, slow gut motility and increase digestive and absorptive capacity and thus re-storing “normal nutrient concentration” in the L-cell environment.

It is of note that there was no significant postprandial GLP-2 response prior to RYGB surgery. As we have previously demonstrated; GLP-2 secretion does not significantly vary between obese and normal weight subjects. It is therefore more likely that the lack of response was due to the relatively small calorie content of the meal. Meal related GLP-2 secretion is known to depend on nutrient composition and calorie content (Xiao *et al.* 1999). In keeping with this, a 500kcal test-meal of similar nutritional composition failed to produce a significant post-prandial response in lean or obese subjects (Chapter 4).

Interestingly, significant disparity in the GLP-2 response between diabetic and non-diabetic subjects is shown early after RYGB. In contrast to the immediately increased postprandial GLP-2 response in non-diabetics, diabetic subjects demonstrated a flat postprandial meal profile, similar to the pre-operative response, on day 2 following surgery. Statistically there was no demonstrable difference between diabetic and non-diabetic subjects at any other time-point, however numerically this difference appeared to reduce only gradually until similar post-prandial secretion profiles were observed on day 42 (Figure 5-4, Figure 5-5; Table 5-2).

The secretion of glucagon-like peptides in diabetic patients has been studied more extensively for the insulinotropic GLP-1. Type-2 diabetic patients have a significantly reduced GLP-1 response to a test meal, compared with non-diabetic controls (Vilsboll et al. 2001; Vilsboll *et al.* 2003). It is thought that the lower GLP-1 response seen in diabetic patients is probably a consequence of type-2 diabetes rather than a primary defect, as normal 24-hour plasma concentration profiles are observed in first-degree relatives of patients with type-2 diabetes (Nyholm et al. 1999). In identical twins, discordant for type 2 diabetes, only the diabetic twin has decreased GLP-1 levels (Vaag et al. 1996). GLP-1 is cleared at similar rates in type-2 diabetic patients and healthy subjects, indicating that the decreased GLP-1 concentrations in type-2 diabetics are not attributable to differences in elimination (Vilsboll et al. 2003). In our cohort, a difference in post-prandial GLP-2 secretion between diabetic and non-diabetic patients can not be shown in the pre-RYGB meal. This is probably due to the relatively small caloric stimulus. Early (on day 2) after bypass surgery, an adaptive postprandial GLP-2 increase occurs in non-diabetics only. In diabetic subjects this early adaptive response appears to be absent. However, postprandial GLP-2 secretion becomes comparable to that of non-diabetics within days. These results suggest that gastric bypass surgery is able to restore a normal L-cell response in diabetics. As this will also result in increased release of GLP-1, this mechanism is likely to contribute towards the improved glucose homeostasis known to occur following gastric bypass.

The GLP-2 related increase of intestinal mass is accompanied by enhanced intestinal digestive and absorptive functional capacity (Section 1.1.7). The reported increased proliferative activity and documented enhanced intestinal mass in humans and rats following intestinal bypass surgery may therefore partly explain the scarcity of calorie malabsorption associated symptoms after RYGB. Given the well known potent intestinotrophic properties of GLP-2, the finding of increased GLP-2 levels post RYGB strongly suggests its contributing role. However, proof of a causative relationship would require studies using GLP-2 blockade or GLP-2 receptor deficiency models.

There have been concerns that high concentrations of growth factors like GLP-2 may stimulate malignant growth (Section 1.1.9). A decrease in the incidence of cancer has been reported after bariatric surgery and specifically no difference in bowel cancer incidence was noted (van der Woude 2007). Cancer of the small bowel is very rare and although exogenous GLP-2 treatment can exert proliferative effects on the large bowel (Drucker *et al.* 1997) it has not been investigated, whether stimulation of endogenous GLP-2 secretion through bariatric procedures also increases crypt cell proliferation in the large intestine.

Summary: An exaggerated GLP-2 response to nutrient ingestion can be demonstrated as early as 2 days following RYGB. Elevation of this growth factor may lead to adaptive changes in the intestinal mucosa that account for the restoration of intestinal function within months after malabsorptive bariatric surgery.

Chapter 6

Glutamine and bile acid induced glucagon-like peptide-2 secretion from L-cells

6.1 Introduction

Glucagon-like peptide-2 (GLP-2) is a potent intestinotrophic hormone that plays an important role in intestinal adaptation following injury or disease. Intake of nutrients is the main stimulus for GLP-2 secretion although the precise mechanisms of its release from enteroendocrine L-cells in the distal small bowel and colon are incompletely understood (Section 1.1.4). Certain dietary components may exert specific effects on structural and functional characteristics of intestinal adaptation. In this context the amino acid glutamine, a vital energy source for enterocytes, has generated interest. In rat models of small bowel resection and TPN induced mucosal atrophy; parenteral glutamine supplementation has been shown to improve the adaptive response (Tamada et al. 1993; Schroder et al. 1995). In patients with short bowel syndrome a glutamine enriched diet may enhance adaptation in conjunction with exogenous growth hormone (Zhou et al. 2005). These effects might be mediated through glucagon-like peptide secretion. Cell culture experiments have suggested that glutamine stimulates GLP-1 secretion from L-cells (Reimann 2004). There is also recent in vitro evidence that bile acids play a role in the release of glucagon-like peptides. Expression of bile acid surface receptor TGR5 has been described in L-cell models (Katsuma *et al.* 2005).

