A Study of Gut Hormones in Type 1 Diabetes

BY

David Simon Hughes

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ABSTRACT

Type 1 diabetes is a chronic autoimmune disease that leads to apoptosis and death of pancreatic islet beta cells. These beta cells are influenced by and contribute to a network of gut derived hormones that regulate glucose homeostasis. Our research explores the effect that type 1 diabetes has on this network of gut hormones. A series of clinical research studies was established to collect samples from both healthy participants and individuals with type 1 diabetes. Methods to measure gut hormones were explored and refined prior to sample analysis. Results from this analysis revealed that the homeostasis of glucagon, active-ghrelin and peptide YY are affected by type 1 diabetes and that glucagon secretion may be controlled by a glucose independent signal derived from the gut. Important lifestyle markers in type 1 diabetes were identified and included a correlation between leptin and body mass index and gastric inhibitory polypeptide and sedentary behaviour. Histopathological analysis of pancreatic slides taken from donors with type 1 diabetes suggest that the islet area stained for glucagon does not correlate with duration of diabetes, but does correlate with age. Together these suggest that type 1 diabetes does affect other gut hormones involved in glucose homeostasis and this can be influenced by lifestyle factors.

DEDICATION

I would like to dedicate this to my parents, wife and children. My parents (Angus William Hughes and Jayne Hughes) provided me the guidance and wisdom that allowed me to safely go forward on the journey of life. My wife (Sarah Hughes) for her endless love and happiness that fills our home. Finally, to my children (Thomas & James) who fill our soul everyday with joy!

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ABBREVIATIONS

AdipoR	Adiponectin Receptors
αMSH	Alpha Melanocyte Stimulating Hormone
AUC	Area Under the Curve
BBC CLRN	Birmingham & Black County Comprehensive Local Research Network
BMI	Body Mass Index
CDRD	Chronic Disease Research into Diabetes study
CI	Confidence Interval
CRH	Corticotrophin releasing hormone peptides
CSII	Continuous Subcutaneous Insulin Infusions
CTLA	Cytotoxic T-lymphocyte Antigen
DAFNE	Dose adjustment for normal eating
DCCT	Diabetes Control and Complications Trial
DMT1	Diabetes mellitus Type 1
DMT2	Diabetes mellitus Type 2
DPP-4	Dipeptidyl peptidase 4
ELISA	Enzyme linked immunosorbent assay
EMA	European Medicines Authority
ExTOD	Exercise in Type One Diabetes Study
FDA	US Food & Drug Administration
GAD65	Glutamic acid decarboxylase
GHRH	Growth hormone-releasing hormone
GIMP	GNU Image Manipulation Program
GIP	Gastric Inhibitory Polypeptide
GLP	Glucagon Like Peptide
GOAT	Ghrelin-O-acyltransferase
GP	General Practitioner
GRLN-R	Ghrelin Receptor
GWAS	Genome-wide association studies

HbA1C	Glycosylated Hemoglobin, type A1c
HENAMI	Hepato-enteral, neuronal, adipo-muscular, Islet endocrine network
HLA	Human leucocyte antigen
IA-2	Islet Antigen - 2
IAA	Insulin antibodies
IFCC	International Federation Clinical Chemist
IFG	Impaired fasting glucose
Ig	Immunoglobulin
IGRP	Islet specific glucose-6-phosphatase catalytic subunit related protein
IGT	Impaired glucose tolerance
Il	Interleukin
JDRF nPOD	Juvenile Diabetes Research Foundation network for Pancreatic Organ donors with Diabetes
JPEG	Joint Photographic Experts Group
MACE	Major Adverse Cardiovascular Endpoints
MODY	Maturity Onset Diabetes of the Young
NASH	Non-alcoholic steatohepatitis
NHS	National Health System
NPY	Neuropeptide Y
OGTT	Oral glucose tolerance test
PACAP	Pituitary adenylate cyclase-activating peptide
PKC	Protein Kinase C
POMC	Proopiomelanocortin
PP	Pancreatic polypeptide
PPAR-γ	Peroxisome proliferator-activated receptor gamma
ppIAPP	Islet amyloid polypeptide precursor protein
PYY	Peptide YY
ROC	Receiver Operated Curve
SAHRP	Streptavidin-horseradish peroxidase
SD	Standard Deviation
SNP	Single nucleotide polymorphisms

SSTR	Somatostatin Receptors
TNF	Tumour necrosis factor
UK	United Kingdom
VIP	Vasoactive Intestinal Peptide
VO2 max	Maximal oxygen consumption
WHO	World Health Organisation

1. INTRODUCTION

Diabetes mellitus is a term for a group of metabolic conditions characterised by a higher than normal glucose level. According to the World Health Organisation ICD-10 classification, there are five forms of diabetes 1) type 1 diabetes mellitus, 2) type 2 diabetes mellitus, 3) Malnutrition related diabetes mellitus, 4) Other specified diabetes mellitus and 5) Unspecified diabetes mellitus [1]. Diabetes mellitus, if left uncontrolled with hyperglycaemia and glycaemic variability, results in increased microvascular and macrovascular complications. Diabetes and its complications are estimated to cost the NHS over £20 billion per year and affect nearly 4 million individuals in the UK alone [2]. Type 1 diabetes is the second most common form of diabetes after type 2 diabetes, with a UK prevalence of ~600 cases per 100,000 population. The prevalence of new cases of type 1 diabetes is approximately 20 per 100,000 [3]. However, the prevalence of type 1 diabetes varies from country to country with the highest incidence in the Scandinavian countries (>50 per 100,000) and the lowest incidence in Far Eastern countries (<5 per 100,000).

The majority of new cases of type 1 diabetes appear around puberty with rapidly declining new cases appearing in adulthood. Symptoms of polyuria and polydipsia, typical of type 1 diabetes are said to occur when approximately 90% of insulin secreting pancreatic beta cells stop functioning [4]. As insulin levels decline, the body's ability to utilise sugars for either metabolism or storage declines. Failure to utilise glucose results in an increase in blood glucose levels (hyperglycaemia). As blood glucose levels increase, glucose enters the urine leading to the osmotic symptom of excessive urination and subsequent thirst. Ultimately, the failure to utilise glucose for energy leads to fatty acid metabolism and formation of ketone bodies. Thus, individuals with new onset type

1 diabetes develop ketosis and dehydration that, without an external source of insulin being administered, could be life threatening [5].

1.1 Historical insight into diabetes

"Diabetes" was coined by Apollonius of Memphis in 250BC, from the Ionian Greek for "passing through", which describes the frequent micturition (polyuria) that leads to dehydration and death. In 1921, Professor John Macleod at the University of Toronto was approached by a Canadian born surgeon Frederick Banting with an idea of extracting anti-diabetic secretions from the pancreas. Working with the English born medical student Charles Best, they were able to isolate pancreatic extract and use it to treat diabetes in a dog. They then went on to use cattle pancreas extract and proved this also was effective treating diabetes in a dog. On the 1st January 1922 they used bovine pancreatic extract in a human diabetic subject, but unfortunately it was unsuccessful. The biochemist James Collip, who was also part of the research team, was able to refine the extraction and purification process of the bovine insulin. On the 23rd January 1922 the first human subject with type 1 diabetes was successfully treated with injectable insulin.

Before the 23rd January 1922 the main treatment for individuals with type 1 diabetes was a severely calorie restricted diet [6]. It was not very effective, with many not surviving more than a year. However, it was successful in a small group of individuals, some of which went on to receive Macleod, Banting, Best and Collip's new bovine insulin. Current treatment strategies for type 1 diabetes have not changed much and include either injectable insulin or using an automated device with an insulin reservoir. Only recently have advances allowed the use of donor islet cells to replace beta cells

lost in type 1 diabetes. The publication of the Edmonton protocol in 2000 heralded a new era in islet cell transplant as it resulted in exogenous insulin independence [7]. It is hoped that better understanding of the pathophysiology of type 1 diabetes may lead to treatments that inhibit beta cell apoptosis and preserve beta cell function.

1.2 Glucose Transport

Glucose transport in and out of cells in humans is controlled through three families of glucose transporter proteins known as the SLC5, SLC2 and SLC50 genetic families [8], [9].

The SLC5 family of glucose transporters is encoded by multiple genes, with the most prominent being the Na/Glucose Like Transporters (SGLT1 and SGLT2). The SGLT2 transporter in the kidney is key in enabling the re-absorption of 90% of glucose through the renal tubules. It is a prominent target of recent drug development in the treatment of type 2 diabetes. SGLT2 inhibition results in improved glycaemia through inhibiting the re-absorption of glucose in the renal tubule. This increases glucose loss in the urine (glycosuria) and lowers plasma glucose levels. However, SGLT2 inhibitors also cause a paradoxical increase in hepatic gluconeogenesis and glucagon secretion. It is thought that the increase in glucagon secretion is due to up regulation of SGLT1 receptors on the alpha cells. SGLT1 inhibitors have been shown to increase GLP-1 which could have benefit in improving glucose sensitivity and increasing insulin secretion [10].

SLC5 family	Substrate	Tissue
5A1 (SGLT1)	Glucose and galactose	Small intestine, trachea, kidney, heart, brain, testes and prostate.
5A2 (SGLT2)	Glucose	Kidney, brain, liver, heart muscle, thyroid and salivary gland.
5A4 (SGLT3)	Sodium	Small intestine (cholinergic neurons), skeletal muscle, kidney, uterus and testis.
5A8 (SMCT1)	Short fatty chain acids	Small intestine, kidney, brain, retina, and muscle.

Table 1.1: Table of SLC5 family of glucose transporters.

The SLC2 family encodes receptors involved in monosaccraharides transport across the cellular membrane via the glucose/hextose transporters (GLUT). The GLUT family of integral membrane proteins can be subdivided into three classes of transporters of similar structure. Every cell of the human body contains a SCL2 genetic family glucose/hextose transporter.

SLC2 family	Substrate	Location
GLUT1	Glucose, galactose, mannose	Red cells, brain, blood-tissue interfaces, Islets of Langerhans, kidney,
GLUT2	Glucose, galactose, fructose, mannose, glucosamine	Liver, intestine, Islets of Langerhans, kidney, brain
GLUT 3	Glucose, galactose, mannose, xylose.	Islets of Langerhans, neurones, testes
GLUT4	Glucose, glucosamine	Adipose tissue, skeletal and cardiac muscle.
GLUT5	Fructose	Small intestine, kidney

Table 1.2 Table of SLC2 family of glucose transporters.

Chronic high levels of monosaccharide and lipids have been shown in rodent models to down regulate the GLUT2 receptors on Beta Cells. It is hypothesised that this results in the down regulation of glucose stimulated insulin secretion. It has also been suggested that GLUT2 down regulation also impacts on hypoglycaemia stimulated glucagon secretion [11], [12]. The GLUT4 transporter is the main transporter involved in increasing glucose uptake into muscle and adipose cells. GLUT4 is insulin sensitive and its cellular expression is also up-regulated by insulin. Thus, defects in GLUT4 expression could contribute to insulin resistance which may form part of the mechanism leading to type 2 diabetes. Reduced expression of GLUT4 has been shown to be induced by the protease inhibitor class of drugs which can induce a form of insulin resistant drug induced diabetes in HIV patients.

The SLC50 class of co-transporters are relatively new and predominantly found in plants. However the SLC50A1 (SWEET1) transport is involved in glucose transport in humans. SCL50A1 is highly expressed in the liver, kidney, exocrine pancreas and Leydig cells. Its importance in glucose homeostasis is still relatively unknown [9].

1.3 Insulin homeostasis

The pancreatic islets are composed of multiple cells that release a variety of hormones. One of the most prominent hormones that they secrete is insulin which is produced in the beta cells. Insulin is a polypeptide hormone composed of two chains (A- and B-chain) [13]. It is initially synthesised as preproinsulin and is converted to proinsulin as it enters the endoplasmic reticulum of the cell. Proinsulin is then cleaved by prohormone convertases and carboxypeptidases into insulin and C-peptide, before being stored as

mature granules which are subsequently released following a stimulus. Insulin release occurs in two phases, the first is dependent on the activation of glucose transporters, the second phase is independent of these glucose transporters. The release of insulin is pulsatile with a frequency of 1 pulse every 3-6 minutes [14]. The target of insulin is the insulin receptor. The insulin receptor is involved in activation of increased expression of GLUT4. GLUT4 increases glucose uptake into hepatocytes where glucokinase (hexokinase in other cells) phosphorylates glucose to glucose-6-phosphate, which can then either enter glycolysis or be polymerized to glycogen for storage.

1.4 Glucagon homeostasis

1.4.1 History of Glucagon

The history of the alpha cell closely mirrors that of the beta cell. In 1869, a 22 year old German medical student, Paul Langerhans, identified an island of clear cells in the pancreas, later named “The Islets of Langerhans” [15], [16]. In 1907 another medical student, Michael Lane discovered that there were two distinct cell types in the islets, which he went on to term the alpha and beta cell [17]. Charles Best, whilst working as a medical student for Dr Fredrick Banting, noted that when the early crude extracts of canine pancreas were administered to diabetic dogs, there was an early rise in blood glucose, before subsequent hypoglycaemia. Finally, in 1923 Charles Kimball, a biochemistry student, isolated a substance from the alpha cell and named it “glucagon” [18]. In 1957 the amino acid sequence was elucidated by a team of biomedical scientists at the Lilly Research Laboratories.

1.4.2 Physiology of alpha cells

Immuno-histochemically, the alpha cell is defined by the presence of glucagon staining,

and the secretion of glucagon is considered to be the primary role of the alpha cell [19]. Glucagon targets the glucagon g-protein coupled receptor that stimulates glycogenolysis and gluconeogenesis, and decreases glycogenesis and glycolysis [20]–[22]. Glycogenolysis of glycogen stores primarily occurs in the liver, but can also occur in muscle. However, the concentrations in the hepatic portal system are significantly higher than those in the systemic circulation. Glycogenolysis involves the breakdown of glycogen stores to glucose-1-phosphate and subsequently to glucose, thus raising blood glucose levels. Gluconeogenesis is an alternative pathway, releasing glucose from non-carbohydrate metabolic stores (see figure 1.1).

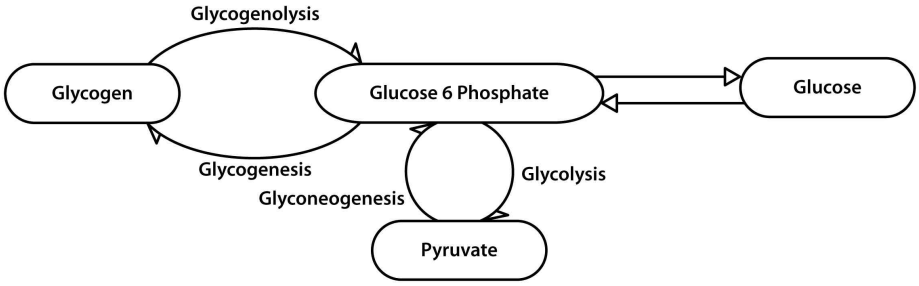


Figure 1.1: Carbohydrate metabolic pathway.

Glucagon is a 29 amino acid sequence peptide involved in glucose homeostasis. It is cleaved from the larger proglucagon peptide by a prohormone convertase. It primarily affects glycogen storage in the liver, with the subsequent release of glucose. Glucagon, along with the Glucagon related peptides, bind specifically to the B1 class G-protein coupled receptor (GPCR) family. This is an evolutionarily conserved receptor family which includes receptors that bind to Glucagon, Gastric Inhibitory Polypeptide (GIP). Glucagon Like Peptide -1 (GLP-1), Glucagon Like Peptide-2 (GLP-2), Vasoactive

Intestinal Peptide (VIP), Secretin, Calcitonin, Corticotrophin releasing hormone peptides (CRH), Pituitary adenylate cyclase-activating peptide (PACAP) and Growth hormone-releasing hormone (GHRH). These receptors have a seven transmembrane structure with a long N-terminal extracellular domain which contains the primary ligand binding site. This group of receptors appear to be influenced by the accessory peptides Receptor Activity Modifying Protein (RAMP). In particular the glucagon receptor is influenced by RAMP2. Stimulation of the glucagon receptor activates adenylate cyclase, resulting in an increase in cAMP that leads to activation of protein kinase A (PKA). Protein kinase A causes stored glycogen breakdown through glycogenolysis in order to release glucose for energy utilisation.

The proglucagon gene was isolated in 1983, four years after the insulin gene was first sequenced [23]. The proglucagon gene encodes glucagon, as well as oxyntomodulin, glicentin and glucagon like peptides 1 and 2 (GLP-1 and 2). The gene is expressed primarily by pancreatic alpha cells, intestinal L-cells as well as some neuronal cells [24]–[26]. However, these cells differ in how the pro-peptide is processed by the enzyme prohormone convertase (PC). PC1/3 is predominantly expressed in the intestinal L-cells and cleaves proglucagon to release GLP-1, GLP-2 and oxyntomodulin. PC2 is predominantly expressed in pancreatic alpha cells and the glucagon secreting enteroendocrine cells of the stomach, and cleaves proglucagon to release glucagon [27]–[32].

Alpha cells release glucagon in response to two distinct pathways: glucose dependent and hormone dependent. The glucose dependent pathway stimulates glucagon release through the formation of calcium channel-SNARE protein complexes resulting in

glucagon granule exocytosis [33]. The intracellular mechanisms involved in the hormone dependent pathway are currently unknown, but probably mediated by Protein Kinase C (PKC) [34], [35]. The hormones involved in this pathway are produced and mediated via a complex hormonal network involving the liver, intestine, nervous system, muscular and adipose tissue (e.g. hepato-enteral, neuronal, adipo-muscular, Islet (HENAMI) endocrine network) (see Figure 1.2). The function of this network is to regulate the storage and utilisation of energy. The Islets of Langerhans are a key part of this network and are composed of five distinct endocrine cells (see Figure 1.3): alpha cells secreting glucagon, glucagon like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP); beta cells secreting insulin and amylin; delta cells secreting somatostatin; epsilon cells secreting ghrelin and the PP cells (also known as F cells) secreting pancreatic polypeptide and small quantities of peptide YY (PYY) [36]–[39]. All islet cells receive and secrete hormones either locally into the islet interstitium (paracrine) or regionally via the intra-islet capillaries into the hepatic portal circulation (endocrine). The islet cells also receive signals from the brain and possibly the intestine via neuronal innervation [40]–[44]. Hormone dependent stimulation of alpha cell secretion of glucagon is principally controlled by paracrine signals from the Islets of Langerhans, in particular from the beta cells and delta cells [45]–[48].