As a therapeutic agent, GLP-2 has shown potential as an adjunct in the treatment of short bowel syndrome and inflammatory bowel disease. Its positive effects on bone metabolism, intestinal barrier function, splanchnic perfusion and mucosal healing could be utilised to expand therapeutic applications to other causes of intestinal injury (Section 1.1.8). Stimulation of endogenous GLP-2 secretion might benefit patients in whom intestinal mucosal regeneration is impaired. This study aimed to evaluate the direct effect of glutamine and bile acids on GLP-2 secretion from enteroendocrine L-cells using the GLUTag cell culture model. A rodent model of papilla of Vater transposition was used to investigate the effect of bilio-pancreatic secretions on GLP-2 release in vivo.

6.2 Methods

6.2.1 Cell culture

These experiments were conducted at the Cambridge Institute for Medical Research and Department of Clinical biochemistry, Addenbrooke's Hospital, Cambridge, UK in collaboration with Dr Fiona Gribble, who supervised the design of the study. Preparation and incubation of the cell cultures was performed by Miss Helen Parker. Cell culture analysis with radioimmunoassay and data interpretation was performed by the author.

GLUTag cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 5.6mM glucose) supplemented with 10% (v/v) FCS, 100µg/ml streptomycin, 100 u/ml penicillin and 4mM glutamine. They were incubated at 37ºC, 5% CO2 and the culture medium was changed every 3 days. When reaching ~70% confluence (~every 5 days) cells were trypsinised with 1X trypsin-EDTA, diluted and reseeded. For secretion experiments, cells were plated in 24-well culture plates coated with Matrigel (BD Bioscience, UK) and allowed to reach 60% confluence (at least 24hrs). Before the experiment, cells were washed twice with saline buffer containing (in mM) 138 NaCl, 5.6 KCl, 4.2 $NaHCO_3$, 1.2 NaH_2PO_4, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES and 0.1% BSA. Experiments were performed by incubating the cells with test reagents (as listed in Table 6-1) and 0.1mM Diprotin A in 500µl of buffer for 2hrs at 37°C. LCA was prepared as a 1000x stock solution in ethanol. Other agents were dissolved directly in the buffer. At the end of the incubation period the media was collected and centrifuged to remove floating cells. Supernatants were immediately frozen and stored at -70°C until analysis.

Secretion was normalised to the baseline release in glucose free medium measured in parallel on the same day.

6.2.2 Ampulla of Vater transposition in rodents

This study was performed in collaboration with Dr Carel Le Roux who developed the experiment. Animal work was performed by Ms Sandra Shurley at Northwick Park Hospital under a licence issued by the Home Office UK. Plasma sample analysis with radioimmunoassay and data interpretation was performed by the author.

Sixteen adult male Wistar rats were randomised to either sham operation or duodenal translocation. The animals received 0.35 ml hypnorm and 0.35 ml diazepam intra muscularly before the procedure. The hair on the ventral surface was removed with an electrical shaver and the skin cleaned with Chlorhexidine. A longitudinal abdominal incision of 5 cm was made and the exposed bowel covered with wet gauze. Intestinal transposition involved excision of a duodenal segment containing the Ampulla of Vater, which allows drainage of both bile and pancreatic juices, and anastomosis to the ileum (10 cm proximal to the caecum). The first part of the duodenum and the first part of the jejunum was then re-anastomosed. The sham operation consisted of transsection of the proximal and distal parts of the duodenum and re-anastomosis. Post operatively gentamicin 8 mg/kg and caprofen 0.01 mL was administered intraperitoneally as prophylaxis for postoperative infection. One rat in the transposition group died. Rats were housed individually and received a normal diet of chow and water ad libitum. The animals were sacrificed 4 weeks post-operatively. 3 cm of un-stretched terminal ileum was collected and weighed. Mixed arterial and venous blood was collected into lithium-heparin tubes containing 5000 Kallikrein inhibitor units (0-2 ml) aprotinin, immediately spun down at 1600g and stored at -70° until analysis.

6.2.3 Radioimmunoassay

Total GLP-2-IR was measured using antiserum FT-17as described in chapter 2. Antiserum FT-17 reacts 100% with human GLP-2. Cross-reaction

with GLP-1 is less than 0.1%. There is no cross-reaction with any other known gastrointestinal or pancreatic hormone (Appendix 1). FT-17 detects intact GLP-2 (1-33), its primary degradation product GLP-2 (3-33) and major proglucagon fragment (MPGF). Detection limit was 25pmol/l; intra-assay CV 8.3%.

6.2.4 Statistics

Cell culture results are presented as fold increase versus control (mean ± SEM). Significance relative to secretion in the absence of nutrients was assessed using Student's one-sample t test. Difference in secretory response between nutrients was assessed with two-tailed student t-test for independent samples. GLP-2 plasma concentration and TI weight in the 2 rat groups are presented as mean (SEM). Groups were compared using the two-tailed student t-test for independent samples. A p-value of less than 0.05 was considered statistically significant for all statistical calculations.

6.3 Results

The release of GLP-2 from GLUTag cells in response to glutamine and bile acids was investigated. GLP-2 secretion following incubation with glucose and glucose/forskolin/IBMX was examined as positive control. The secretory response to the different reagents is summarized in Table 6-1.

Maximum stimulation of the cAMP/PKA pathway with glucose/forskolin/IBMX led to a 17.5 fold increase of GLP-2 in the culture medium. Glutamine (10mmol/l) increased the GLP-2 concentration in the culture medium 2.18 fold. This response tended to be higher compared to stimulation with glucose (2 or 10 mmol/), however this difference was not significant (Figure 6-1).

GLP-2 release in response to bile acids was diverse. DCA and LCA significantly increased GLP-2 secretion at 30mcM (2.36 and 1.96 fold respectively) but not at 1 mcM concentration. There was no difference in potency between DCA and LCA. There was a trend for GLP-2 secretion to increase after incubation with 30 mcM CDCA. DHCA and HDCA had no secretory effects (Figure 6-2 a). Addition of low dose glucose (2mmol/l) to either DCA or LCA had an additive effect on GLP-2 secretion compared with glucose or bile acids alone (Figure 6-2 b).

Rats with transposition of bilio-pancreatic drainage to the ileum had an approximately 2-fold elevation of fasting GLP-2 plasma levels compared with sham operated controls: 83.3 pmol/l (74.2-112.6) vs. 165.6 pmol/l (134.2-183.0); p=0.04 (Figure 6-3a). The bowel mucosa distal to the anastomosis was noted to be hypertrophic on post-mortem examination. Medium wet weight of the terminal ileum (3cm segment) was greater in the transposition group compared with the sham-operated group: 310g (255-315) vs. 200g (170-205) respectively; p=0.0006 (Figure 6-3b).

Test Reagent	Concentration (mmol/l)	Wells (n)	Change (fold control)	SEM	p
Glucose+ Forskolin+ IBMX	10 0.01 0.01	4	17.5	3.6	0.02
Glucose	2	8	1.77	0.11	0.0002
	10	6	1.88	0.19	0.005
Glutamine	10	10	2.18	0.17	0.0001
DCA	0.001	6	1.13	0.14	0.42
DCA	0.03	10	2.36	0.23	0.0002
LCA	0.001	6	1.06	0.12	0.6
LCA	0.03	12	1.96	0.19	0.0003
CDCA	0.001	4	1.01	0.12	0.97
CDCA	0.03	4	1.27	0.24	0.35
HDCA	0.03	4	0.60	0.04	0.003
DHCA	0.03	3	0.72	0.11	0.12

Table 6-1: **Secretory responses of GLUTag cells**

tGLP-2-IR, measured in culture medium after 2-hour incubation with the test reagents and compared with control cultures, containing no active reagents

Statistical significance was assessed using one-sample t test.

(IBMX: 3-isobutyl-1-methylxanthine; LCA: lithocholic acid; DCA deoxycholic acid; CDCA: chenodeoxycholic acid; HDCA: hyodeoxycholic acid; DHCA dehydrocholic acid).

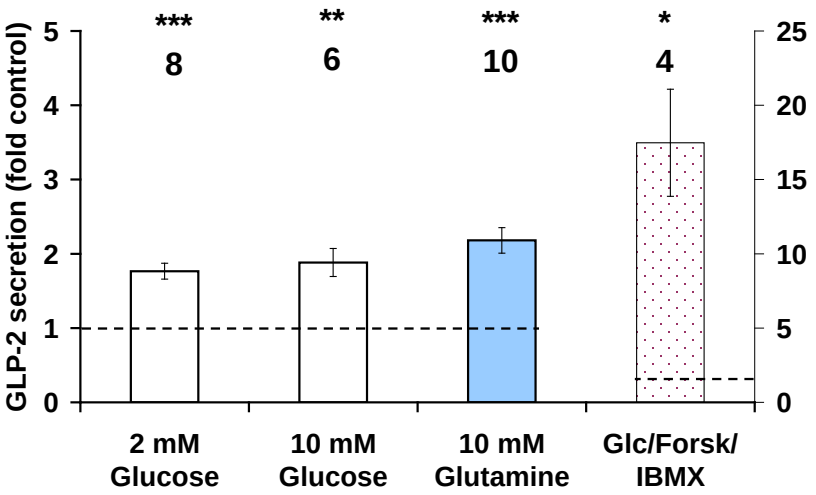
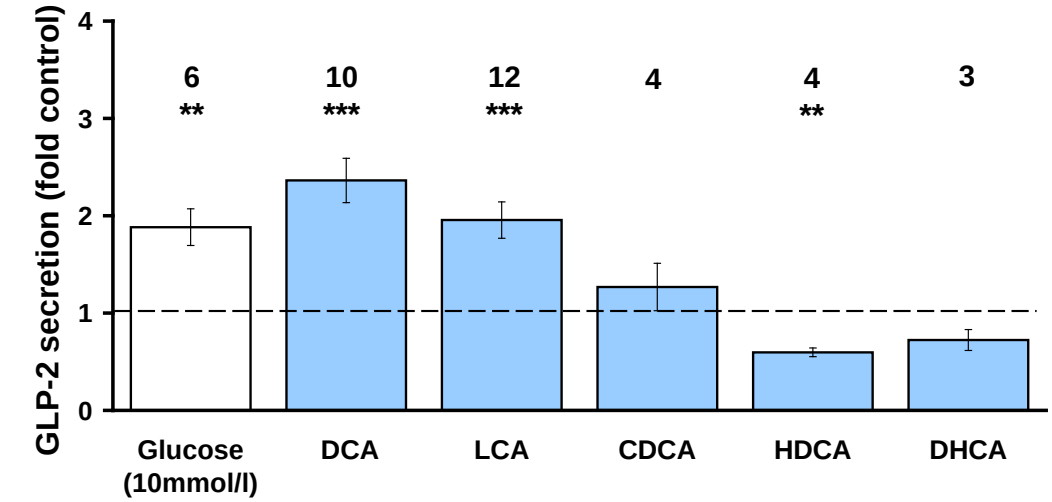


Figure 6-1: **GLP-2 response to glutamine** (solid bar) in comparison to glucose (empty bars), stimulation with IBMX (dotted bar, secondary y axis) and controls (dotted line).