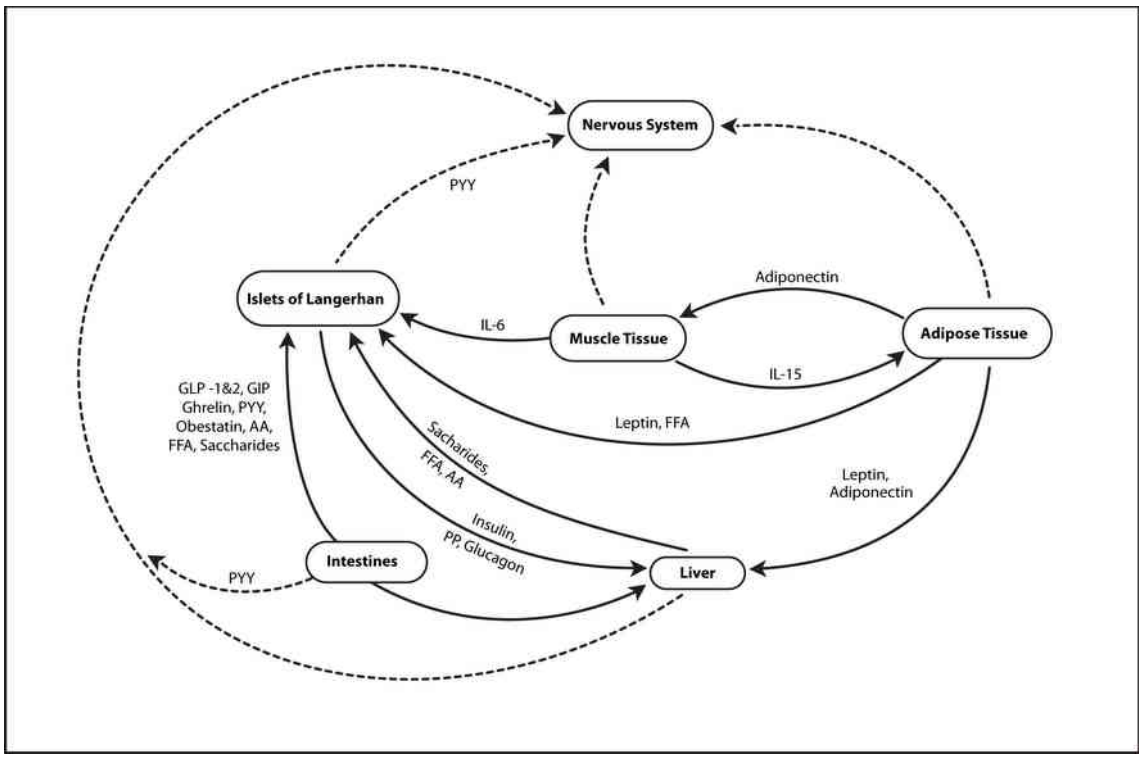


Figure 1.2: HENAMI – (hepato-enteral, neuronal, adipo-muscular, islet) hormonal network.

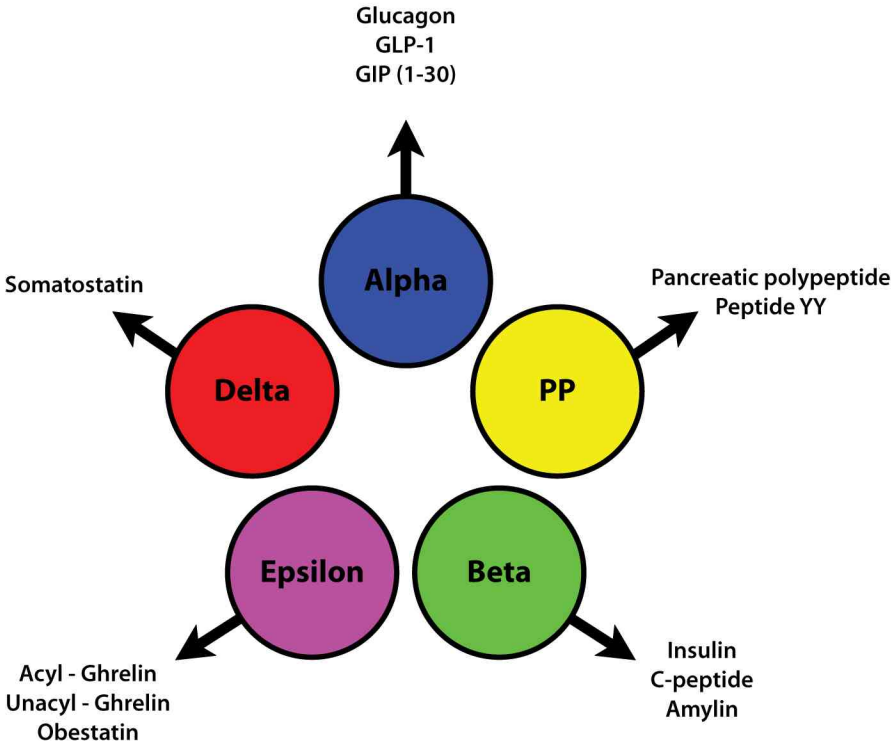


Figure 1.3: Cells of the Islets of Langerhans

In health, a postprandial rise in blood glucose activates both a glucose mediated and beta cell derived hormone mediated inhibition of glucagon release from the alpha cells. This in turn inhibits hepatic glucose release[20]. Conversely, low blood glucose inhibits the beta cell derived inhibition of glucagon secretion, thus the alpha cell releases glucagon, resulting in hepatic glucose release.

The intestine also secretes other hormones involved in the HENAMI network. The intestinal L cells secrete glucagon like peptide -1 (GLP-1) and glucagon like peptide – 2 in equimolar concentrations. GLP-1 inhibits alpha cell secretion of glucagon. It also stimulates beta cell secretion of insulin and inhibits beta cell apoptosis. GLP-2

stimulates both intestinal cell growth and alpha cell secretion of glucagon [49]–[54]. The mechanism that controls the balance between GLP-1 and GLP-2 signalling in alpha cells is unknown. The intestinal K cells secrete GIP which stimulates alpha cell secretion of glucagon and possesses anti-apoptotic beta cell properties [55]–[57]. In healthy humans GIP administration results in a dose dependent increase in glucagon secretion from alpha cells [58]. The PC2 expressing alpha cells have also been found to secrete a truncated form of GIP [59]. Both forms of GIP are biologically active, but their mode of action may vary.

The intestinal P/D1 and X/A-like cells found in the stomach's oxyntic glands express the proghrelin peptide [39], [60]–[63]. These cells cleave proghrelin and secrete it as acylated-ghrelin, unacylated-ghrelin and obestatin [64], [65]. Acylated-ghrelin can bind to receptors on the alpha cell to stimulate glucagon secretion and act on the brain to stimulate hunger [66]. Leptin is secreted by adipocytes and has the opposite action to ghrelin by inhibiting alpha cell secretion of glucagon and decreasing hunger [67]–[71]. Leptin is closely related to IL-6 which is released from muscle tissue in response to exercise [72], [73]. Il-6 can also stimulate alpha cells to secrete a small quantity of GLP-1, which stimulates beta cell secretion of insulin [74].

1.4.3 Glucagon function in type 1 diabetes

In type 1 diabetes the loss of beta cells results in failure to inhibit alpha cell secretion of glucagon. This results in persistent hyperglucagonaemia that drives the release of glucose from hepatic stores [75], [76]. In severe insulin deficient hyperglycaemia the systemic blood glucagon concentration causes the release of glucose from muscle stores and stimulates lipolysis in adipose tissue. This can lead to ketone formation and

clinically to diabetic ketoacidosis [77]–[79].

In paediatric case studies of new onset type 1 diabetes, there is a 45% decline in meal-stimulated C-peptide, and a reciprocal 37% rise in glucagon levels within the first year [80]. Other studies have found that whilst glucagon suppression to an intravenous glucose challenge remains intact in type 1 diabetes, there is a 0-30 minute increase in glucagon secretion before suppression to an oral glucose challenge [81]–[83]. This suggests that intestinal derived factors influence glucagon secretion in type 1 diabetes, but not in individuals with preserved beta cell function. Conversely a study in type 1 diabetes found that insulin induced hypoglycaemia failed to significantly stimulate glucagon release at a median of 8 months after diagnosis, whilst arginine stimulated glucagon release remained intact. The pathophysiology for this remains unknown, but may be due to desensitisation in the glucose dependent mechanism that controls glucagon exocytosis. Clinically this leads to an impairment in response to hypoglycaemia in individuals with type 1 diabetes [84]–[86].

1.4.4 Current drugs that target glucagon secretion.

High dose glucagon is used in clinical practice to treat severe disabling hypoglycaemia in type 1 diabetes and historically in treating beta blocker overdose [87]. In a trial of 15 participants with type 1 diabetes the use of a combined glucagon and insulin closed loop delivery system improved glycaemic control, decreased episodes and time in hypoglycaemia [88], [89]. Unfortunately, currently purified glucagon peptide degrades rapidly and forms amyloidogenic fibrils, but synthetic glucagon analogues have the potential to last longer [90]. In the future synthetic glucagon analogues have the potential to be used in dual hormone closed loop systems [91].

Activation of the g-protein coupled GLP-1 receptor on some pancreatic alpha cells results in a reduction in glucagon secretion [14], [92]. GLP1 stimulation of beta cells, and the paracrine effects of the resulting insulin secretion on adjacent alpha cells lead to a decrease in glucagon secretion. Thus, clinically approved drugs that target the incretin pathway (i.e. GLP-1 agonists and DPP-4 inhibitors) exert some of their glycaemic effects through the reduction in alpha cell secretion of glucagon, thus reducing release of hepatic glucose. These drugs have now established themselves as important agents in the treatment of type 2 diabetes.

Exploratory trials of the use of GLP-1 analogues in individuals with type 1 diabetes suggest there may be glycaemic benefit (see Figure 1.4) [93]–[96]. This benefit persists in the absence of detectable C-peptide, suggesting GLP-1 agonists exert a direct inhibitory effect on the alpha-cells or through an as of yet unexplored mechanism. These trials also show reduced insulin requirements in individuals with type 1 diabetes, without an increase in hypoglycaemic episodes, as well as weight loss. GLP-1 analogues are not currently licensed in type 1 diabetes by the US Food & Drug Administration (FDA) or European Medicines Authority (EMA). However, a large international trial into the effects of GLP-1 agonists in type 1 diabetes is under way. Drugs that inhibit the enzyme DPP-4 from degrading endogenous GLP-1 are also widely used in clinical practice for type 2 diabetes. Vildagliptin and Sitagliptin have undergone trials in individuals with type 1 diabetes resulting in a reduction in glucagon levels and improvements in postprandial glycaemia (see Figure 1.5) [97]–[107]. DPP-4 inhibitors are not currently licensed in type 1 diabetes by the FDA or EMA.

Study	*Study Design*	*Intervention*	*HbA1c(%) Effect*	*Meal stimulated Glucagon effect*
Varanasi 2011	24 week open labelled,cohort study (n=8)	Liraglutide variable dose	Baseline vs 24 weeks: 6.5 vs 6.1, p<0.05	N/A
Kielgast 2011	4 week open-labelled, cohort study (n=29)	Liraglutide 1.2mg OD	Baseline vs 4 weeks: C-peptide +ve on liraglutide (n=10) 6.6+/-0.3 vs 6.4+/-0.3, p<0.05; C-peptide -ve on liraglutide (n= 9) 7.5+/-0.2 vs 7.0+/-0.1, p<0.05; C-peptide -ve not on liraglutide (n=10) 7.1+/-0.3 vs 6.9+/-0.2, p>0.05	N/A
Raman 2010	Single Dose double-blinded, randomised, placebo controlled study (n=8)	Exenatide 1.25µg – 2.5µg or Placebo	N/A	No statistical difference

Table 1.3 GLP-1 inhibitors in type 1 diabetes

Study	*Study Design*	*Intervention*	*HbA1c(%) Effect*	*Meal stimulated Glucagon effect*
Garg 2012	16 week double-blind, randomized placebo-controlled parallel study (n=125)	Sitagliptin 100mg OD or Placebo	Change from baseline: Sitagliptin 100mg vs Placebo; -0.07 +/- 0.7 vs -0.12 +/- 0.75, p>0.05	4 hrs AUC (pg/ml.min) Sitagliptin vs Placebo: 84.7 ± 23.6 vs 84.9 ± 35.3, p>0.05
Farngren 2012	4 week double-blind, randomized, placebo-controlled crossover study (n=28)	Vildagliptin 50mg BD or Placebo	Difference between 4 weeks: Vildagliptin vs Placebo; -0.32 +/- 0.09%, p<0.005	2hr AUC (nmol/l.min) Vildagliptin vs Placebo: 2.4 +/- 0.2 vs. 2.6 +/- 0.2, p<0.05
Ellis 2011	4 week double-blind, randomized, placebo-controlled crossover study (n=20)	Sitagliptin 100mg OD or Placebo	Difference between 4 weeks: Sitagliptin vs Placebo; -0.27 +/- 0.11%, p<0.05	N/A
Foley 2008	4 week double blind, randomised, placebo-controlled, cross-over trial (n=12)	Vildagliptin 100mg BD or Placebo.	N/A	1hr AUC (mcg/l.min) Vildagliptin vs Placebo: -0.2+/-0.6 vs 1.9+/- 0.6, p<0.05

Table 1.4: DPP-4 inhibitors in type 1 diabetes

1.4.5 Future drugs that might target glucagon secretion

The number of identified target receptors that regulate alpha cell function has increased over the last decade. The development of novel dual GLP-1/Glucagon receptor and GLP-1/GIP receptor targeted peptides have been shown to treat hyperglycaemia in mice. Ghrelin antagonists which decrease alpha cell secretion of glucagon in type 1 diabetes have been shown to improve glycaemic control in non-human studies [108]–[111]. The

infusion of a leptin related peptide in mice models of type 1 diabetes prevented hyperglucagonaemia and improved glycaemic control [112]. The leptin analogue, Metreleptin has now entered phase 1 clinical trials in patients with type 1 diabetes. A group of novel drugs that target the cannabinoid receptor GPR119 have shown promise in protecting mice alpha and beta cells from streptozotocin induced apoptosis that leads to type 1 diabetes [113].

Tocilizumab, an Il-6 receptor monoclonal antibody, showed an improvement in glycaemic control when used in rheumatoid arthritis patients with concomitant diabetes [114]–[116]. Il-6 is also a marker of inflammation and a constant low grade inflammatory process may accelerate the micro-vascular and macro-vascular complications of type 1 diabetes [117]. Acute changes in Il-6 may act through increasing gut expression of GLP-1 [118]–[121]. Thus, there may be future scope in using the Il-6 receptor in regulating alpha cell secretion of glucagon and reducing rates of micro-vascular complications.

Probably the most exciting new drugs in development are those that act directly on glucagon receptor signalling. Lee *et al*, subjected glucagon receptor knock-out mice and wild-type mice to two doses of streptozotocin [122]. The wild-type mice developed hyperglycaemic ketoacidosis, whilst the glucagon receptor knock-out mice maintained normal fasting glucose levels and did not develop ketoacidosis nor diabetes. This opens the possibility of novel treatments through disruption of glucagon receptor signalling to prevent the hyperglycaemia of type 1 diabetes [123], [124]. Similarly non-diabetic monkeys given glucagon receptor blocking antibodies showed a reduced blood glucose level in a dose dependent manner [125]. The antibody was also given in higher doses to

ob/ob mice and was successful in suppressing their hyperglycaemia [124]–[126].

1.5 Other Gut Hormones

1.5.1 Neuropeptide Y Family

Neuropeptide Y family of peptides are potent orexigenic hormones. The family consists of three hormones, Neuropeptide Y (NPY), Peptide YY (PYY) and Pancreatic Polypeptide (PP) and were first identified in 1975 [127]–[129]. In humans there are at least four functional Y receptors (Y1R, Y2R, Y4R & Y5R) [130]. The receptors have different binding affinities to NPY, PYY & PP. NPY preferentially binds to Y1R and Y5R, whilst PP binds Y4R. PYY and NPY equally bind with high affinity to Y2R. Y2R is mainly found in the peripheral nervous system supplying both blood vessels and intestine. Y4R is mainly expressed in the gastrointestinal tract, skeletal muscle, heart and thyroid. It has been shown to be involved in pancreatic secretion, gallbladder contraction and Luteinising Hormone secretion. Y1R and Y5R are mainly located in the CNS, but Y1R is also found in adipose tissue and smooth muscle innervation. Agonists of Y2R and Y4R receptors have been developed and are currently undergoing investigation as potential targets for weight management drugs.

Neuropeptide Y (NPY) is a neurotransmitter and is mainly localised to the brain and the autonomic nervous system. It is involved in a multitude of processes including regulation of feeding, blood pressure, addiction, memory and inflammation. Release of NPY increases food intake, and the storage of energy as fat. NPY antagonists are in early clinical trials and hope to significantly reduce food intake and suppress appetite and thus cause weight loss.