Results are presented as mean ± SEM.
The number of wells is indicated above each bar.
Significance of change relative to secretion in the absence of nutrients
*p<0.05, **p<0.01, ***p<0.001 (Student's one-sample t test)



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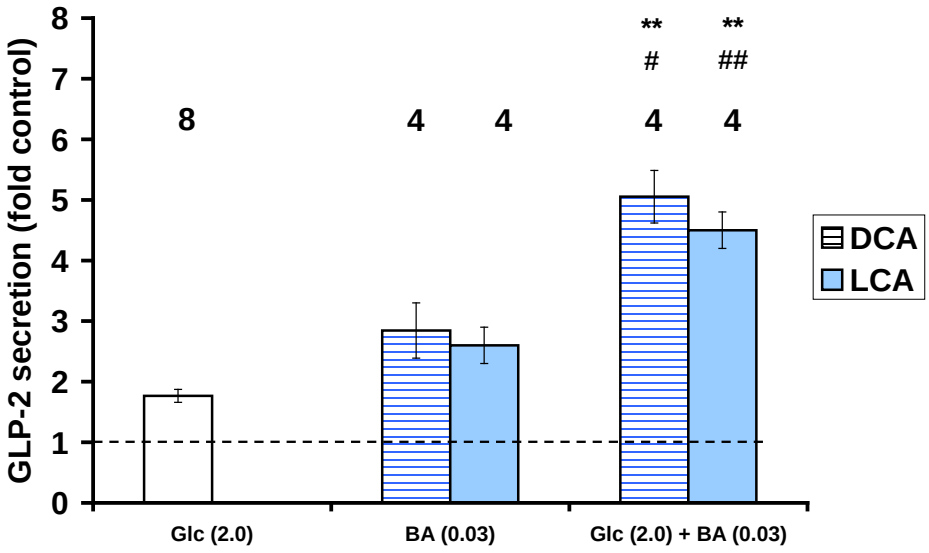
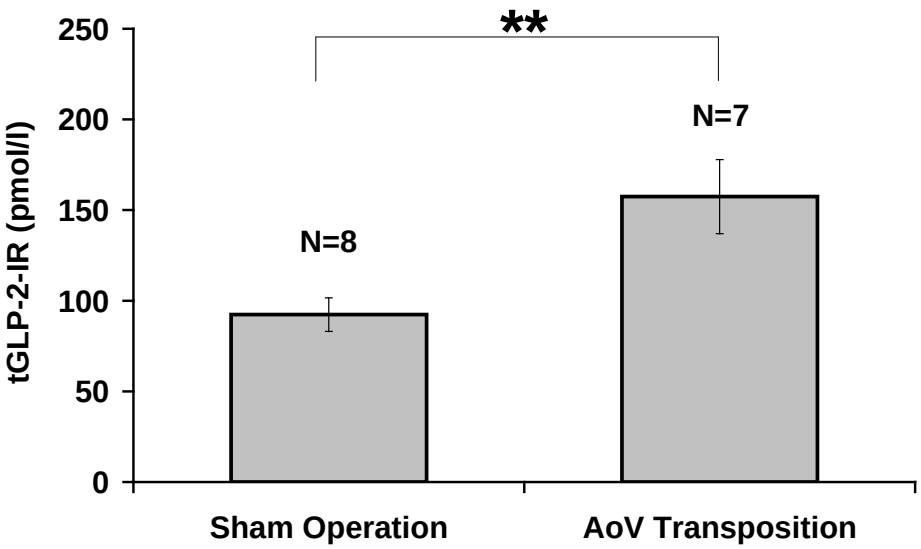


B

Figure 6-2: **GLP-2 response bile acids (BA)** in comparison to glucose (empty bars) and. controls (dotted lines).The number of wells is indicated above each bar. Results are presented as means ± SEM.

A: Bile acid response: DCA deoxycholic acid; LCA lithocholic acid; CDCA chenodeoxycholic acid; HDCA hyodeoxycholic acid; DHCA dehydrocholic acid; BA concentration (30µmol/l).
Significance of change relative to secretion in the absence of nutrients
**p<0.01; ***p<0.001 (Student's one-sample t test)

B: Additive effect of co-stimulation with glucose: Combination of low dose glucose (Glc; 2mM/l) and bile acids (BA; 0.03mM/l) promotes stronger GLP-2 secretion from GLUTag cells than either agent alone.
Statistical significance was assessed using two tailed student-t test
(** p<0.01: increase vs. glucose; # p<0.05; ## p<0.01: increase vs. BA alone)



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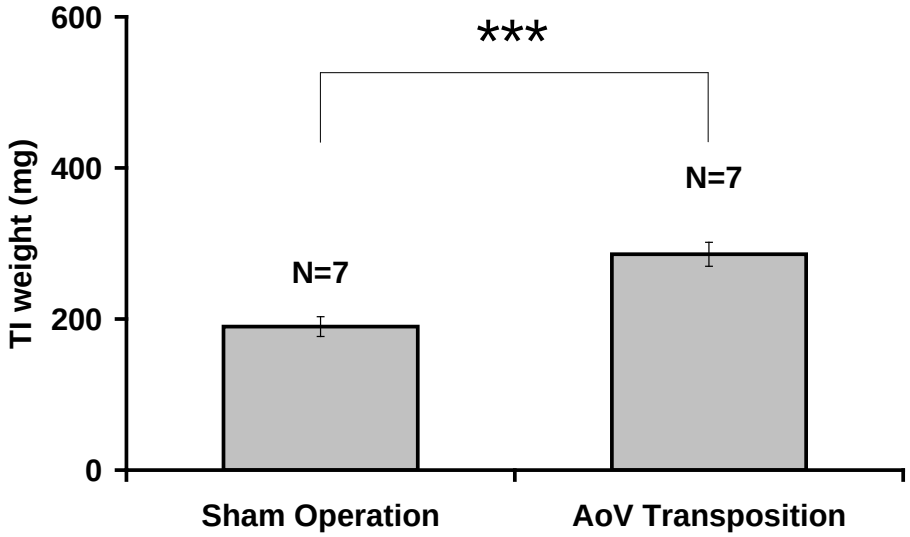

B

Figure 6-3: **Ampulla of Vater (AoV) transposition in rats**

Week 4 post AoV transposition to the distal ileum versus sham operated controls.

A: Plasma tGLP-2-IR concentration

B: Weight of a 3cm unstretched segment of terminal ileum (TI)

Values are mean (SEM)
** p<0.01; ***p<0.001 (two tailed student-t test)

6.4 Discussion

It has been demonstrated that glutamine and bile acids are effective GLP-2 secretagogues in vitro, using the GLUTag L-cell model.

Because enteroendocrine L-cells represent only a small proportion of epithelial cells and are difficult to isolate in purity, GLUTag cells were used to investigate the L-cell secretory response. This cell line is a well-recognized L-cell model, derived from a colonic tumour in a transgenic mouse expressing SV 40 large T antigen. These cells express the glucagon gene and secrete the glucagon-like peptides in a regulated manner and are therefore frequently utilized for the analysis of GLP-1 and GLP-2 secretagogues (Drucker et al. 1994).

Proglucagon gene expression in the intestine has been shown to be regulated by a cAMP-dependent pathway in rodent tissue culture (Drucker et al. 1989). Work on GLUTag cell cultures has provided additional evidence that cAMP elevation also directly triggers GLP-1 release and enhances the secretory response to other stimuli by modulating hyperpolarisation-activated currents and the background potassium current (Simpson et al. 2007). In keeping with these results this study shows that forskolin and phosphodiesterase inhibitor IBMX, commonly used to maximise intracellular cAMP accumulation, also led to a substantial increase in GLP-2 concentration in the culture medium, indicating that the cAMP pathway in the cultured cells was intact.

Glutamine is not only a key metabolic fuel for enterocytes, it has also been shown to regulate the expression of many genes related to metabolism, signal transduction, cell defence and repair (Curi et al. 2005). In animal models, glutamine improves intestinal morphology in parenteral nutrition induced mucosal atrophy (Schroder et al. 1995) and enhances adaptation after massive intestinal resection (Tamada et al. 1993). These findings have encouraged glutamine supplementation in patients with short bowel

syndrome where it has shown to be of possible clinical benefit when used in combination with low-dose human growth hormone and a modified diet (Zhou et al. 2005). Other potential clinical uses of glutamine include intensive care settings, where it might reduce serious sepsis (Andrews et al. 2002). Glutamine supplementation might also reduce complications following haematopoietic stem cell transplantation in children (Kuskonmaz et al. 2008).

The mechanisms of glutamine action in the intestine are poorly understood. It is demonstrated here that Glutamine is a GLP-2 secretagogue in vitro. It could therefore be hypothesised that the intestinotrophic properties of glutamine might in part be mediated through the release of GLP-2. Increased secretion of glucagon-like peptides following glutamine supplementation might also be relevant in the treatment of sepsis as GLP-2 has been shown to reduce bacterial translocation and boost immune responses in rats (Chance *et al.* 2001; Kouris *et al.* 2001), whereas GLP-1 improves glycaemic control in animal models and human clinical studies (Todd *et al.* 2007). Glutamine induced secretion of GLP-2 from L-cells has not yet been described. Reimann et al. have previously demonstrated that glutamine dose-dependently triggers and potentiates secretion of GLP-1 from GLUTag cells (Reimann et al. 2004). This data is strengthened by the documentation of co-secretion of GLP-2. To optimize substrate delivery to the cultured cells, pharmacological concentrations of glutamine (10mmol/l) were used in this study. However, Reiman et al have previously shown that glutamine-induced GLP-1 secretion from GLUTag cells also occurs at 1mmol/l (Reimann *et al.* 2004), which resembles the physiological extracellular concentration of glutamine (0.7 mmol/l) (Newsholme et al. 2003) more closely.