Pancreatic polypeptide is a 36 amino acid sequence peptide involved in energy homeostasis. It is produced by the PP cells (also known as F cells) in the islets of Langerhans and is a member of the neuropeptide Y hormone family. In the fasting state levels of PP are low, but increase within minutes of being stimulated by food [131]. Its release follows activation of the efferent vagal nerve and in particular it is sensitive to foods with mixed nutrient composition. Thus, PP can be used as a marker for vagal afferent activation. Vagal afferent activation causes hepatic glucose production to decline and increases insulin secretion. In individuals with anorexia, baseline PP levels are chronically elevated, but are chronically suppressed in individuals that overeat. In studies where PP is infused into healthy volunteers, it resulted in both a reduced appetite and decreased calorie intake. Thus, it is hypothesised that PP binds to the neuropeptide Y4 receptors located in the brain stem and hypothalamus to suppress appetite. The effects of PP last around 6hours, thus helping to regulate intervals between mealtimes.

PYY is released from enteroendocrine cells located in the gut and some specialized hypothalamic neuronal cells in response to food intake. PYY is released as PYY1-36 which bind to Y1R, Y2R and Y5R, but then is degraded by DPP-4 to PYY 3-36 which can still bind to Y2R. The Y2R is commonly found in the nerves supplying the gut, thus PYY reduces appetite and decreases gastric motility. When PYY binds to NY2 neurones in the arcuate nucleus of the hypothalamus, it acts as a competitive inhibitor to NPY. Thus it removes the inhibitory effect that NPY has on the release of GABA neurotransmitters in the hypothalamic Proopiomelanocortin (POMC) neurones. In turn this release of GABA neurotransmitters stimulates alpha Melanocyte Stimulating Hormone (αMSH) production which is has a potent inhibitory effect on appetite [132].

1.5.2 Somatostatin

Somatostatin secreting cells are found in the stomach, pancreas (delta cells), hypothalamus and intestine. Somatostatin acts on at least 5 distinct g-protein coupled somatostatin receptors (SSTR1-5). Somatostatin primarily acts in an inhibitory capacity on other endocrine cells and can inhibit the secretion of insulin, glucagon, GLP-1, GIP, VIP, Cholecystokinin, Motilin, Gastrin, Secretin, Thyroid Stimulating Hormone, Growth Hormone and the exocrine pancreatic enzymes. Somatostatin release is sensitive to glucose concentrations, with a sudden rise in glucose resulting in a pulsatile release of somatostatin, as well as glucagon and insulin from the Islets of Langerhams.

Somatostatin secreting cells do not appear to be affected by type 1 diabetes. However, in pre-pubertal individuals they can maintain a pleuripotent ability and transform themselves into beta cells [133]. The role and interest in somatostatin as a potential drug target has increased due to both its ability to regulate glucagon and insulin, as well as being a potential reservoir for beta cell regeneration. In preliminary animal studies, the inhibition of insulin has been shown to occur via the SSTR5 and inhibition of glucagon via SSTR2 [134]. There are currently a number of SSTR2 antagonists in early phases of development that manipulate glucagon secretion which may help to prevent recurrent hypoglycaemia in individuals with diabetes [135] .

1.5.3 Incretin Hormones

Incretin describes a group of gut peptides which are released on oral food consumption and enhance insulin secretion that would otherwise be absent with matched intravenous infusion. They therefore promote the efficient uptake and storage of energy. GLP-1 is a member of the incretin family and is produced as Proglucagon which is cleaved by PC-

1/3 in intestinal L-cells to produce GLP-1 and GLP-2. Active GLP-1 (7-36) rises acutely in response to food consumption (in particular fatty acids and glucose). Its release potentiates insulin release and is estimated to account for up to 50% of the postprandial stimulated insulin secretion. It also appears to inhibit glucagon secretion via a somatostatin dependent paracrine effect, thus further increasing insulin sensitivity. Unfortunately, it has a very short half life and is quickly degraded by Dipeptidyl peptidase-4 (DPP-4), so less than 10% of the total secreted from intestinal L-cells actually reaches the circulation. However, with the advent of GLP-1 agonists, it has been shown that it has a mechanism of action beyond its effect on insulin secretion. GLP-1 agonists also decrease glucagon secretion, delay gastric emptying, improve non-alcoholic fatty liver disease and reduce appetite.

There may also be a role for GLP-1 agonists in cardiovascular protection in type 2 diabetes, but this still remains controversial. In the “Evaluation of Lixisenatide in Acute Coronary Syndrome” (ELIXA) trial there was no significant difference in major adverse cardiovascular endpoints (MACE) in the lixisenatide arm compared to placebo arm of the trial [136]. However, in the “Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results” (LEADER) trial there was a significantly lower event rate in the liraglutide arm compared to the placebo arm for MACE [137]. Early data from the Harmony series of clinical trials involving albiglutide have suggested a small but significant increase in risk of atrial fibrillation in the albiglutide arm compared to the placebo arm, but without a significant difference in MACE [138]. Cardiovascular endpoint trials for dulaglutide are not due until 2019. However, there are outcome data from three trials involving DPP-4 inhibitors whose mechanism of action is to increase circulating levels of GLP-1. In the “Saxagliptin Assessment of Vascular Outcome

Recorded in Patients with Diabetes Mellitus – Thrombolysis in Myocardial Infarction S3" (SAVOR-TIMIS3) trial there was no significant difference in the saxagliptin arm compared to placebo arm of the trial for cardiovascular death, non-fatal myocardial infarction and non-fatal stroke, but there was a significant increase in hospital admissions for heart failure [139]. In both the "Examination of Cardiovascular Outcomes with Alogliptin vs Standard of Care" (EXAMINE) trial and the "Trial Evaluating Cardiovascular Outcomes with Sitagliptin" (TECOS) there were no significant differences in MACE outcomes compared to placebo [140], [141]. Thus suggesting that whilst there is no cardiovascular protective advantage from DPP-4 inhibitor drugs, there may be an advantage of certain GLP-1 agonists in terms of cardiovascular protection. It is unlikely that we will know whether GLP-1 agonists as a class provide a clinically meaningful level of cardiovascular protection until a complete meta-analysis of all trials is performed.

GIP is also a member of the incretin family of peptides and is produced from the K cells in the small intestine [55]. It is also degraded rapidly by DPP-4 and potentiates insulin release following oral consumption of food. GIP is typically described as a stimulant of insulin secretion that works in a glucose dependent manner. Glucose can potentiate its effect on GIP signalling through stimulating GIP receptor expression and inhibiting DPP-4 expression. This mechanism is mainly lost in individuals suffering from type 2 diabetes through the development of GIP resistance. GIP has been shown to stimulate glucagon secretion, which is in sharp contrast to GLP-1. Thus, there is a potential for GIP to actually worsen glycaemic control. GIP has also been proposed to have wider actions on adipocytes to stimulate fatty acid metabolism, osteoclastic activity to reduce bone re-absorption and in the adrenal secretion of cortisol.

1.5.4 Ghrelin

Ghrelin is a 28 amino acid peptide secreted in the gastrointestinal tract and central nervous system [142]. It is involved in energy homeostasis and is known as an orexigenic hormone. Enteroendocrine cells in the stomach and duodenum are the principle sources of ghrelin secretion. The consumption of food stretches the gastric lining which inhibits ghrelin section, whilst an empty stomach stimulates ghrelin secretion. Preproghrelin is cleaved by the enteroendocrine cells by prohormone convertases 1/3 to form proghrelin and obestatin. Proghrelin is then catalysed by ghrelin-O-acyltransferase (GOAT) to the principal active hormone “acylated ghrelin”. The preproghrelin gene is able to produce three distinctly biologically active hormones: acylated ghrelin, desacylated ghrelin and obestatin. An increase in ghrelin stimulates the central nervous system to increase appetite and consumption of food. It decreases under physiologically stressful conditions (e.g. infection, trauma and physical exertion) and on intravenous administration of either somatostatin (SST2 specific) agonist or GLP-1 or insulin. The ghrelin receptor (GRLN-R) in humans is localised in the pituitary, adipose tissue, adrenal gland, pancreas and brain [143].

1.5.5 Leptin

Leptin is a 167 amino acid peptide which shares similar chemical characteristics to cytokines. It is mainly secreted from adipocytes in white fat, but can also be produced from the placenta, bone marrow and ovaries. Leptin levels are closely correlated to total adipose mass [144]. Leptin levels follow a circadian rhythm with their peak levels at midnight and lowest at midday. This is notably opposite to the circadian rhythm of cortisol. Circulating leptin primarily stimulates hypothalamic neurones to inhibit hunger

through inhibiting NPY and Agouti related peptide (AgRP) neurones and activating Pro-opiomelanocortin (POMC) neurones. Mice that are leptin deficient (ob/ob) demonstrate obesity, hyperphagia, increased susceptibility to infections, postural hypotension, partial hypothalamic failure (growth hormone deficient, hypothyroid and hypogonadism) and features of the metabolic syndrome. Mice that carry a leptin receptor mutation (db/db) similarly demonstrate obesity, hyperphagia, but develop type 2 diabetes with fasting hyperglycemia and hyperinsulinemia. The ob/ob mice models along with the leptin resistant (db/db) mice models have been crucial in furthering the understand of the physiological role of leptin and its role in insulin resistance.

In humans, congenital leptin deficiency is a rare cause of childhood morbid obesity that can be treated with subcutaneous injections of leptin analogues. These leptin analogues have also been shown to be effective at partially restoring hypothalamic function and reducing features of the metabolic syndrome in both obese individuals and those with very low body fat (eg lipodystrophy). Animal models have suggested that exogenous leptin administration may also be an effective treatment in type 1 diabetes [145]. The mechanism of action was originally thought to involve the suppression of glucagon, but this has now been refuted. Therefore, it is hypothesized that leptin restores euglycaemia in type 1 diabetes through altering lipolysis [146].

Outside of the field of glucose homeostasis, leptin has been demonstrated to influence inflammation, vascular constriction and the reproductive system. The Ob-R leptin receptor is found on leucocytes and shares structural similarities to class 1 cytokine receptors, such as Il-6R and granulocyte colony-stimulating factor [147]. Activation of this leptin receptor enhances leucocytic phagocytosis, stimulates CD8+ and B

lymphocyte cell proliferation. Thus, leptin could be considered a pro inflammatory cytokine. Animal and human models have demonstrated the presence of raised leptin levels in auto-immune conditions such as psoriasis, rheumatoid arthritis, systemic lupus erythematosus and inflammatory bowel disease. This tendency to develop autoimmune disease is found to be significantly reduced in both the ob/ob and db/db mice models.

Leptins have also been shown to activate neuronal pathways in the hypothalamus that stimulate the sympathetic nervous system [148]. This was demonstrated through the infusion of leptin into rodent models leading to both an increase in blood pressure and heart rate [149]. The administration of drugs that blocked the sympathetic nervous system reversed the effect. Leptin also stimulates endothelin-1 secretion in the smooth muscle of blood vessels leading to vasoconstriction and release of oxygen free radicals which leads to the progression of atherosclerosis [150]. This helps to explain why the chronic hyperleptinaemic state is associated with the early onset of cardiovascular disease. Leptin is also hypothesised to effect uterine smooth muscle and research into this field may help advance the understanding of pre-eclampsia.

Finally, leptin has been shown to influence the human reproductive system and in particular the onset of puberty. Precocious puberty is the premature onset of secondary sexual characteristics in children and is commonly associated with the hyperleptinaemic state of obesity. The role of the arcuate nucleus in the hypothalamus appears to be the key to the signalling pathway that links leptin and the release of early spike in gonadatrophins. Within the arcuate nucleus both the signalling pathways of “αMSH-kisspeptin-Gonadotropin-releasing hormone” and the “leptin-αMSH” co-exist. It is hypothesized that these two pathways are to some way linked to form a “leptin-αMSH–

kisspeptin-Gonadotropin-releasing hormone" pathway which could lead to precocious puberty.

1.5.6 Adiponectin

Adiponectin is a 224 amino acid collagen like protein that is derived from adipocytes. It has anti-inflammatory and insulin sensitizing properties [151]. Compared to most adipocyte derived hormones, adiponectin is inversely proportional to body mass index with low levels recorded in obesity. It is able to suppress hepatic gluconeogenesis and stimulates fatty acid oxidation in the liver. There are two distinct adiponectin receptors AdipoR1 and AdipoR2. AdipoR1 is predominantly located in skeletal muscle and AdipoR2 is predominately located in the liver [152]. Adiponectin also been shown to be secreted by the placenta, and levels are lower in non-pregnant females than males. Adiponectin concentrations have also been found to be higher in males than in females. High levels of adiponectin increase glucose uptake, decreases gluconeogenesis and triglyceride clearance. It is noted that in non-alcoholic steatohepatitis (NASH) the expression of both receptors is reduced. In individuals with insulin resistance the levels of adiponectin are significantly reduced. It is also used as biomarker of inflammation and endothelial dysfunction [153]. However, in a recent meta-analysis it was not found to be strongly linked to cardiovascular disease [154], [155]. Low levels of adiponectin are also associated with dyslipidaemia, through modulating lipoprotein lipase activity.

1.5.7 Il-6

Il-6 is a cytokine mainly produced from adipose tissue, but is also released from skeletal muscle in response to acute exercise. Il-6 acts both as a pro-inflammtoary cytokine in macrophages and anti-inflammatory myokine through inhibiting TNF alpha and Il-1

[115]. IL-6 levels are found to be chronically raised in obese subjects and those with type 2 diabetes [74]. Post exercise, Il-6 release leads to increased secretion of GLP-1 from intestinal L-cells and pancreatic alpha cells, thus improving insulin resistance [156]. Il-6 also inhibits alpha cell apoptosis and its proliferation. Il-6 appears to have adaptive mechanisms that are overall beneficial in individuals with type 2 diabetes.

1.6 Clinical Characteristics of Diabetes

Diabetes mellitus describes a common clinical characteristic that is an endpoint of multiple aetiologies. Diabetes is characterised by hyperglycaemia along with the classical clinical triad of symptoms: polydipsia, polyuria and weight loss. These classical clinical symptoms can occur to a varying degree depending on the aetiology and the degree of hyperglycaemia. Diagnostic criteria for Diabetes were not standardised until 1965 when the WHO produced their Expert Committee findings [157]. This was later consolidated and simplified in the WHO second Expert Committee report in 1980, to allow for the absence of symptoms in the diagnosis of diabetes [158]. In the absence of symptoms then two separate biological samples that confirm hyperglycaemia (fasting glucose ≥7.0mmol/l, or two-hour OGTT glucose ≥ 11.1mmol/l, or random glucose ≥11.1mmol/l or HbA1c ≥ 6.5%) are required to confirm the diagnosis.

Type 1 diabetes commonly presents with acute onset of the classical clinical triad of symptoms alongside hyperglycaemia. However, biochemical features may have been present for many years before the onset of symptoms. The loss of the first phase insulin response occurs prior to the onset of symptoms leading to an increase in glucose

variability [159].

1.6.1 Type 1 Diabetes Pathology

Type 1 diabetes is histo-pathologically characterised by the lymphocytic infiltration of the Islets of Langerhans [160]. Type 1 diabetes is a chronic autoimmune disorder with the onset of clinical features dependent on the degree of insulin insufficiency caused by the percentage of beta cell dysfunction and also related to the duration of dysfunction, percentage of islets affected and rate of beta cell destruction. Histological analysis of pancreases from individuals newly diagnosed with type 1 diabetes show specific loss of insulin expressing beta cells but preservation of other islets with occasionaly mild alpha cell hyperplasia [161], [162]. Pancreatic atrophy then develops as the process continues and is even seen as early as symptom onset. Atrophy progresses over time so that individuals with long standing diabetes have a pancreas which is frequently 50% smaller than normal. It is speculated that the atrophy results from the autoimmune process spilling over and damaging the acinar cells that surround the inflamed Islets [163], [164].

1.6.2 Histopathology

Insulinitis is described microscopically as the infiltration of pro-inflammatory cells (cytotoxic T cells, T and B lymphocytes and macrohpages) into Islets of Langerhans that contain beta cells. The degree of infiltration correlates to earlier age of onset of type 1 diabetes and presence of multiple monoclonal antibodies targeting islet related antigens. The target antigens that have been identified include: insulin, tyrosine phosphatase-like protein (IA-2), islet specific glucose-6-phosphatase catalytic subunit related protein (IGRP), preproinsulin, glutamic acid decarboxylase (GAD65) and islet

amyloid polypeptide precursor protein (ppIAPP) [165], [166]. Thus, the more aggressive disease occurs in the pre-pubertal population with many of these antigens targeted. Inflammation is more commonly seen in individuals with recent onset of type 1 diabetes, but may be present prior to symptom onset. The degree of infiltration varies between each individual Islet and may be absent from the majority of islets in older individuals with less aggressive inflammatory responses.

1.6.3 Genetic and environmental susceptibility

The genetic susceptibility of developing type 1 diabetes was first demonstrated through the use of twin studies in 1972 [167], [168]. Further studies demonstrated that if one mono-zygotic twin has type 1 diabetes the incidence of developing type 1 diabetes in the other twin is approximately 40% and in di-zygotic twins the incidence is approximately 20%. However, in the background population it is only 0.2%. Genetic susceptibility involves multiple genes primarily involving the HLA class of genes. Genome-wide association studies (GWAS) in the last decade have helped to identify numerous single nucleotide polymorphisms (SNPs) linked to type 1 diabetes. These SNPs can either increase or decrease an individual's susceptibility to type 1 diabetes. In particular the DR3/4-DQ8 haplotype (DR3=DRB1*03-DQB1*0201, DR4=DRB1*04-DQB1*0302, DQ8=DQA1*0301, DQB1*0302) occurs in >20% of individuals with type 1 diabetes [169], [170]. If these SNPs are found in a newborn, then they have approximately 20% chance of developing islet antibodies by the age of 2 years compared to a 2.7% background rate, which could lead on to type 1 diabetes.

Individuals who develop type 1 diabetes are thought to have a genetic susceptibility, which is then triggered by environmental factors [3]. Environmental agents that

precipitate type 1 diabetes are thought most likely to enter the body via the gastrointestinal tract triggering the mucosal immune system. Environmental agents that have been put forward as possible triggers include enterovirus, gut bacteria, cows milk or wheat. Whilst these agents are suspected, causation for all these triggers has not yet been proven.