The essential role of bile acids for the absorption of dietary lipids is well described. More recently it has become apparent that bile acids are also signalling molecules, influencing bile acid synthesis, lipid metabolism and energy homeostasis (Houten et al. 2006) and may confer gut mucosal protection against bacteria (Hofmann et al. 2006). Bile acids regulate expression of various transport proteins and enzymes through binding and activating nuclear receptors such as farnesoid X receptor α. It is now

understood that bile acids are also ligands for a membrane based G-protein-coupled receptor; TGR5. TGR5 has been identified in the L-cell models STC-1 and GLUTag. Its activation induces rapid intracellular cAMP accumulation (Maruyama *et al.* 2002; Kawamata et al. 2003).

Increased gut glucagon-like immunoreactivity after exposure of a canine ileal loop to bile and following intracolonic infusion of deoxycholic acid in humans has previously been reported (Namba et al. 1983; Adrian *et al.* 1993). More recently bile acid mediated stimulation of GLP-1 secretion though TGR5 was demonstrated in STC-1 cultures (Katsuma *et al.* 2005). Since GLP-1 and GLP-2 are co-secreted it could be assumed that GLP-2 release will follow the same pattern in this cell line. The results of the work presented here support this data by demonstrating bile acid induced GLP-2 secretion in an alternative L-cell model.

It has been noted that the agonistic activities of bile acids on the TGR-5 receptor appear to increase in accordance with hydrophobicity (Kawamata *et al.* 2003). The findings of this study are consistent with this. Strongly hydrophobic bile acids like LCA and DCA acted as GLP-2 secretagogues whilst hydrophilic bile acids like HDCA and DHCA elicited no measurable response. Low concentration (1mcM) DCA and LCA failed to stimulate quantifiable secretion, in keeping with the dose dependent response previously described for GLP-1 (Katsuma *et al.* 2005). A stimulatory effect on glucagon-like peptide-1 secretion from GLUTag cells has been described for other hormones that act via G-protein coupled receptors, coupled to adenylate cyclase. Glucose-dependent insulinotrophic peptide (GIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) dose-dependently trigger GLP-1 release in the presence of 10 mmol/l glucose, with a maximum stimulation of 1.8- to 2-fold (Simpson *et al.* 2007). The magnitude of this response compares with the documented increase of GLP-2 in response to bile acid stimulation.

Effects on cell cultures might not always indicate physiological relevance as pathways identified in vitro, might be overridden by other regulatory signals.

A contributory role of bile acids in intestinal growth and adaptation in vivo is controversial. Small intestinal mass remained unchanged in response to bile infusion in pythons, an established model of intestinal adaptation (Secor et al. 2002). In rat models of small intestinal resection, additional diversion of biliary juices did not hamper development of compensatory hypertrophic changes in the small intestine (Fenyo 1977; Ulshen et al. 1984). Nonetheless, an increase of mucosal mass in distal ileal segments following transplantation of the duodenal papilla into the ileum has been previously described (Weser et al. 1977). This is in keeping with the observations in our rat model. Hypertrophic distal ileal mucosa was noted in all the rats following the diversion of bilio-pancreatic juices to the distal ileum that contains a large proportion of L-cells. There was a 50% increase in weight in the terminal ileum. We have also shown that this is associated with higher levels of plasma GLP-2. It is therefore conceivable that, whilst the adaptive response following intestinal resection might be chiefly dependent on other stimuli like nutrient exposure, bilio-pancreatic secretions are also capable of inducing local intestinal hypertrophy, possibly mediated through stimulation of GLP-2 release. Alternatively, the increased release of GLP-2 might be explained by delivery of greater amounts of poorly digested foods to the distal L-cells.

In vivo, L-cells are exposed to a large variety of nutritional, hormonal and neural stimuli. It is likely that some of these have additive or even synergistic effects. In this study the combined stimulation of GLUTag cells with glucose and bile acids was more effective than with either agent alone. When exploring augmentation of endogenous GLP-2 secretion for therapeutic purposes, combined agents might therefore prove more effective. General caution should be exercised in the long-term use of intestinal growth factors. Stimulatory effects on colonic proliferation and a possible role in colon carcinogenesis has been described for bile acids (Reddy et al. 1977; Lapre et al. 1992) and GLP-2 (Thulesen et al. 2004; Masur et al. 2006).

Summary: Glutamine and bile acids stimulate secretion of GLP-2 from L-cells in several experimental models. Diversion of bilio-pancreatic secretions to the distal ileum is associated with increased plasma GLP-2 and local

intestinal hypertrophy in rats. It is proposed that the intestinotrophic properties of these substances might in part be mediated through the release of GLP-2.

Chapter 7

Final discussion and conclusions

The link between increased secretion of glucagon-related peptides and the development of intestinal villus hyperplasia was first established in 1971, in a case report of patient, presenting with an “enteroglucagon”-producing renal tumour and massive small bowel enlargement (Gleeson et al. 1971). In a landmark study more than 20 years later, Daniel Drucker and colleagues were able to link these potent intestinotrophic properties to glucagon-like peptide 2 (GLP-2) (Drucker *et al.* 1996). Although basic research into the physiological role of GLP-2 has been hampered by difficulties in developing effective GLP-2 deficiency models, there is rapidly growing understanding of the central role of GLP-2 for intestinal growth and adaptation. The most recent scientific progress relates to the identification of downstream mediators of GLP-2 action, in particular the pivotal role of IGF-1.

The ability to determine GLP-2 concentration in tissue and bodily fluids accurately has furthered the understanding of GLP-2 secretion and actions and given insights into the regulation of intestinal adaptation to injury or disease. It has also paved the way towards exploration of therapeutic GLP-2 supplementation in a variety of intestinal conditions, culminating in a recent large scale clinical trial of GLP-2 in patients with short bowel syndrome. However, measurement methods for GLP-2 are not widely available and commercial assays are often poorly validated.

This thesis has shown that a radioimmunoassay using antiserum FT-17 provides a sensitive and specific method for the measurement of GLP-2 in plasma samples and tissue extracts. Although the antiserum is not specific for measurement of biologically active GLP-2 (1-33), accurate estimations of active GLP-2 concentrations can be made when the secretory response to a physiological stimulus is measured. The finding that fasting t-GLP-2-IR concentrations do not correlate well with active GLP-2 (1-33) concentration is very important in the interpretation of study results. The poor correlation of results obtained when using a commercially available GLP-2- ELISA kit in comparison with the current gold-standard method of measurement (GLP-2-RIA using antiserum 92160), emphasises the need to validate any analytical method in order to interpret test-results accurately.

Alteration of gut hormone secretion from enteroendocrine L-cells has been demonstrated in a variety of pathological conditions, in particular following intestinal resection. In keeping with previous studies, it has been demonstrated here that secretion of proglucagon derived peptides (GLP-1 and GLP-2) as well as PYY is reduced in patients with short bowel syndrome and jejunostomy. However, it has been shown for the first time that SBS patients with a retained colon, despite increased L-cell secretion of PYY, can be GLP-2 deficient. This has led to the conclusion that, despite their co-localisation in enteroendocrine L-cells, the production and/or secretion of PYY and proglucagon derived peptides may be regulated differentially and could vary depending on the residual intestinal anatomy. Following intestinal resection, an adaptive increase of PYY secretion might mainly occur in the colonic L-cells, whereas L- (or other endocrine) cells in the residual jejunum might be primarily responsible for the adaptive increase in GLP-1 and GLP-2 that was previously shown in this patient group (Jeppesen *et al.* 2000). These results would tie in with the relatively novel concept of multipotent enteroendocrine cells that produce peptides according to stimuli in the metabolic or biochemical milieu (Theodorakis *et al.* 2006).

A similar disparity between regulation of PYY and glucagon-like peptide secretion may also occur in obesity. The results presented in this thesis show that, in contrast to the previously demonstrated attenuated postprandial PYY response in the obese (Le Roux *et al.* 2006), no reduction of glucagon-like peptide secretion is found. However, the limited subject number might have concealed a small concentration difference between obese and normal weight subjects.

Measurement of gut hormone secretion in patients following gastric bypass procedures has revolutionized our understanding of the mechanisms of weight loss following bariatric surgery. It is now thought that, in addition to malabsorption of nutrients due to reduced surface area, a gut hormone mediated decrease of appetite and energy intake, is a major contributor to achieving sustained weight reduction. Results presented in this thesis document for the first time an exaggerated GLP-2 response to nutrient

ingestion as early as 2 days following RYGB that is sustained for at least 2 years. It is hypothesised that the elevation of GLP-2 may lead to adaptive changes in the intestinal mucosa that account for the restoration of intestinal function after malabsorptive bariatric surgery. The interesting additional finding of an apparently delayed adaptive response in diabetic subjects deserves further investigation as the resolution of type-2 diabetes has been closely linked with increase of glucagon-like peptide secretion.

Enhancing endogenous GLP-2 secretion might be an effective therapeutic strategy for patients in whom intestinal mucosal regeneration is impaired. This thesis demonstrates that glutamine and bile acids stimulate secretion of GLP-2 from model L-cells and proposes that the intestinotrophic properties of these substances might in part be mediated through the release of GLP-2. However, the limited interpretability of results obtained in artificial cell models, which might not always indicate physiological relevance, has to be taken into consideration.

Far from simply being a place of nutrient digestion and absorption, the gastrointestinal tract is increasingly appreciated as an important endocrine (and immunological) organ. Although so far mostly studied in isolation, it is a fundamental consideration that GLP-2 will act in concert with other gut hormones. The mechanisms of such interaction constitute an important field of future research in order to understand the physiological consequences and maximise the benefits of therapeutic GLP-2 supplementation.

In the development of future treatments for intestinal failure, GLP-2 might become instrumental in the development of tissue engineered neo-intestine. Although still experimental, this technique emerges as a possible alternative to long-term parenteral nutrition or intestinal transplantation. The response of tissue-engineered neo-intestine to exogenous GLP-2 includes mucosal growth and enhanced SGLT-1 expression (Ramsanahie *et al.* 2003). Measurement of GLP-2 in neo-intestine could serve as marker of enteroendocrine differentiation.

Finally; it has been very exciting to witness the progress of GLP-2 from relative obscurity to its recognition as an important physiological mediator and promising therapeutic agent. I hope that my work has contributed towards the understanding of this interesting peptide.

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Wallis K, Walters JRF, Gabe, SM
Current Opinion in Clinical Nutrition and Metabolic Care, 2009 Sep; 12(5):526-32

Promoting intestinal adaptation
Wallis K, Lloyd DAJ, Gabe SM
British Journal of Hospital Medicine, 2007 Jan; 68(1):11–14

Glucagon-like peptide 2 - Current applications and future directions
Wallis K, Walters JRF, Forbes A
Aliment Pharmacol Ther. 2007 Feb; 25(4):365-72.