1.6.4 Current Treatments of Type 1 Diabetes

The primary treatment of type 1 diabetes is exogenous insulin administration. The goal is to relieve symptoms, reduce hyperglycaemia, reduce glucose variability and so reduce the risks of complications (e.g. nephropathy, neuropathy, retinopathy, hypoglycaemia and atherosclerotic disease). In order to reduce glucose variability, exogenous insulins and their administrative devices have been designed to mimic a normal endogenous response. The ability to produce human analogue insulins has decreased the requirements of pancreas extract from pigs and cows. The ability to alter the speed of insulin absorption has provided us with two main classes of insulin; the rapid acting insulin to treat postprandial hyperglycaemia and the long acting insulin that provides a continuous low level background dose of insulin. Insulin delivery devices such as disposable dose adjustable insulin pens has improved accuracy of insulin dose delivery. In particular, continuous subcutaneous insulin infusions (CSII) devices that are programmable have made a great impact. They help to reduce glucose variability by permitting the user to program hourly variations in their background insulin rate and make temporary changes to their infusion rate depending on their need.

Individuals with type 1 diabetes can now better monitor their glucose levels through

pocket sized glucometers and the newer continuous blood glucose monitors. The ability to easily monitor capillary glucose in response to diet and exercise provides important biofeedback to the individual. The individual can then appropriately adjust rapid acting insulin doses to decrease glucose variability in response to diet and exercise. These devices now incorporate mini computers that can calculate insulin dosage bolus advice once pre-programmed with coefficients for diet and insulin sensitivity. In the most up to date devices this can then be transmitted to a CSII using short range wireless technology (e.g. bluetooth).

For those individuals that do not reduce their rate of hypoglycaemia through these devices, then continuous glucose monitoring devices can be provided. These help the individual to monitor their glucose variability more frequently and can be linked to CSII devices to make necessary changes to their insulin administration. For those few individuals that continue to suffer from recurrent disabling hypoglycaemia, islet cell or whole pancreatic transplantations can sought, when available, to relieve symptoms [171]–[173].

Patient education is another key cornerstone of treatment for individuals with type 1 diabetes. The internationally recognised DAFNE (dose adjustment for normal eating) course has provided structure and content to facilitate optimum use of rapid acting insulin and glucometers [174]. The DAFNE course and similar education courses have been shown to significantly improve an individual's quality of life as well as improve glycaemic control [175].

1.6.5 Future Immuno-modulation treatments

Since the 1980's clinical trials have explored the theory of preserving beta cell function through suppressing the immune system in type 1 diabetes [176], [177]. These trials involved immunosupressive therapy more commonly seen in organ transplant recipients to prevent acute rejection, but unfortunately showed mixed results. Currently there are many therapeutic interventions being investigated to both prevent and inhibit the inflammatory response seen with the onset of type 1 diabetes and insulinitis. The anti-CD3 monoclonal antibody, teplizumab, was the first new agent with preliminary data showing significantly reduced inflammation, delay in the progression of type 1 diabetes and preservation of beta cell function [178]. The fusion molecule CTLA4-Ig (Abatacept), has also been shown in early phases of research to significantly delay the progression of type 1 diabetes by preventing the antigen presenting cells from activating T-cells [179]. Both these drugs focus on reducing the inflammatory process of insulinitis to delay the progressive loss of insulin secretion. An alternative immuno-modulating approach is to identify individuals at risk of type 1 diabetes, and repeatedly present a self-antigen to maintain immunological tolerance. Trials using repeated doses of oral insulin in individuals that have developed the insulin antibodies (IAA) are under way with preliminary data suggesting a delay of onset and progression of type 1 diabetes [180].

1.7 Type 2 Diabetes

Diabetes mellitus type 2 is less likely to present acutely than type 1 diabetes and is very rarely associated with ketoacidosis. However, similar to type 1 diabetes, there is a pre-diabetic period in which glucose variability increases prior to the onset of symptoms. This can manifest itself as an impaired fasting glucose (IFG) or impaired glucose

tolerance (IGT). In type 2 diabetes there is also a homoeostatic imbalance between the availability of excess glucose and its utilisation. However, unlike in type 1 diabetes where there is a decrease in insulin production, in type 2 diabetes it is usually due to an increase in insulin resistance. The excess availability of glucose is usually due to either overconsumption of carbohydrates or excessive release of carbohydrates from glycogen stores in the liver and muscle. The reduced utilisation of glucose is due to increased sedentary behaviour and it is the resistance to insulin that reduces the synthesis of glycogen. Pharmacological treatment of type 2 diabetes is principally targeted towards reducing and overcoming insulin resistance to facilitate storage of excess glucose. Significant weight loss through lifestyle changes or bariatric surgery can help to reduce insulin resistance and can lead to remission of type 2 diabetes. There are a variety of drugs which target insulin resistance that are used in the treatment of type 2 diabetes including biguanides, peroxisome proliferator-activated receptor gamma (PPAR-γ), DPP-4 inhibitors and GLP-1 agonists. Other drugs target the availability of glucose through either decreasing its absorption or by stimulating glucose excretion e.g. SGLT2 inhibitors, intestinal alpha glucosidase inhibitors and intestinal lipase inhibitors. If insulin resistance cannot be reduced, then it can be overcome by exogenous insulin administration.

1.8 Other types of Diabetes

Non type 1, non type 2 diabetes encompasses a spectrum of less prevalent aetiological causes of diabetes, including gestation induced, genetic abnormalities, non beta cell endocrine disorders, exocrine disorders of the pancreas, infections and drug induced diabetes.

Gestational diabetes occurs commonly during the second trimester due to hormonal changes (e.g. cortisol, progesterone, oestradiol and human placental lactogen levels) that increase insulin resistance. GLUT1 receptors on the placenta facilitate the transfer of maternal glucose to the fetus. Increasing maternal glucose levels increases fetal glucose levels which are then utilised by the fetus and stored as adipose tissue which can result in a macrosomic baby.

Genetic abnormalities leading to diabetes are numerous, but the most common occurring abnormalities are found in a group called 'Maturity Onset Diabetes of the Young' (MODY). MODY is a group of autosomal dominant monogenetic abnormalities that disrupt insulin secretion. They can present with hyperglycaemia at any age, but only occur in 1% of all individuals with diabetes. Usually they are initially diagnosed following a strong family history of phenotypically similar diabetes. They do not typically display insulin resistance or have detectable anti-islet cell antibodies. All forms of MODY that exhibit hyperglycaemia, except for MODY 3 and Neonatal Diabetes Mellitus, will eventually require insulin therapy. However, MODY 3 and Neonatal Diabetes Mellitus tend to initially be responsive to sulfonylureas.

1.9 Summary

Type 1 diabetes is a chronic autoimmune disease with long term micro-vascular and macro-vascular sequelae caused by hyperglycaemia and glycaemic variability. A functioning network of hormones is key to glycaemic homeostasis. In individuals with type 1 diabetes, beta cell function is impaired and there is evidence that this disrupts the network of hormones. Pancreatic endocrine cells appear to sit in the centre of a hormonal network regulating storage and utilisation of carbohydrates. Determining

which other hormones in this network are disrupted could prove effective as novel targets for new drugs. If alpha cell function did decline this may have a knock on effect on the other hormones in the endocrine network such as GIP, GLP-1, Peptide YY, PP, Leptin and Ghrelin.

1.10 HYPOTHESIS AND AIMS

Hypothesis:

- Gut Hormones are affected by type 1 diabetes

Aims:

- Establish a clinical research network for the recruitment and study of gut hormones in type 1 diabetes
- Explore the networks of gut hormones in individuals with type 1 diabetes.
- Explore the effects of lifestyle on the networks of gut hormones in type 1 diabetes.
- Explore the histopathological effect of type 1 diabetes on glucagon secreting cells in the pancreas.

2. ESTABLISH A CLINICAL RESEARCH NETWORK FOR THE RECRUITMENT AND STUDY OF GUT HORMONES IN TYPE 1 DIABETES.

2.1 Introduction

To study human gut hormones it was important to establish a network of clinical research studies in order to recruit participants. This was achieved by developing links to an established study and by designing a new study. The priority was not only to gain access to samples, but to ensure these samples were both in the fasting and fed state, in a reproducible manner, as gut hormone levels vary between the two states. Thus, all studies in the network ensured participants undertook a fast followed by a standardised meal stimulated test. To calculate an appropriate sample size to ensure statistical significance, a search of published literature was undertaken. Unfortunately, there was a paucity of data available that made determining an appropriate sample size impossible. So, in order to detect a significant difference in gut peptides in type 1 diabetes compared to healthy volunteers, it was decided to attempt to recruit at least twelve participants with a) early onset type 1 diabetes, b) chronic type 1 diabetes and c) healthy volunteers.

2.2 Exercise in Type One Diabetes Study (ExTOD)

In order to establish a network of clinical research studies, the study coordinator of a recently set up lifestyle intervention study was approached. The 'Exercise in Type One Diabetes (ExTOD)' study was recruiting individuals with new onset type 1 diabetes at the University of Birmingham. The chief investigator and study coordinator of this study kindly agreed to adapt the study's protocol in order to allow access to samples

from participants undergoing meal stimulated tests. This provided a means to facilitate the collection of samples for future measurement of gut hormone levels in individuals with new onset type 1 diabetes. As part of the collaboration I agreed to facilitate the set up of a second wave of study sites wishing to recruit to the ExTOD study. The study had initially begun recruiting in November 2011 with 5 centres: United Bristol Healthcare NHS Trust, Taunton and Somerset NHS Trust, North Bristol NHS Trust, Gloucestershire Hospitals NHS Trust and University Hospitals Birmingham Foundation NHS Trust. The study sites involved in the second wave that I was involved in setting up commenced recruiting between October 2012 – February 2013 and included: East & North Herts NHS Trust, Oxford University Hospitals NHS Trust, Mid Yorkshire Hospitals NHS Trust, Royal United Hospital Bath NHS Trust and Yeovil District Hospital NHS Foundation Trust. The process that I undertook involved: 1) screening the site, 2) setting up the site and 3) initiating the site.

1. The screening process started with the identification of a site and a principal investigator. The site was then screened to ensure the appropriate staff and equipment were in place to run the study.
2. Setting up the site involved gaining study site approval from local research and development departments and coordinating the signing of contracts by the Study Sponsor and the representatives of the study site.
3. Initiation of the site then followed after approval by both the sponsor and local Research and Development department. This was the final practical process required before the site could commence the study. For the ExTOD study this involved identifying a provider to educate the local research staff on motivational interviewing, show them how to use the activity monitors, and

finally talk through the study visits.

2.2.1 Protocol outline for the 'Exercise in Type One Diabetes study (ExTOD)'

The Exercise in Type One Diabetes (ExTOD) Study (www.birmingham.ac.uk/extod) has three main aims:

1. To determine whether motivation interviewing can increase the amount of exercise taken by an individual with newly diagnosed type 1 diabetes.
2. To act as a pilot study to determine whether an increase in the quantity of exercise an individual with newly diagnosed type 1 diabetes undertakes can reduce the rate of decline in c-peptide.
3. To provide feasibility data for an extended study by measuring: the enrolment period, site count, patient recruitment rate, screen failures, drop out rates and completion rates.

The "Exercise in Type One Diabetes study (ExTOD)" was funded by a "Research for Patient Benefit" grant from the National Institute of Health Research and supported through the Birmingham & Black Country Comprehensive Local Research Network (UKCRN ID: 12984). The chief investigator was Dr Parth Narendran and the University of Birmingham acted as the sponsor (ref: RG_09-123). Ethics approval was granted by the Birmingham, East, North & Solihull research ethics committee in February 2010 (ref: 10/H1206/4). University Hospitals Birmingham Foundation NHS Trust - Research and Development department approved the study in March 2010 (ref: RRK3950). The ethics committee submissions, NHS R&D submissions, study protocol, standard operating protocols and all documentation associated with the study was authored by Dr

Parth Narendran and Dr Amy Kennedy.

Screening for the study commenced in early November 2011 with the first participant recruited to the study in mid November 2011.

Study inclusion criteria:

1. Individuals between 16-60 years old and within twelve weeks of a clinical diagnosis of type 1 diabetes.
2. Individuals able and willing to self-monitor blood glucose and record their results.
3. Individuals who are considered safe to exercise.
4. Individuals willing and able to take multiple dose injection insulin.
5. Individuals able to increase their current exercise levels.

Study exclusion criteria:

1. Individuals who are pregnant or planning a pregnancy within the time frame of the study.
2. Individuals with a blood pressure greater than 180/100 mm Hg.
3. Individuals taking medication that affects heart rate (e.g. beta blocker, calcium channel antagonist, etc.)
4. Individuals with either ischaemic heart disease or psychiatric or physical disease that would prevent exercise.
5. Individuals who are due to undergo major surgery which would prevent exercise for more than 6 weeks within the time frame of the study.
6. Individuals participating in another type 1 diabetes related clinical trial.

Following recruitment (Visit 1) into the study, participants were questioned about their medical history, underwent anthropometric measurements, and asked to wear an activity monitor. The study involved a total of 12 study visits in the control group and 17 in the intervention (motivational interviewing) group:

- 1 enrolment visit
- 1 visit (baseline) review by a dietician.
- 3 visits (baseline, 6 months & 12 month) involved meal stimulated test.
- 3 visits (baseline, 6 months & 12 month) involved exercise fitness test.
- 4 visits (3 between baseline-6 months, 1 between 6-12months) involved clinical assessment by diabetes specialist nurse
- Intervention group - 5 extra visits (3 between baseline-6 months, 2 between 6-12months) involved motivational interviewing by a trained specialist nurse.

All participants recruited in Birmingham underwent the meal stimulated test and exercise fitness test at the Wellcome Trust Clinical Research Facility at the Queen Elizabeth Hospital Birmingham.

2.3 Chronic Disease Research into Diabetes study

As the Exercise in Type One Diabetes (ExTOD) study only provided access to individuals with newly diagnosed type 1 diabetes, an alternative study was required to recruit patients with long standing type 1 diabetes. A research grant from Novo nordisk to develop a biological repository of individuals with chronic diabetes facilitated the set up of the 'Chronic Disease Research into Diabetes' study. Unlike other repositories, this study would have matched anthropometric data, data from validated questionnaires and

biological samples on participants with diabetes. The combination would provide researchers with a powerful resource to screen for biomarkers of disease and gain insight into the chronic disease burden of diabetes.

The study ethics submission, protocol, participant documents and standard operating protocols were all written by Dr David Hughes (author of this thesis). The application for comprehensive research network portfolio adoption was initiated and promoted by myself. Once portfolio adoption was gained an application to utilise the Wellcome Trust Research Facility was submitted by Dr David Hughes and subsequently granted. Thus facilitating access to research staff to aid in recruiting and processing participants samples. However, during the initial year of the study most participants were recruited by Dr David Hughes (author of this thesis).

In order to facilitate gut hormone sample collection a substantial protocol amendment was submitted. In essence this amendment established a sub-study within the Chronic Disease Research into Diabetes study. The two key objectives of the sub-study were: 1) to facilitate the use of meal stimulated tests on study participants to measure gut hormone levels, 2) to enable the recruitment of a group of healthy volunteers to undergo meal stimulated test in order to act as a control group. Once, this amendment had been approved, participants recruited from the Queen Elizabeth Hospital Birmingham site were invited to undergo the meal stimulated test.

The process of recruiting participants into the study evolved rapidly in the first few months after study initiation. At the start, suitable participants who were attending an annual diabetes clinic appointment were identified and sent a letter through the post. As

this failed to generate significant interest both myself and another member of the research team attended these clinics which further increased recruitment. Finally, electronic means of communication via electronic newsletter, websites and text messaging were employed. This involved me developing a short email advert that was distributed through the help of the communication teams at the University of Birmingham and Queen Elizabeth Hospital. Then using NHS email facilities to send patients SMS text messaged about the study as well as working with the University of Birmingham web team to develop a web-page for the study (http://www.birmingham.ac.uk/research/activity/mds/trials/bctu/trials/primary-care/cdr/). These methods of recruiting participants enabled me to recruit the majority of participants to the study from the Queen Elizabeth Hospital Birmingham site.

The study was initially set up as a single site, but subsequent amendments facilitated the role out of the study to multiple sites in the Midlands. These amendments to the study involved key changes and innovations that were initiated and continued by Dr David Hughes (author of this thesis) and facilitated the expansion of the study:

1) Development of a secure website to enable electronic uploading of data and communication with researchers from all study sites.

2) A reduction in the study visits and initiating online follow ups.

3) Collaboration with the Birmingham & Black County Comprehensive Local Research Network (BBC CLRN) to invest in research nurse time to support the set up of new sites.

2.3.1 Protocol outline for the "Chronic Disease Research into Diabetes study" (CDRD)

The "Chronic Disease Research into Diabetes study" (www.birmingham.ac.uk/cdr-diabetes) is an observational study and has two aims:

1. To develop a repository of data and biological samples for the detection of novel biomarkers.
2. To investigate the chronic disease burden of diabetes on individuals with diabetes.

The "Chronic Disease Research into Diabetes study" was funded by a research grant from the pharmaceutical company Novo Nordisk and supported through the Birmingham & Black Country Comprehensive Local Research Network (UKCRN ID: 12984). The chief investigator was Dr Parth Narendran and the University of Birmingham acted as the sponsor (ref: RG_12-061). Ethics approval was granted by the West Midlands – Staffordshire regional ethics committee on April 2012 (ref: 12/WM/0089). University Hospitals Birmingham Foundation NHS Trust - research and development department approved the study in August 2012 (ref: RRK4441). The ethics committee submissions, NHS R&D submissions, study protocol, standard operating protocols and all documentation associated with the study was authored by Dr David Hughes (author of this thesis).

Screening for the study commenced in early September 2012 with the first participant recruited to the study in mid September 2012. In April 2013 the study was adopted at Russells Hall Hospital (Dudley), City Hospital (Birmingham), Good Hope Hospital

(Sutton Coldfield), Walsall Manor Hospital and Heartlands Hospital (Birmingham).

Study inclusion criteria:

1. Individuals who fulfilled the World Health Organisation (WHO) 2011 classification for a diagnosis of either diabetes, or impaired fasting glucose, or impaired glucose tolerance.

Sub-study inclusion criteria:

1. Maximum of 24 healthy participants to undergo a meal stimulated test to determine normal gut hormone levels.

Major exclusion criteria included:

1. Unable to provide full informed consent.
2. Significant illness (including psychological, psychiatric, developmental or physical disease) that may prevent a study visit attendance.
3. The principal investigators opinion that participation would not be in the individuals best interest, due to the potential of psychological harm.
4. Participation in an intervention study.

Following enrolment into the study, participants were questioned about their medical history, before undergoing anthropometric measurements:

- Blood pressure and pulse were measured from the non-dominant arm.
- Height and weight were measured.
- Hip circumference was taken from the widest point at the level of the greater trochanter.
- Waist circumference was taken at the halfway point between the greater trochanter and xiphoid process of the sternum.

- Thigh circumference was taken from the non-dominant leg at the widest level of the thigh just below the groin.
- Neck circumference was taken during exhalation at the level of the thyroid cartilage.

All measurements were taken by a qualified healthcare professional who had undergone training. After all anthropometric measurements had been completed the participants were asked to complete a series of questionnaires (see Appendix 2) which included:

- Socio-demographic information: gender, age, ethnicity, level of academic attainment, employment status.
- Lifestyle information: smoking, alcohol consumption (measured by the validated questionnaire "Alcohol Use Disorders Identification Test Consumption" (AUDIT-C)), sedentary time and quality of life (measured by the validated questionnaire "EuroQol five dimensions questionnaire" (EQ-5D)) [181], [182].
- Depression (measured by the validated questionnaire "Patient Health Questionnaire 9 point" (PHQ-9)) [183].
- Eating behaviour (measured by the validated questionnaire "Three Eating Factors Questionnaire – 18 point" (TEFQ-18)) [184].

On completion of the baseline visit, participants were asked whether they wished to attend an optional extra visit to complete a meal stimulated test at the Wellcome Trust Research Facility, Queen Elizabeth Hospital, Birmingham. Participants were provided with a separate participant information sheet concerning the meal stimulated test visit before attending this optional visit. All these meal stimulated tests were arranged and supervised by Dr David Hughes (author of this thesis).

Recruitment of participants at the Queen Elizabeth Hospital, Birmingham occurred through the distribution of the “participant information sheets” to individuals attending a diabetes clinic. Healthy volunteers were recruited through advertising via the University of Birmingham's weekly e-mail newsletter and through discussions with healthy relatives of participants recruited to the CDRD study. Dr David Hughes (author of this thesis) distributed the participants information sheets, organised the email advertising and recruited the majority of participants into the CDRD study during 2012-2014. Dr David Hughes (author of this thesis) also worked closely with CLRN research staff in order for the study to continue to actively recruit participants both at Queen Elizabeth Hospital Birmingham and later at multiple sites across the Midlands.

2.4 Demographics of Participants recruited from the 'ExTOD' and 'CDRD' studies.

The combination of both the 'Exercise in Type One Diabetes' and 'Chronic Disease Research into Diabetes' facilitated the collection of samples from participants with: a) early onset type 1 diabetes, b) chronic type 1 diabetes and c) healthy volunteers. All samples were then stored at -80°C in preparation for analysis. Listed below are the gender, age and body mass index (BMI) of all 'ExTOD' participants whose samples were collected and stored ready for analysis (see Table 2.1). All participants recruited from the ExTOD study had a duration of type 1 diabetes of under one year.

ID	*Gender*	*Age*	*Body Mass Index*
ex**	Male	30	28
EX001	Female	29	24
EX002	Male	29	26
Ex003	Male	21	22
Ex004	Male	24	27
Ex005	Male	22	25
Ex006	Male	27	21
EX007	Female	24	23
EX008	Male	18	24
EX009	Male	26	28
EX011	Male	18	25
EX012	Male	22	27
EX013	Female	29	34
EX014	Male	27	28
EX016	Male	39	23
EX017	Male	38	28
EX018	Female	31	22
EX019	Male	30	23
EX020	Female	17	27
EX021	Female	26	26
EX022	Male	33	25
EX023	Male	31	29
EX025	Male	20	20
EX026	Female	22	24
EX027	Female	54	35
EX031	Female	27	18
EX032	Male	44	24

Table 2.1: Demographics of participants recruited from the Exercise in Type One Diabetes Study.

Listed below are the gender, age, body mass index (BMI) and duration of diabetes of all healthy 'CDRD' participants whose samples were collected and stored ready for analysis (see Table 2.2).

ID	*Gender*	*Age*	*Body Mass Index*
101	Male	35	26
103	Male	46	20
105	Female	63	28
106	Female	43	19
107	Female	43	25
108	Female	57	21
110	Female	48	23
111	Female	32	22
112	Male	38	20
113	Female	57	36
114	Male	26	22
115	Female	47	28
116	Male	56	26
117	Female	41	20
118	Female	28	21
122	Female	48	37
123	Female	32	27
126	Male	30	23
127	Male	71	28
128	Male	21	19
129	Male	25	25
130	Female	50	28
131	Male	76	21
134	Female	36	22

Table 2.2: Demographics of healthy volunteers recruited from the Chronic Disease Research into Diabetes study.

Listed below are the gender, age and body mass index (BMI) of all 'CDRD' participants with type 1 diabetes whose samples were collected and stored ready for analysis (see table 2.3).

ID	*Duration of Type 1 Diabetes*	*Gender*	*Age*	*Body Mass Index*
6	37	Male	52	49
12	64	Male	76	27
13	7	Male	26	21
16	14	Male	65	27
22	5	Male	26	22
23	57	Female	57	26
27	30	Female	41	28
28	21	Female	54	32
30	22	Female	33	20
32	39	Female	58	28
34	38	Female	49	30
36	37	Male	37	26
40	39	Female	59	23
47	23	Male	62	24
49	52	Male	75	27
53	21	Male	23	28
56	31	Female	52	23
61	1	Male	18	24
62	21	Male	35	27
64	26	Female	46	31
67	19	Female	50	23

Table 2.3: Demographics of participants recruited from the Chronic Disease Research into Diabetes study with type 1 diabetes.

2.5 Discussion

The success of this research hinged on the ability to recruit participants with type 1 diabetes to studies that would faciliate access to standardised samples in both the fasted and fed state. This was achieved through collaborating with researchers from the

'ExTOD' study as well as setting up a new study 'CDRD'. The target of collecting at least 12 participants samples from 1) healthy volunteers, 2) individuals with acute onset type 1 diabetes and 3) individuals with chronic type 1 diabetes was also met and exceeded. In total 24 healthy volunteers, 28 participants with a duration of diabetes <2 years and 20 participants with a duration of type 1 diabetes >2 years were recruited and undertook a meal stimulated test.

3. OPTIMISE SAMPLE COLLECTION AND ASSAYS FOR GUT HORMONE ANALYSIS

3.1 Introduction

At the start of the study there was no departmental protocol for sample collection or processing for human gut hormones. In order to develop a sample collection protocol, I undertook a review of available literature to gain an insight into assays and their methods used for sample collection. The key focus was to find low cost assays that had low concentration detection ranges for gut hormones, in particular glucagon and active-GLP-1. Gut hormone assay instruction manuals from websites of commercial biotechnology companies as well as peer reviewed journal methods were included in the search. Please see Table 3.1 for the results of the assay search and Table 3.2 for the results of the sample collection methods.

Kit	Cost	Detection Range	Pro	Cons
RayBiotech Human Glucagon EIA	£220 per 96 well	Glucagon = 1-1,000 pg/ml	Low cost assay with lowest glucagon detection range	Instructions appear complicated
Mercodia Glucagon ELISA	£525 per 96 well plate	Glucagon = 5 – 453 pg/mL	Very simple instructions with pre made standards. Second lowest detection range.	New assay. Medium ranged price
Millipore Glucagon RIA	£300 per 250 tubes	Glucagon = 20–400 pg/mL	Low cost assay with plenty of publications.	Would need specialist training and no one currently available to supervise me.
Phoenix Pharmaceuticals Glucagon EIA kit	£400 per 96 well plate	Glucagon = 160-3740 pg/ml	Medium cost	Worst detection range out of glucagon assays
R&D High Sensitivity Il-6 ELISA kit	£400 per 96 well plate	Il-6 = 0.2 - 10 pg/mL	Medium cost. Very low detection range. Recommended by team at Bristol & Warwick	None
RayBiotech Active GLP-1	£220 per 96 well	Active GLP-1 = 1.17 - 1,000 pg/ml	Low cost assay	Assay protocol not straightforward
Millipore High Sensitivity GLP-1 Active ELISA Kit, Chemiluminescent	£400 per 96 well plate	Active GLP-1 = 0.46-330 pg/mL	Medium cost. Very low detection range	None
MSD Human Active GLP-1, Insulin, Glucagon, Leptin Kit	£1200 per 96 well plate	Glucagon = 21-10000pg/ml; Active-GLP-1 = 1.0-10000pg/ml; Il-6 = 13.7-10,000 pg/mL	Multi-analyte assay which is very cost efficient. Recommended assay by the team at Cambridge.	Company informs me that nearest serviced machine is based in Warwick.
Millipore MAP Human Metabolic Hormone Panel - (C-Peptide, Ghrelin (Active), GIP (Total), Leptin, PP, PYY (Total)	£1080 per 96 well plate	Glucagon = 13.7-10,000 pg/mL; Active GLP-1 = 2.7-2,000 pg/mL	Multi-analyte assay which is very cost efficient. Serviced machine at Uni of Bham.	Would need to contribute to service costs of machine.

Table 3.1: Review of potential commercial assays with pros and cons.

Kit	*Hormone*	*Collection Method*
Mercodia Glucagon ELISA	Glucagon	Method 1: Collect samples in EDTA tubes stored at room temperature for <2 hours Aliquot plasma and freeze at -80°C.
		Method 2: Collect samples in Becton Dickinson (BD) P800 tubes containing a cocktail of protease, esterase and DPP-IV Inhibitors stored at room temperature for <6 hours. Aliquot plasma and freeze at -80°C.
Millipore Glucagon RIA	Glucagon	Collect samples in EDTA tubes containing 250 KIU Aprotinin per mL of whole blood. Aliquot plasma and freeze at -20°C - -70°C.
Millipore MAP Human Metabolic Hormone Panel	C-Peptide, Ghrelin (Active), GIP (Total), Leptin, PP, PYY, Amylin, Il-6	Collect samples in EDTA tubes, then add DPPIV inhibitor, protease inhibitor cocktail, Aprotinin and AEBSF.
MSD Human Active GLP-1, Insulin, Glucagon, Leptin Kit	Active GLP-1, Insulin, Glucagon, Leptin	Collect samples in EDTA tubes containing DPP-4 inhibitor & 500 KIU Aprotinin per mL of whole blood. Aliquot plasma and freeze at -80°C
Millipore High Sensitivity GLP-1 Active ELISA Kit, Chemiluminescent	Active-GLP-1	Collect sample in EDTA tubes, immediately (<30 seconds) after collection add DPP-IV inhibitor.Aliquot plasma and freeze at -80°C.
Millipore Human Ghrelin (active) ELISA	Active-Ghrelin	Collect samples in EDTA tubes treated with Pefabloc or AEBSF. Then acidify plasma samples with HCl to a final concentration of 0.05N. Aliquot plasma and freeze at -80°C.

Table 3.2: Review of collection methods from assay kits.

The review of assays and collection methods highlighted an important aspect concerning freeze-thaw cycles. Performing multiple assays would increase the number of freeze thaw cycles and thus the degradation of the gut hormone in the sample. Assays that could be performed at the same time would help reduce the number of freeze thaw cycles and prevent sample degradation. This meant that there was one assay that was a clear forerunner as it was both low cost and could measure multiple analytes simultaneously. The 'Human Metabolic Hormone Magnetic Bead Panel Luminex kit (#HMHMAG-34K)' provided by EMD Millipore Corporation (Billerica, MA, USA) provided a means to assay multiple gut hormones using luminex multiple analyte profiling technology, that would provide a cost saving over traditional ELISAs and

would keep freeze thaw cycles to a minimum. The kits literature described a sample collection method that was similar to the other collection methods. The collection method included adding DDP-4 inhibitors to prevent degradation of active-GLP-1, AEBSF to prevent degradation of actyl-ghrelin, aprotinin to prevent degradation of glucagon and protease inhibitor cocktail to prevent degradation of amylin. As no members of my lab had ever measured gut hormones before, I discussed this selection with researchers from the field of gut peptides at the University of Bristol, University of Warwick and University of Cambridge. They all commented on the difficulty of measuring glucagon and advised me to collect a separate sample containing DPP-4 inhibitor and aprotinin for glucagon analysis in case the detection range of the Human Metabolic Hormone Magnetic Bead Panel Luminex kit was not good enough. Therefore, I decided to use two collection methods in the hope that one sample collection method would be good enough.

3.2 Methods and materials for preparing gut hormone blood collection tubes

The two methods selected for preparing blood collection tubes and cryovials were given the terms 'Tube A' and 'Tube B'. Tube A preparation instructions were taken from MSD and Millipores sample collection methods for analysing single glucagon and active-glp-1 hormones. Tube B preparation instructions were taken from the sample collection method as provided by the Human Metabolic Hormone Magnetic Bead Panel Luminex kit (#HMHMAG-34K) with the use of a protease inhibitor cocktail containing AEBSF, Aprotinin, Bestatin, E-64, Leupeptin, Pepstatin A . Below are the methods and materials used to prepare these two gut hormone blood collection tubes.

Tube A: 2ml EDTA Vacuette tube (#454087, Greiner Bio-one GmbH, Frickenhausen, Germany) of which 20 µl DPP-4 inhibitor (#DPP4, EMD Millipore Corporation, Billerica, MA, USA) and 1000 kIU Aprotinin (#616371, Merck KGaA, Darmstadt, Germany) was added.

Tube B: 2ml EDTA Vacuette tube (#454087, Greiner Bio-one GmbH, Frickenhausen, Germany) of which 20 µl DPP-4 inhibitor (#DPP4, EMD Millipore Corporation, Billerica, MA, USA) and 20 µl of 100mM Pefabloc® SC (#76307, Sigma-Aldrich, St. Louis, MO, USA) was added. Tube B also had an accompanying cryovial for Tube B: 1.5ml Sterile Natural Cryovial (#V4381, Sigma-Aldrich, St. Louis, MO, USA) of which 5 µl of Protease Inhibitor Cocktail (#P8340, Sigma-Aldrich, St. Louis, MO, USA) was added to every 0.5ml serum.

As these sample collection tubes and cryovials needed to be prepared in advance, they were stored at -20°C for up to one month. This time frame was selected as it was shorter than the time frame given by the commercially available gut peptide collection tubes provided by BD Bioscience (Franklin Lakes, New Jersey, U.S.A) i.e. P700 and P800 collection tubes. The decision to prepare my own collection tubes compared to purchasing these commercially available gut peptide collection tubes was based on cost alone (own collection tubes = £3 each vs P700/P800 tubes = £12 each).

3.3 Meal stimulated test and sampling protocol

As gut hormone levels vary with ingestion of food, it is important to use a method such as the meal stimulated test in order to standardise samples. All participants were

recruited from the Queen Elizabeth Hospital Birmingham for both the 'ExTOD' study and those healthy volunteers and participants with type 1 diabetes from the 'CDRD' study. Participants were asked to attend the Wellcome Trust Clinical Research Facility at the Queen Elizabeth Hospital Birmingham between 8:45 – 9:30am in a fasted state having consumed nothing except for plain water for 10 hours. All short/rapid acting anti-diabetic medication was omitted prior to the test. Participants who had a mild hypoglycaemic episode during the fast that required only a single dose of rapid acting sugars (eg dextrose tablet, lucozade, jelly babies, etc) could proceed with the test as long as this occurred >4 hours prior to the first blood sample. A pre-test capillary glucose reading was taken using the participant's own glucose monitor. If capillary glucose was <4mmol/L then they were treated for hypoglycaemia and the test rescheduled. If glucose was >15mmol/L the test would continue under medical supervision, if the participant felt well and had not developed ketosis.

At the start of the meal stimulated test a peripheral cannula was inserted in order to collect multiple blood samples during the test. Blood samples were taken at time points: -10min, 0min, 15min, 30min, 60min, 90min & 120min and kept in an ice bucket until centrifuged. Blood samples were collected into a 4ml EDTA Vacuette tube (#454021, Greiner Bio-one GmbH, Frickenhausen, Germany), a 2ml Fluoride Oxalate (#454061, Greiner Bio-one GmbH, Frickenhausen, Germany), and the chilled hormone collection tubes. Immediately after the 0min blood sample was taken, each participant was asked to drink 240ml of standard fortisip (#18475, Nutricia Ltd, Trowbridge, UK) within the subsequent 5 minutes.

Blood samples were centrifuged within 45 minutes of sample collection at 1100-1300g

at 4°C for 10 minutes. Then 0.5ml of plasma from each sample collection tube was transferred into two cryovials. All samples were initially frozen at -20°C for up to 2 weeks before being transferred to a -80°C storage.

After the 120min blood sample was taken, a capillary blood glucose test was taken before the cannula was removed. Participants were reviewed by a clinician and advised what medication to take before being provided with a meal.