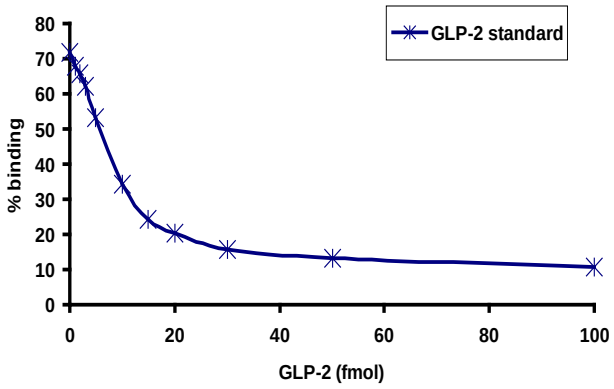
Abstracts:

Differential gut hormone secretion from enteroendocrine L-cells in patients with short bowel syndrome
Wallis K, Le Roux CW, Ghatei MA, Holst JJ, Forbes A, Gabe SM, Walters JRF
Oral presentation; World congress Gastroenterology, London 2009

Bile acids stimulate glucagon-like peptide- 2 secretion from L-cells; possible mediation through membrane receptor TGR5.
Wallis K, Parker H, Le Roux CW, Ghatei, MA, Forbes A, Gabe, SM, Gribble F, Walters JRF
Poster presentation; Digestive Disorders Week, San Diego 2008

Exaggerated postprandial GLP-2 response after Roux-en-Y gastric bypass might be causative for post surgical intestinal adaptation
Wallis K, Le Roux CW, Aylwin SJB, Patel AG, Welbourn R, Bloom SR, Forbes A, Ghatei MA, Walters JRF
Oral presentation; Digestive Disorders Week, Washington DC 2007

Appendix 1: Cross-reactivity of antiserum FT-17



GLP-2 (fmol)	GLP-2 (pmol)	% binding
0	0	71.8
1	0.001	67.9
2	0.002	65.6
3	0.003	62
5	0.005	53.1
10	0.01	34.2
15	0.015	24.4
20	0.02	20.5
30	0.03	15.8
50	0.05	13.1
100	0.1	10.8

Peptide concentration	**% binding (1pmol)**	**% binding (10pmol)**	**% binding (100pmol)**
alpha CGRP	72.2	70.6	71.1
AVP	69.5	70.1	70.5
Bombesin	69.5	68.7	70
CRF	71.2	71.4	70.7
Galanin	68.5	70.3	70.3
GIP	71	70.2	65.7
GLP-1	***65.5***	***38.4***	***12.6***
Glucagon	69.9	68.7	69.4
GRF	70.9	70	69.8
LH-RH	68.8	67.5	70.5
Neurotensin	70.9	69.2	71.2
NPY	69.1	68.8	73.6
PACP	70.4	71.5	72.6
PHM	72.3	72.7	75.4
PYY	65.4	67.8	69.3
Secretin*	*70.3	*70.1	*70
VIP	71.5	71.9	71.4

FT-17 RIA: GLP-2 standard curve and cross-reactivity of with other peptide hormone standards. Minimal cross-reactivity of with GLP-1 (0.01%) is demonstrated. Laboratory data from Prof. M. Ghatai 1997 (unpublished).
** Tested standard concentrations of secretin are 0.2pmol; 2pmol and 20pmol.*

Appendix 2: Solutions

- **Dextran coated charcoal:**

2.4g of charcoal and 0.24g of dextran were added to 100ml of phosphate buffer with gelatine and mixed for twenty minutes at 4oC

- **Phosphate buffer (GLP-2 and PYY):**

48g of Na2HPO4.2H2O, 4.13g KH_2PO_4, 18.61g EDTA ($C_{10}H_{14}H_2O_8Na_2.2H_2O$, 2.5g NaN_3 are dissolved in 5l of distilled water that had been boiled and allowed to cool, the pH was measured to confirm it was 7.4±0.1. The buffer was stored at 4^oC

- **Veronal buffer (GLP-1)**

10.1g of Barbitone Sodium ($C_8H_{12}N_2O_3$) and 0.5g of NaN_3 are dissolved in 1l of distilled water that had been boiled and allowed to cool. Solution was titrated with concentrated HCL until pH was 8.0 and stored at 4^oC

- **Sephadex G50:**

8g fine grade Sephadex G-50 beads (diameter: 20-80μm) (Sigma), 2ml 100x TE and 0.1ml 20% SDS are added to 200ml water and autoclaved to expand beads. Column buffer was 0.06 M phosphate buffer containing 0.2 M NaCl and 0.3% BSA

- **Freeze-dry solution**

12.5g Lactose, 3ml BSA (30%), 0.5g citric acid, 0.25g L-cysteine hydrochloride, 1.06ml formic acid and 2ml Trasylol dissolved in 250 ml distilled water

Appendix 3: Table of amino acids

Amino Acid	Codes	
	Three letter	**Single letter**
Glycine	Gly	G
Proline	Pro	P
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Cysteine	Cys	C
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Tryptophan	Trp	W
Histidine	His	H
Lysine	Lys	K
Arginine	Arg	R
Glutamine	Gln	Q
Asparagine	Asn	N
Glutamic Acid	Glu	E
Aspartic Acid	Asp	D
Serine	Ser	S
Theronine	Thr	T