3.4 Human Metabolic Hormone Magnetic Bead Panel Luminex Assay

The Human Metabolic Hormone Magnetic Bead Panel Luminex kit (#HMHMAG-34K) was provided by EMD Millipore Corporation, Billerica, MA, USA. The 96 microplate was washed with assay buffer and then tapped dry. Standards, controls and plasma samples were added to each well of the microplate. Magnetic detection beads were re-suspended in bead diluent before being added to each well. The microplate was incubated overnight at 4°C on a plate shaker. The microplate was washed using a magnetic plate washer (#Bio-Plex Pro Plate Washer, Bio-Rad Laboratories, Hercules, CA, USA). Then human metabolic panel detection antibodies were added to each well and incubated for 30 minutes at room temperature on a plate shaker. Streptavidin-Phycoerythrin was then added to each well, incubated for a further 30 minutes at room temperature on a plate shaker. Finally, the microplate was washed using a magnetic plate washer, before being analysed on a Bio-Plex 200 machine (Bio-Rad Laboratories, Hercules, CA, USA). The reader was gated for each magnetic bead, set at a 50 bead count with a 60 second time out.

3.5 Results comparing the two gut hormone sample collection methods

The first three pairs of samples using the two different sample collection methods were compared for all gut hormones in the panel using the Human Metabolic Hormone Magnetic Bead Panel Luminex kit (#HMHMAG-34K). The results for each analyte are shown below.

	Tube A			Tube B		
	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C
C-peptide	10082	917	0	12424	398	47
Acetyl-Ghrelin	771	3270	318	1883	15689	3160
GIP	17049	7725	5816	13826	6778	7304
GLP-1	1939	497	115	2729	1392	773
Glucagon	3117	0	0	1823	0	0
Leptin	30142	221326	128634	29288	199625	134967
PP	20127	11713	21310	21395	15759	28825
PYY	17860	2831	0	18803	7409	1330
Il-6	0	0	0	0	0	0
Amylin	0	0	0	0	0	0

Table 3.3: Results from three participant samples comparing sample collection methods for an array of gut hormones measured by the Human Metabolic Hormone Magnetic Bead Panel Luminex kit reported as the Area Under the Curve for 0-120 minutes.

The results obtained show that levels of Il-6 and amylin were too low to be detected in the samples analysed. This suggests that either there were problems with the sensitivity of the assay used in analysing Il-6 and amylin or in the collection method. However, as two independent collection methods had been used, it was felt that it was more likely to be a problem with the sensitivity of the assay especially in relation to Il-6. There was also concern regarding the sensitivity of the assay in analysing glucagon and active GLP-1. Two of the three glucagon samples had recorded undetectable levels and the active GLP-1 results were only just above the manufacturers stated lower limits of detection. Thus, it was decided to find alternative assays for detecting glucagon, active

GLP-1, Il-6 and amylin.

In order to determine which sample collection method had provided the greatest gut hormone yields the combined total yields for each collection method was calculated and the difference between the sample collection methods calculated (see table 3.4).

	Total Tube A	Total Tube B	% Difference
C-peptide	10999	***12868***	15
Acetyl-Ghrelin	4359	***20732***	21
GIP	***30590***	27908	9
GLP-1	2551	***4893***	52
Glucagon	***3117***	1823	42
Leptin	***380101***	363880	4
PP	53150	***65979***	19
PYY	20692	***27542***	25

Table 3.4: Total yields of the two sample collection methods from three participant samples. The highest yield is in **bold**. The percentage difference is that between the lowest and highest yield collection method.

These results confirm that sample collection method using Tube B generated a greater hormone yield for all hormones except for Glucagon, GIP and Leptin. The difference in yields for leptin (4% higher yield in Tube A) and GIP (9% higher yield in Tube A) were felt to be small enough that these hormones could still be analysed using collection tube B. However, as the difference in yield for glucagon was 42% higher in Tube A than collection Tube B, then this method would continue to be used for glucagon. The conclusion of this analysis was that two collection methods would continue to be used in all participants. Tube A would be used for glucagon analysis and tube B would be used for all other gut hormone analysis.

3.6 Selection of alternative assays

The process of selecting an alternative assay for glucagon, amylin, Il-6 and active GLP-1 started by reviewing the detection ranges for each available commercial assay. The priority was to find a suitable glucagon and active GLP-1 assay as there was significant scientific interest in alpha cell function and the incretins in type 1 diabetes. RayBiotech glucagon assay and Mercodia AB glucagon assay were short listed following review of available literature (see Table 3.1). The two active GLP-1 assays that were also short listed were RayBiotech active GLP-1 assay and Millipore High Sensitivity active-GLP-1 assay. The only short listed Il-6 assay was R&D Systems High Sensitivity Il-6 assay, but no high sensitivity amylin assay was short listed. The UK distributor for RayBiotech then offered a significant discount off the list price for both their active-GLP-1 and glucagon assay which made these assays significantly cheaper than their competitors. As both the RayBiotech Glucagon and active-GLP-1 assays used the same methods then it was possible to use them in tandem, reducing freeze-thaw times. Therefore, the RayBiotech assays were selected and samples were processed using these assays.

3.7 Method of sample processing using RayBiotech Glucagon and GLP-1 EIA

Plasma samples from collection tube B collected during the meal stimulated test at time points: -10min, 0min, 15min, 30min, 60min, 90min & 120min were taken out of -80°C storage and allowed to thaw on a workbench. The Glucagon and GLP-1 EIA kits provided by RayBiotech (Norcross, Georgia, USA) were stored at -80°C until used. The microplate in the kits were pre-coated with secondary anti-rabbit IgG antibody. When preparing the assay then either anti-glucagon or anti-glp-1 antibodies were added to the

microplate, incubated overnight at 4°C on a plate shaker before being washed. Plasma samples were diluted four fold into micro-centrifuge tubes and combined with either biotinylated-glucagon or biotinylated-GLP-1. The mixed samples were added to each well alongside standards and controls before being incubated overnight at 4°C on the plate shaker. During this phase the plasma glucagon or GLP-1 in each sample competes with their respective biotinylated-peptide for the binding sites on the microplate. The microplate was then washed and streptavidin-horseradish peroxidase (SAHRP) added to produce a colour reaction with the biotinylated moiety that is bound to the plate. The colour intensity of each well was measured at 450nm absorbance on a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). The intensity of colorimetric signal was inversely proportional to the amount of plasma Glucagon or GLP-1 peptide in the four fold diluted plasma samples.

3.8 Experience of using RayBiotech Glucagon and GLP-1 EIA

As samples were processed it became evident that whilst the method of measuring levels of glucagon and active-glp-1 with these assays was valid, it was time intensive and prone to preparation errors. Also, there was concern that the intra-assay variability may be greater than published. Thus the confidence in the assay was adversely affected and concerns about reproducibility of our results led us to abandon these assays. We therefore turned to the more expensive Mercodia Glucagon assay and the Millipore High Sensitivity active-GLP-1 assay and were immediately impressed with these assays. The Mercodia glucagon assay came with both its own pre-prepared controls and standards that reduced possible preparation errors. It was also simpler and quicker to use than the RayBiotech assay. The Millipore High Sensitivity active-GLP-1 assay was also

less time intensive and by coming with two prepared control samples it increased our confidence in its results. During this process of assay optimisation both time and money had been lost, which led us to abandon our aim of optimising an amylin and Il-6 assay.

3.9 Method of sample processing using Mercodia Glucagon ELISA assay.

Plasma samples from collection tube A collected during the meal stimulated test at time points: -10min, 0min, 15min, 30min, 60min, 90min & 120min were taken out of -80°C storage and allowed to thaw on a workbench. The glucagon enzyme immunoassay was provided by Mercodia AB, Uppsala, Sweden. The microplate in the kits were pre-coated with an anti-glucagon antibody. Plasma samples and calibration samples were transferred in duplicate to wells on the microplate. Peroxidase conjugated anti-glucagon antibody solution targeting a second binding site was then added to the each well. The microplate was incubated for 18-22hrs at 4C on a plate shaker. The microplate was washed and 3,3',5,5'-tetramethylbenzidine (TMB) solution was then added to produce a colour reaction. The colour intensity of each well was measured at 450nm absorbance on a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). The intensity of colorimetric signal was proportional to the amount of plasma Glucagon in each sample.

3.10 Method of sample processing using Millipore active GLP-1 ELISA assay

The active GLP-1 ELISA (#EZGLPHS-35K) was provided by EMD Millipore Corporation, Billerica, MA, USA. The microplate in the kits were pre-coated with an anti active-glp-1 antibody. The microplate was washed with buffer and then tapped dry.

Standards, controls and plasma samples were added to each well of the microplate. GLP-1 capture antibody solution was then added to each well. The plate was incubated for two hours at room temperature on a plate shaker. The plate was washed, before a detection antibody was added to each well and then incubated for one hour at room temperature on a plate shaker. The plate was washed and Enzyme Solution added to each well before being incubated for 30 minutes at room temperature on a plate shaker. The plate was washed and substrate solution added to each well. The microplate was immediately read at 425nm on a Centro LB 960 microplate luminometer (Berthold Technologies GmbH, Bad Wildbad, Germany).

3.11 Discussion

The aim of optimising both sample collection methods and assays was not a straightforward process, but was finally achieved. In order to measure gut hormone levels, two sample collection methods were compared to find the method which provided the highest yield. The results showed the need to use both collection methods as the yields varied depending on the gut hormone analysed.

The initial intention to use Millipores luminex multiple analyte profiling technology to analyse all gut hormones resulted in failure to detect levels of Il-6, amylin, glucagon and active GLP-1 in the cohort of samples. Our second choice of assay for glucagon and active-glp-1 analysis using RayBiotech's assays turned out to be plagued with problems as plates were repeatedly spoiled due to preparation errors, internal quality control sample results outside of acceptable limits, and so the confidence in the reproducibility of the results these assays produced waned. This led us to use Mercodia's glucagon

assay and Millipore's high sensitivity active-GLP-1 assay. Both these assays were easy to prepare, with internal control samples repeatedly within acceptable limits that ultimately led to our confidence in their use for all our samples. Unfortunately, the time and cost taken in optimising assays and sample collection meant that Il-6 and amylin levels were not measured.

On reflection, it would have been worth spending more time before sample analysis to validate the methods of sample collection and analysis. The process of validating these could have occurred with the help of an academic from another laboratory. The first step would have required taking matched samples of blood from a volunteer as per our sampling methods and stored according to protocol. Reference samples of gut peptides with known concentrations would need to be obtained, undergo serial dilution and then split into pairs. Half the samples (volunteer and diluted reference samples) would then be simultaneously analysed on our selected assays at two laboratories. The results could then be analysed to determine within laboratory variations of the assays and reproducibility of the assays between laboratories for both the reference samples and the human samples. By repeating the simultaneous analysis at two laboratories we could analyse repeatability (intra-assay precision) and determine which collection method produced the greatest gut peptide yield as well as lowest sample degradation. Unfortunately, these steps were not performed due to the fact that most samples had already been collected by the time the problem had been identified. The cost both in time for repeating sample collection and monetary value of the assays prohibited our ability to complete the validation process during this research project.

The use of three assays and two collection methods was not ideal in practical terms, but

it did lead to an increase in confidence in our results. Ideally, we would have preferred to use one sample collection method in order to reduce preparation time and cost of consumables. However, one of the advantages of using two methods, was the ability to use sample collection methods that better matched the assay manufacturers recommended sampling protocols. Another strength of using two sample collection methods was that it prevented the need for repeated freeze-thaw cycles that could have led to sample degradation. The decision to prepare our own collection tubes also had the advantage of keeping costs down compared to purchasing commercially available collection tubes. This needed to be offset against the disadvantage of longer preparation time.

In terms of our assays, again it would have been ideal to have found one multi analyte assay that could have accurately measured all the gut hormones simultaneously. Millipore's multi-analyte assay came very close to being ideal, as it accurately measured multiple hormones simultaneously. This ability saved both significant time and cost compared to using multiple individual assays making it very economically efficient. The Millipore multi-analyte assay also had the advantage of coming with multiple internationally standardised quality controls that the analyser automatically checked to confirm validity of our results. One disadvantage was that the assay could only run on one specialised analyser, which was rarely used. This meant that I had to learn to maintain and organise the servicing of the equipment. A key weakness of the Millipore multi-analyte assay was its inability to detect the low concentrations of active GLP-1 and glucagon in our samples. This led us to use Millipore's high sensitivity GLP-1 assay and Mercodia's glucagon assay. Their strengths were their lower detection ranges compared to Millipore's multi-analyte assay. Both Millipore's high sensitivity GLP-1

assay and Mercodia's glucagon assay also came with multiple international standardised quality control samples which ensured validity of our results. The Mercodia's glucagon assay also had the advantage of being read on a standard colour intensity plate reader which was readily available and regularly maintained. Both assays were easy to set up, but unfortunately were relatively more expensive. Millipore's high sensitivity GLP-1 assay was read on a luminometer plate reader, of which there was only one in our department. This meant that it was frequently in use, so a degree of coordination was required to ensure timing assay preparation and access to the analyser. In the future, as multi-analyte assays become more popular, I hope that the advantages of all three assays can be combined into one bespoke multi-analyte assay. This could either occur through combining analyte beads from other assays or by developing bespoke 'in house' glucagon and active-GLP-1 analyte beads that can be used on Millipore's multi-analyte assay.

4. EXPLORE GUT HORMONE AXIS IN PATIENTS WITH TYPE 1 DIABETES

4.1 Exploring progression of Type 1 diabetes and effect on other gut hormones

The DCCT study in the 1980's firmly established the link between the decline in C-peptide levels and the duration of type 1 diabetes [185]. Subsequent studies have also shown that glucagon levels slowly increase over the first year following diagnosis of type 1 diabetes [80]. However, there is very little research exploring the effect of duration of type 1 diabetes on other gut hormones. The incretins were of particular interest as the physiological potential of incretins to stimulate insulin secretion in a glucose dependent manner had been demonstrated in insulin resistant type 2 diabetes, but there was limited research into type 1 diabetes. The other group of gut hormones of interest were those that influence satiety. PP, PYY, Ghrelin and Leptin are markers of adiposity and satiety. In individuals with type 1 diabetes there is a need to administer exogenous insulin to utilise and store carbohydrates in adipose tissue. It is unknown whether the decline in beta cell function has any effect on these networks of hormones that control adipose tissue and satiety.

4.2 Method used to explore gut hormones in type 1 diabetes

Samples were collected according to the optimised collection method as described in chapter 3 from participants from the CDRD study and ExTOD study. Samples were stored at -80°C and then analysed using the Millipores human metabolic hormone magnetic bead panel luminex kit for C-peptide, active ghrelin, GIP, Leptin, PYY and PP; Millipore high sensitivity active-GLP-1 assay and Mercodia Glucagon assay.

Statistical analysis of the results of samples taken at 0min, 30min, 90 minute, alongside calculated results for 90 minute Area Under the Curve (AUC 90min) were performed using GraphPad software (Prism 6, GraphPad Software Inc, USA). Participants were divided into the following three groups according to their duration of type 1 diabetes:

Group 1) healthy individuals

Group 2) individuals with diabetes for less than 2 years

Group 3) individuals with diabetes for more than 2 years

4.3 Demographic results of the groups in the analysis

The demographics of the groups according to duration of diabetes was analysed to explore any potential significant confounding factors (see table 4.1)

Mean +- SD	**Healthy Controls (n=23)**	**DMT1 - duration<2yrs (n=28)**	**DMT1 - duration>2yrs (n=21)**
Age	43yrs +- 13.5	28yrs+-8.4 ***	50yrs+-15.8
Duration of DMT1	na	0.5yrs+-0.3	30yrs+-15.3
BMI	24kg/m2+-4.8	25kg/m2+-3.7	27kg/m2+-5.9
% female	61%	32%	48%

Table 4.1: Demographics of the participants by analysis groups. Statistical analysis with Kushmal-Wallis and Chi-squared. [*** P<0.001]

Analysis of the groups revealed a significant difference (P<0.001) in the age of those participants with type 1 diabetes for <2yrs duration compared to the other two groups.

This difference would introduce a confounding factor that will need to be taken into consideration when interpreting subsequent results. There was no significant difference in age between the healthy controls and those with type 1 diabetes for >2yrs. There was no statistical difference in female percentages between all groups. Also, there was no statistical difference in body mass index (BMI) between all groups.

4.4 Results of gut hormone analysis in individuals with type 1 diabetes

4.4.1 Analysis of C-peptide

All samples collected from participants who undertook a standard volume mixed meal stimulated test were processed using Millipores human metabolic hormone magnetic bead panel luminex kit to detect levels of C-peptide. Results were then analysed using Graphpad software.

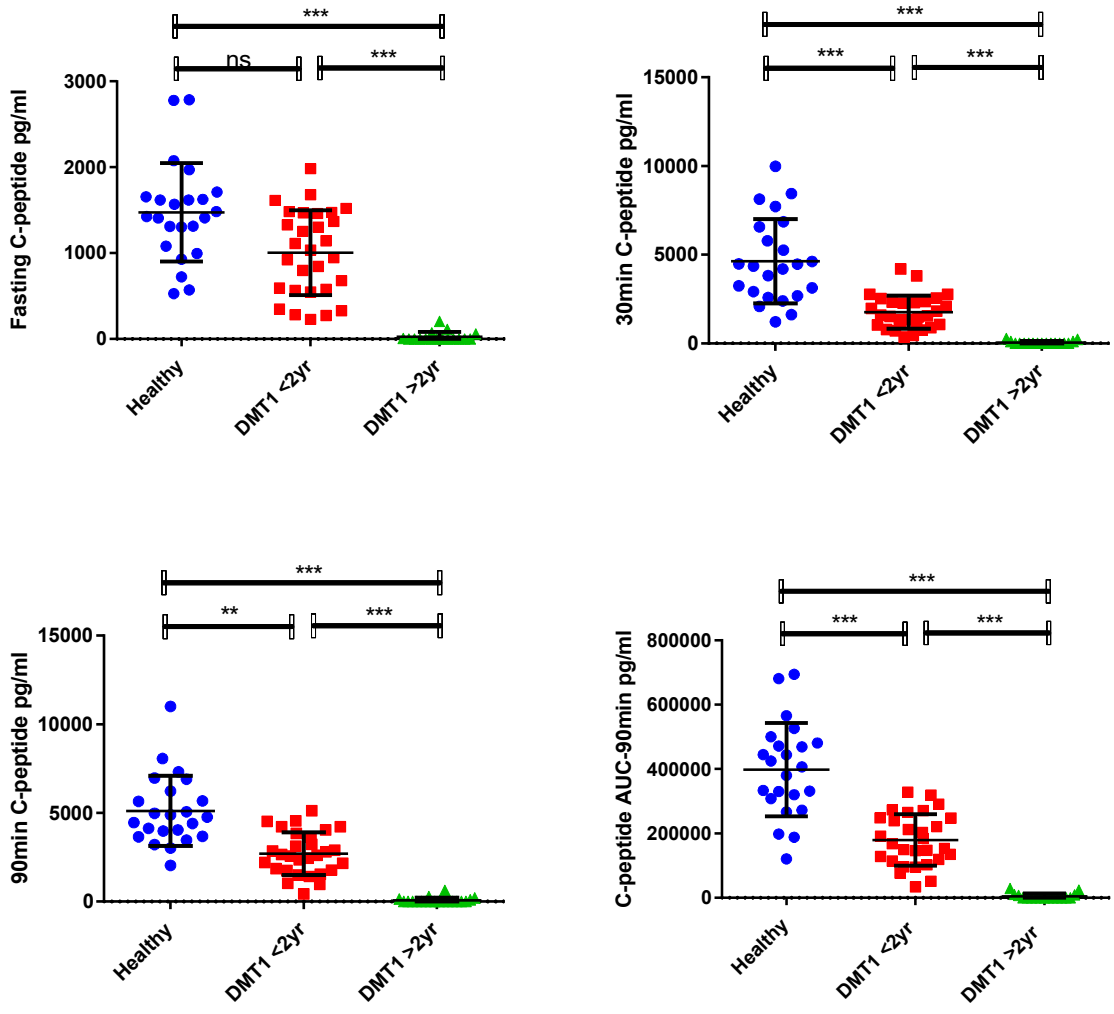


Figure 4.1 : C-peptide mean and standard deviations for the three groups [healthy controls, subjects with duration of type 1 diabetes <2yrs, subjects with duration of type

1 diabetes >2yrs] (a) fasting, (b) 30 minutes, (c) 90 minutes, (d) Area Under Curve 0-90minutes. Statistical analysis with Kruskal–Wallis was utilised to find significant differences [legend: (ns ($p>0.05$), * ($p<0.05$), **($p<0.01$), ***($p<0.001$)].

The results showed a significant decline in C-peptide with duration of diabetes. Further statistical analysis was undertaken to determine the confidence intervals and suitability of C-peptide to differentiate between healthy controls and those with a diagnosis of type 1 diabetes (see Table 4.2). The data from these groups was also analysed with receiver operated curves (ROC) to determine sensitivity and specificity of both the 30 minute or 90 minute stimulated C-peptide levels for the healthy compared to duration of diabetes <2yrs (see Figure 4.2).

	Healthy	DMT1 – duration <2yr	DMT1 – duration >2yr
Fasting Mean (SD) [95% CI] (pg/nl)	1470 (570) [1230 - 1720]	1000 (490) [820 - 1190]	27 (60) [0-56]
30min Mean (SD) [95% CI] (pg/nl)	4630 (2370) [3610-5660]	1760 (940) [1400-2120]	42 (85) [0-85]
90min Mean (SD) [95% CI] (pg/nl)	5120 (1970) [4260-5970]	2700 (1200) [2250-3160]	68 (150) [0-140]

Table 4.2: C-peptide mean, standard deviation and 95% confidence intervals for healthy volunteers, those with duration of <2yrs and duration >2yrs of type 1 diabetes.

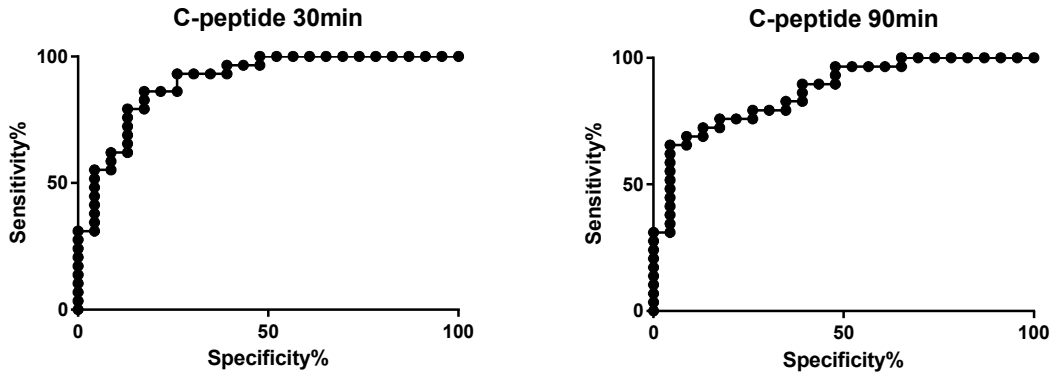


Figure 4.2 : C-peptide ROC graph comparing the two groups [healthy controls vs subjects with type 1 diabetes with duration <2yrs]. (a)30minute C-peptide (pg/ml) ROC area=0.90 with a cut off value of <1600 pg/ml providing a specificity of 95% and sensitivity of 55%. (b) 90minute C-peptide (pg/ml) ROC area=0.87 with a cut off value of <2960 pg/ml providing a specificity of 95% and sensitivity of 65%.

The results from these analysis suggest that both a 30 minute (ROC=0.90) and 90 minute (ROC=0.87) meal stimulated C-peptide level have good diagnostic accuracy to differentiate between healthy controls and those with type 1 diabetes.

4.4.2 Analysis of GIP

All samples collected from participants who undertook a standard volume mixed meal stimulated test were processed using Millipores human metabolic hormone magnetic bead panel luminex kit to detect levels of GIP. Results were then analysed using Graphpad software.

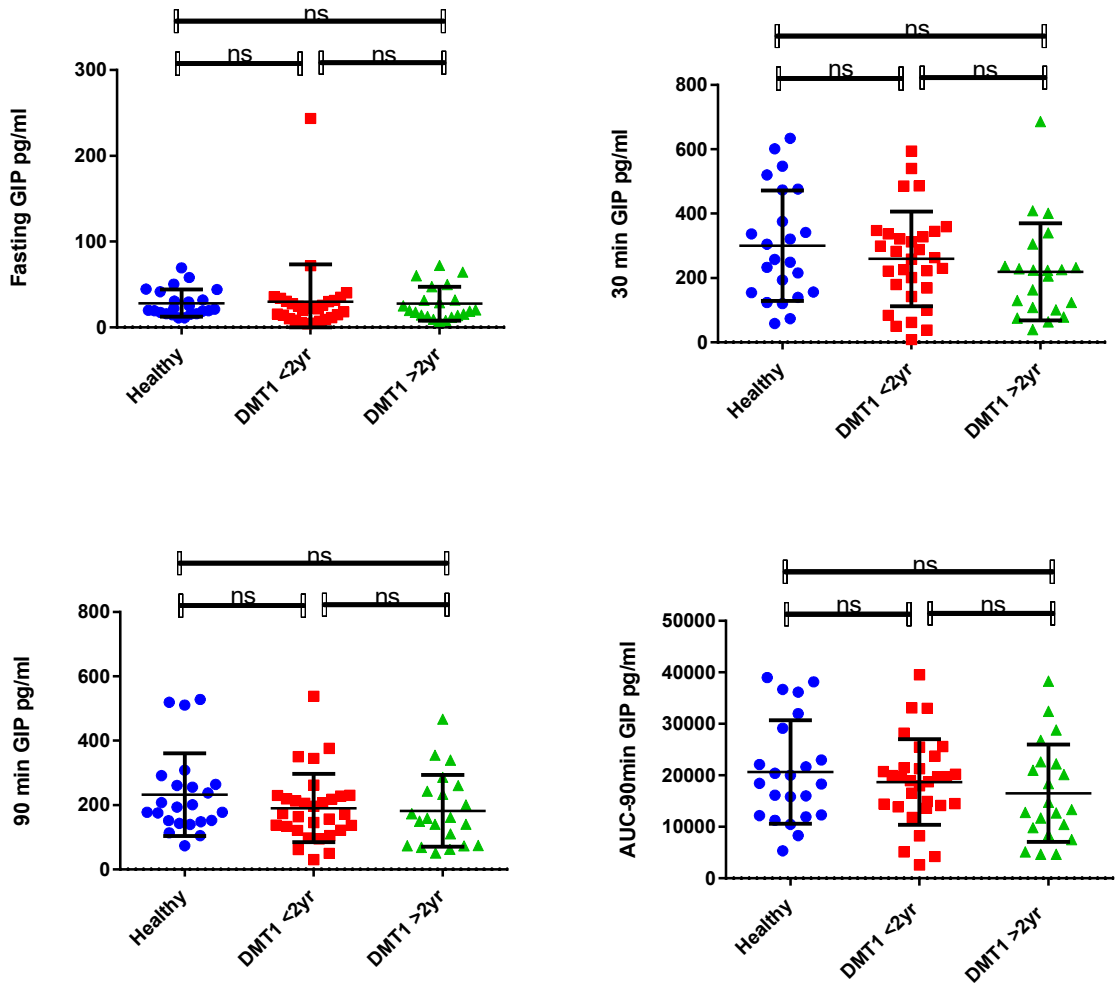


Figure 4.3 : GIP means and standard deviations for the three groups [healthy controls, subjects with duration of type 1 diabetes <2yrs, subjects with duration of type 1 diabetes >2yrs] (a) fasting, (b) 30 minutes, (c) 90 minutes, (d) Area Under Curve 0-90minutes. Statistical analysis with Kruskal–Wallis was utilised to find significant differences

[legend: (ns ($p>0.05$), * ($p<0.05$), **($p<0.01$), ***($p<0.001$)].

These results suggest there is no statistical difference in GIP levels between the three groups at all time points as well as Area Under the Curve 0-90minutes.

4.4.3 Analysis of GLP-1

All samples collected from participants who undertook a standard volume mixed meal stimulated test were processed using Millipores high sensitivity active-GLP-1 ELISA to detect levels of active-GLP-1. Results were then analysed using Graphpad software.

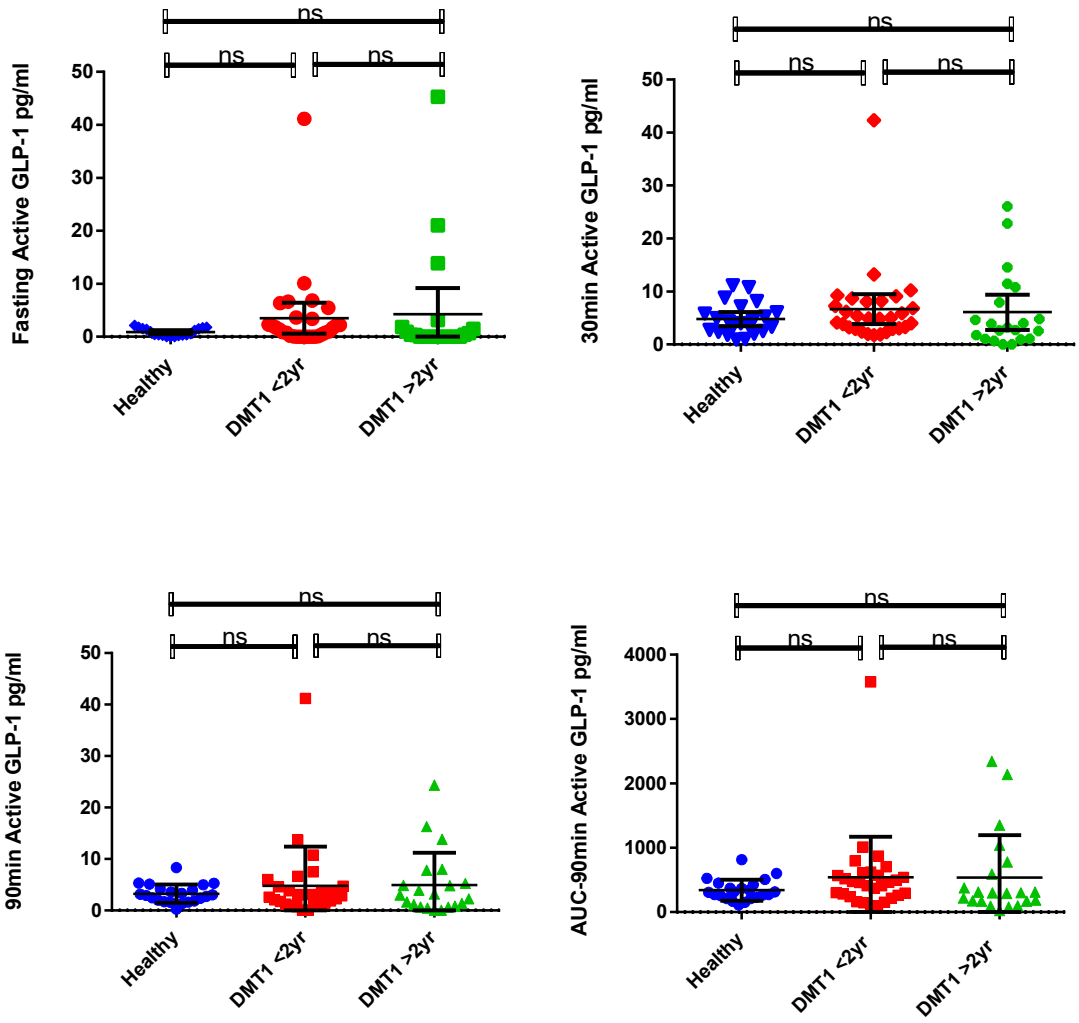


Figure 4.4 : Active GLP-1 means and standard deviations for the three groups [healthy controls, subjects with duration of type 1 diabetes <2yrs, subjects with duration of type 1 diabetes >2yrs] (a) fasting, (b) 30 minutes, (c) 90 minutes, (d) Area Under Curve 0-90minutes. Statistical analysis with Kruskal–Wallis was utilised to find significant

differences [legend: (ns ($p>0.05$), * ($p<0.05$), **($p<0.01$), ***($p<0.001$)].

These results suggest there is no statistical difference in active-GLP-1 levels between all three groups at all time points as well as Area Under the Curve 0-90minutes.

4.4.4 Analysis of Leptin

All samples collected from participants who undertook a standard volume mixed meal stimulated test were processed using Millipores human metabolic hormone magnetic bead panel luminex kit to detect levels of leptin. Results were then analysed using Graphpad software.

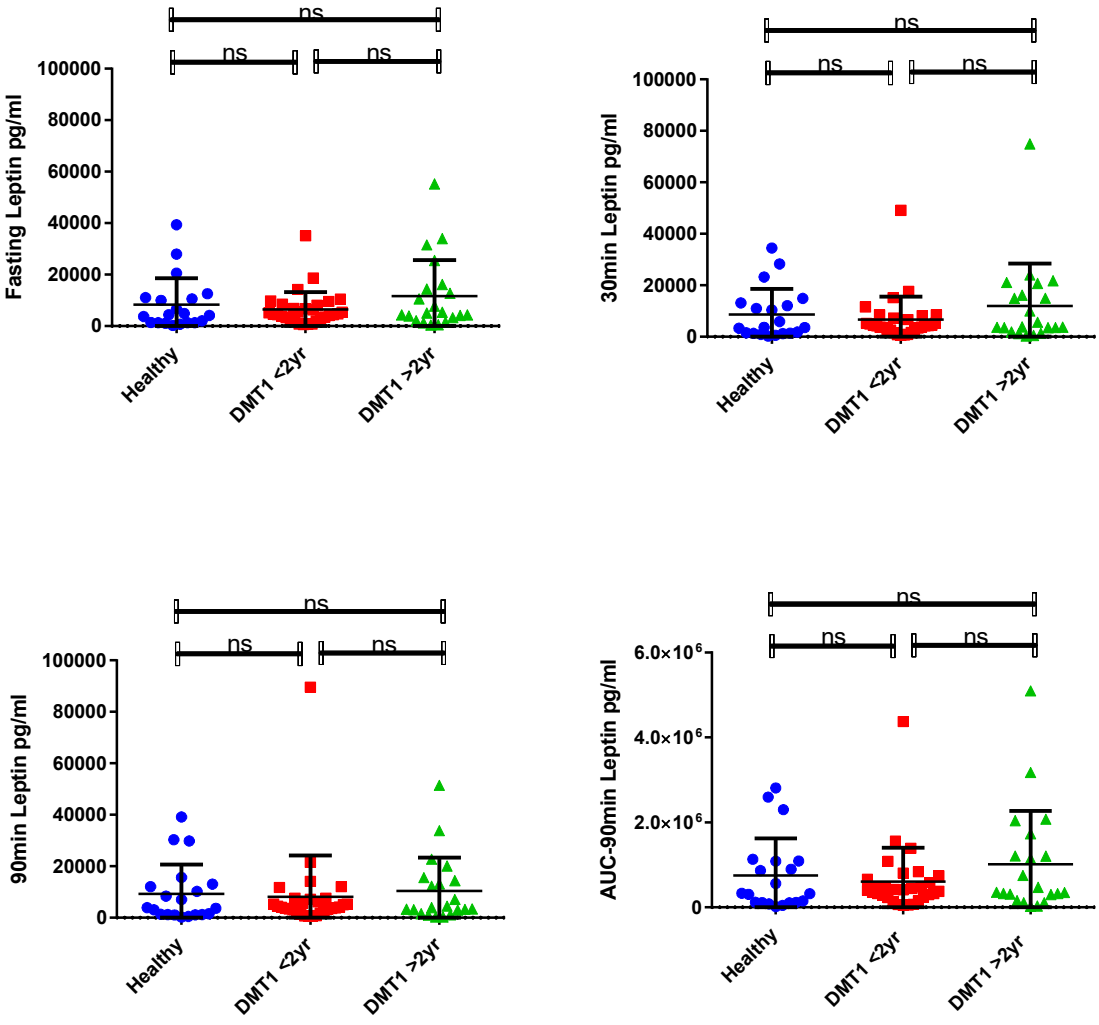


Figure 4.5 : Leptin means and standard deviations for the three groups [healthy controls, subjects with duration of type 1 diabetes <2yrs, subjects with duration of type 1 diabetes

>2yrs] (a) fasting, (b) 30 minutes, (c) 90 minutes, (d) Area Under Curve 0-90minutes. Statistical analysis with Kruskal–Wallis was utilised to find significant differences [legend: (ns ($p>0.05$), * ($p<0.05$), **($p<0.01$), ***($p<0.001$)].

These results suggest there is no statistical difference in leptin levels between all three groups at all time points as well as Area Under the Curve 0-90minutes.

4.4.5 Analysis of Pancreatic Polypeptide

All samples collected from participants who undertook a standard volume mixed meal stimulated test were processed using Millipores human metabolic hormone magnetic bead panel luminex kit to detect levels of pancreatic polypeptide (PP). Results were then analysed using Graphpad software.

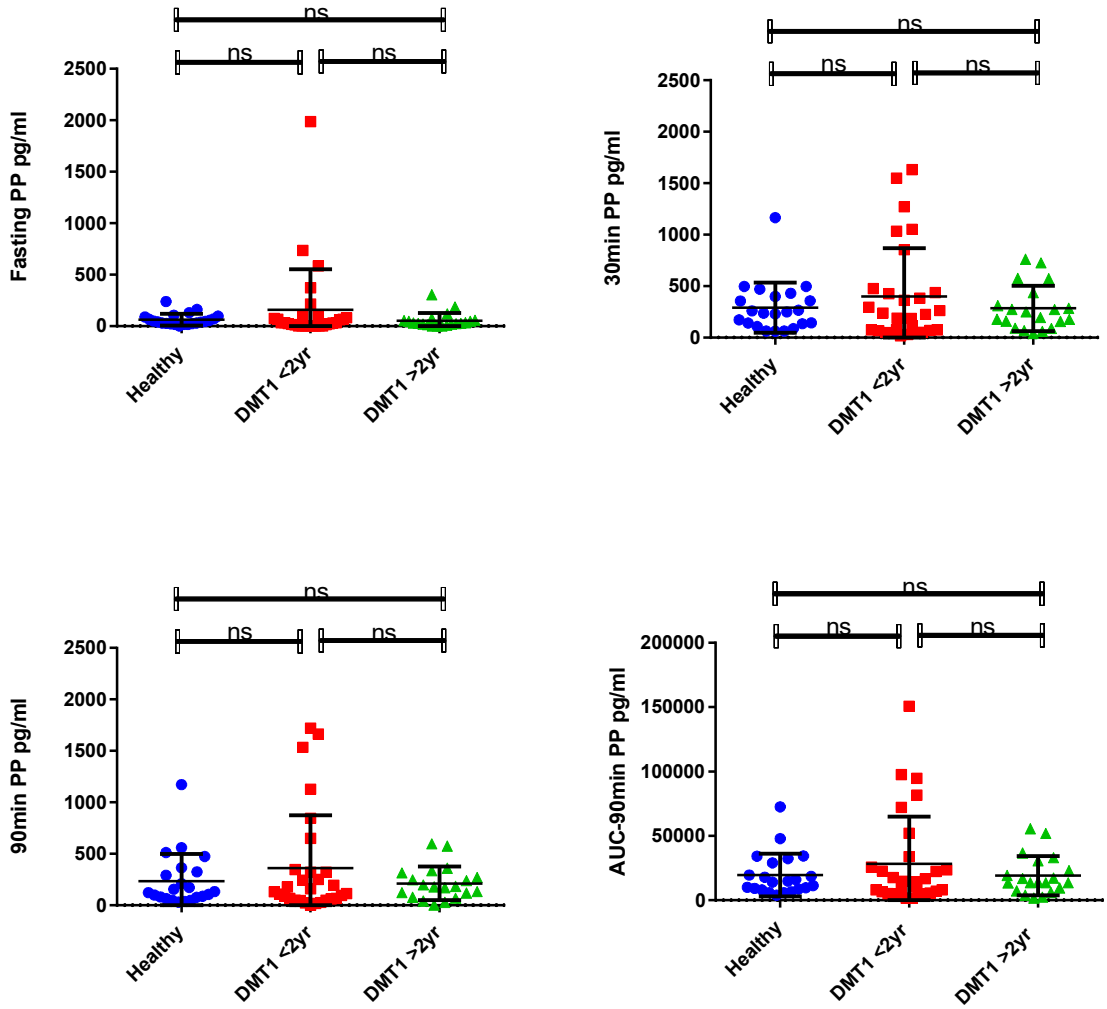


Figure 4.6: Pancreatic Polypeptide means and standard deviations for the three groups [healthy controls, subjects with duration of type 1 diabetes <2yrs, subjects with duration of type 1 diabetes >2yrs] (a) fasting, (b) 30 minutes, (c) 90 minutes, (d) Area Under Curve 0-90minutes. Statistical analysis with Kruskal–Wallis was utilised to find

significant differences [legend: (ns ($p>0.05$), * ($p<0.05$), **($p<0.01$), ***($p<0.001$)].

These results suggest there is no statistical difference in PP levels between all three groups at all time points as well as Area Under the Curve 0-90minutes.

4.4.6 Analysis of PYY

All samples collected from participants who undertook a standard volume mixed meal stimulated test were processed using Millipores human metabolic hormone magnetic bead panel luminex kit to detect levels of PYY. Results were then analysed using Graphpad software.

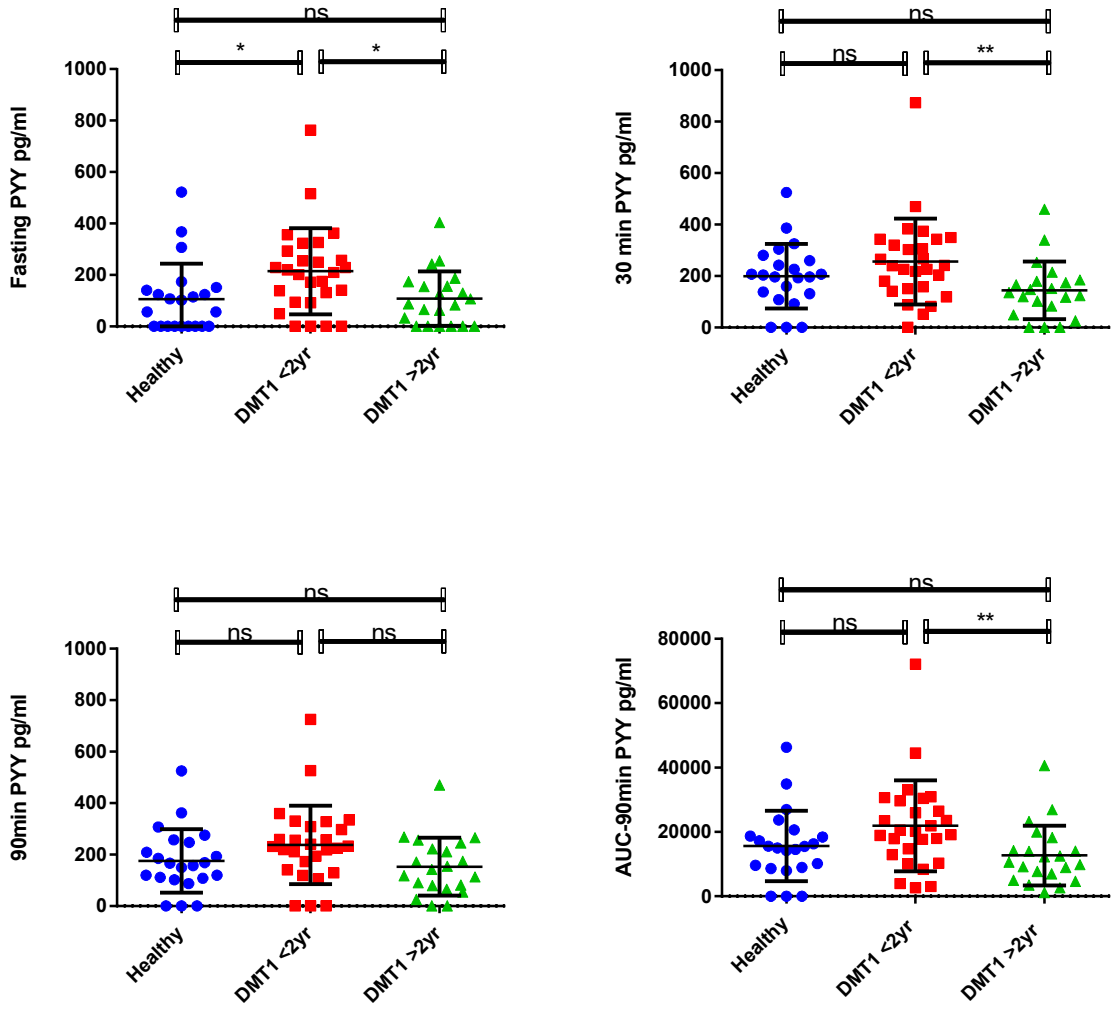


Figure 4.7: Peptide YY means and standard deviations for the three groups [healthy controls, subjects with duration of type 1 diabetes <2yrs, subjects with duration of type

1 diabetes >2yrs] (a) fasting, (b) 30 minutes, (c) 90 minutes, (d) Area Under Curve 0-90minutes. Statistical analysis with Kruskal–Wallis was utilised to find significant differences [legend: (ns ($p>0.05$), * ($p<0.05$), **($p<0.01$), ***($p<0.001$)].

These results suggest there is a statistically lower level of PYY in the group of individuals with type 1 diabetes group for >2yrs compared to those with diabetes <2yrs at time points 0min, 30min and AUC 0-90min and only just being non significant at 90 minutes ($p=0.55$). Level of PYY at 0min were also statistically lower in the healthy controls compared to those with duration of type 1 diabetes <2yrs, but no different to those with type 1 diabetes for >2yrs.

4.4.7 Analysis of Active Ghrelin

All samples collected from participants who undertook a standard volume mixed meal stimulated test were processed using Millipores human metabolic hormone magnetic bead panel luminex kit to detect levels of active ghrelin. Results were then analysed using Graphpad software.

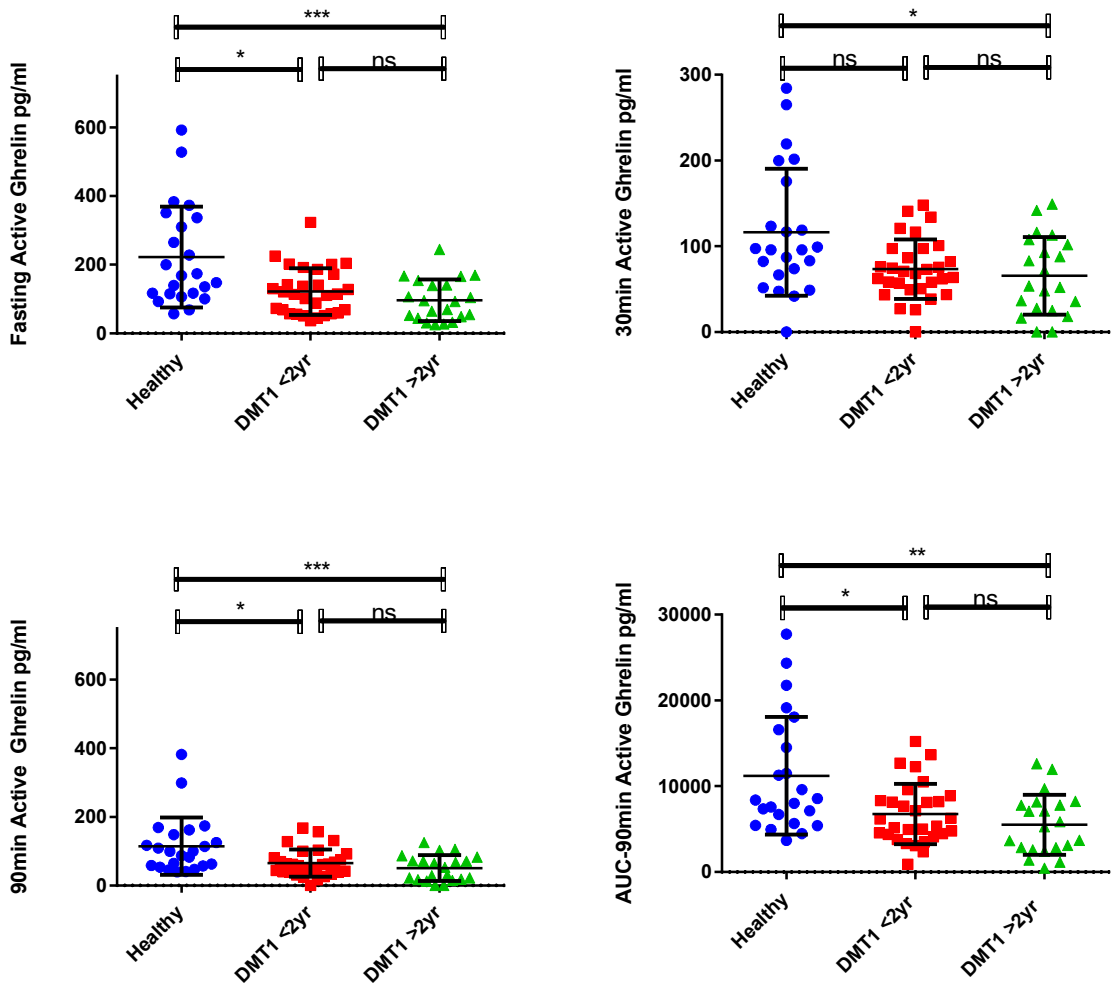


Figure 4.8: Active ghrelin mean and standard deviations for the three groups [healthy controls, subjects with duration of type 1 diabetes <2yrs, subjects with duration of type 1 diabetes >2yrs] (a) fasting, (b) 30 minutes, (c) 90 minutes, (d) Area Under Curve 0-90minutes. Statistical analysis with Kruskal–Wallis was utilised to find significant

differences [legend: (ns ($p>0.05$), * ($p<0.05$), **($p<0.01$), ***($p<0.001$)].

The results showed a significant decline in active ghrelin with the duration of diabetes in a similar fashion to that seen in the C-peptide analysis. Therefore further analysis was undertaken to determine if active ghrelin could also aid differentiation between healthy controls and those within two years of diagnosis of type 1 diabetes. Table 4.3 shows the analysis of the groups and their confidence intervals. The data was then analysed with receiver operated curves (ROC) to determine sensitivity and specificity of a 0 minute or 90 minute stimulated active ghrelin (see figure 4.9).

	Healthy	DMT1 – duration <2yr	DMT1 – duration >2yr
Fasting Mean (SD) [95% CI] (pg/ml)	222 (147) [159 - 286]	122 (68) [97 - 147]	97 (61) [69-124]
30min Mean (SD) [95% CI] (pg/ml)	116 (74) [84-148]	74 (35) [60-86]	66 (45) [45-86]
90min Mean (SD) [95% CI] (pg/ml)	115 (84) [79-151]	65 (40) [50-80]	51 (37) [34-68]

Table 4.3: Active ghrelin mean, standard deviation and 95% confidence intervals for active ghrelin in healthy volunteers, those with duration of type 1 diabetes <2yrs and duration >2yrs.

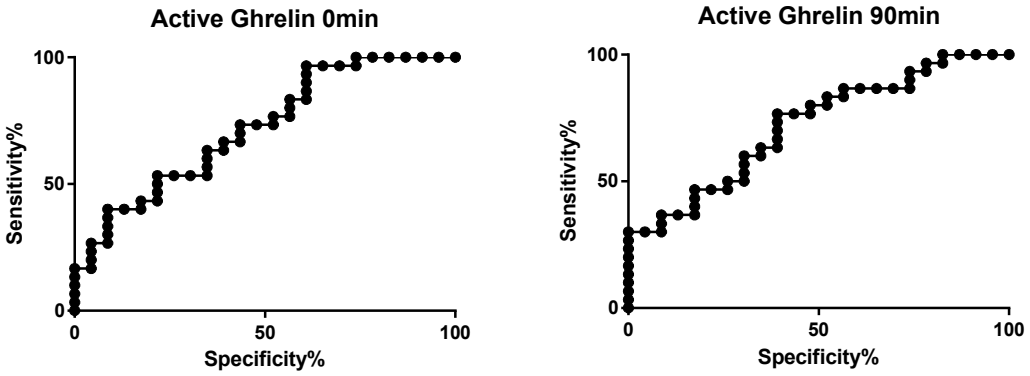


Figure 4.9: Active ghrelin ROC graphs compares the two groups [healthy controls vs subjects with type 1 diabetes with duration <2yrs]. (a)0minute active ghrelin (pg/ml) ROC area=0.72 with a cut off value of <64pg/ml providing a specificity of 95% and sensitivity of 27%. (b) 90minute active ghrelin (pg/ml) ROC area=0.72 with a cut off value of <40pg/ml providing a specificity of 95% and sensitivity of 30%.

Results from the ROC curves suggest that both a 0 minute and 90 minute meal stimulated active ghrelin levels have only a fair diagnostic accuracy in differentiating between healthy volunteers and those with duration of type 1 diabetes <2yrs. Therefore these results were not as accurate as those for C-peptide and would not be recommended for use in clinical practice.

4.4.8 Analysis of Glucagon

All samples collected from participants who undertook a standard volume mixed meal stimulated test were processed using Mercodia Glucagon ELISA to detect levels of glucagon. Results were then analysed using Graphpad software.

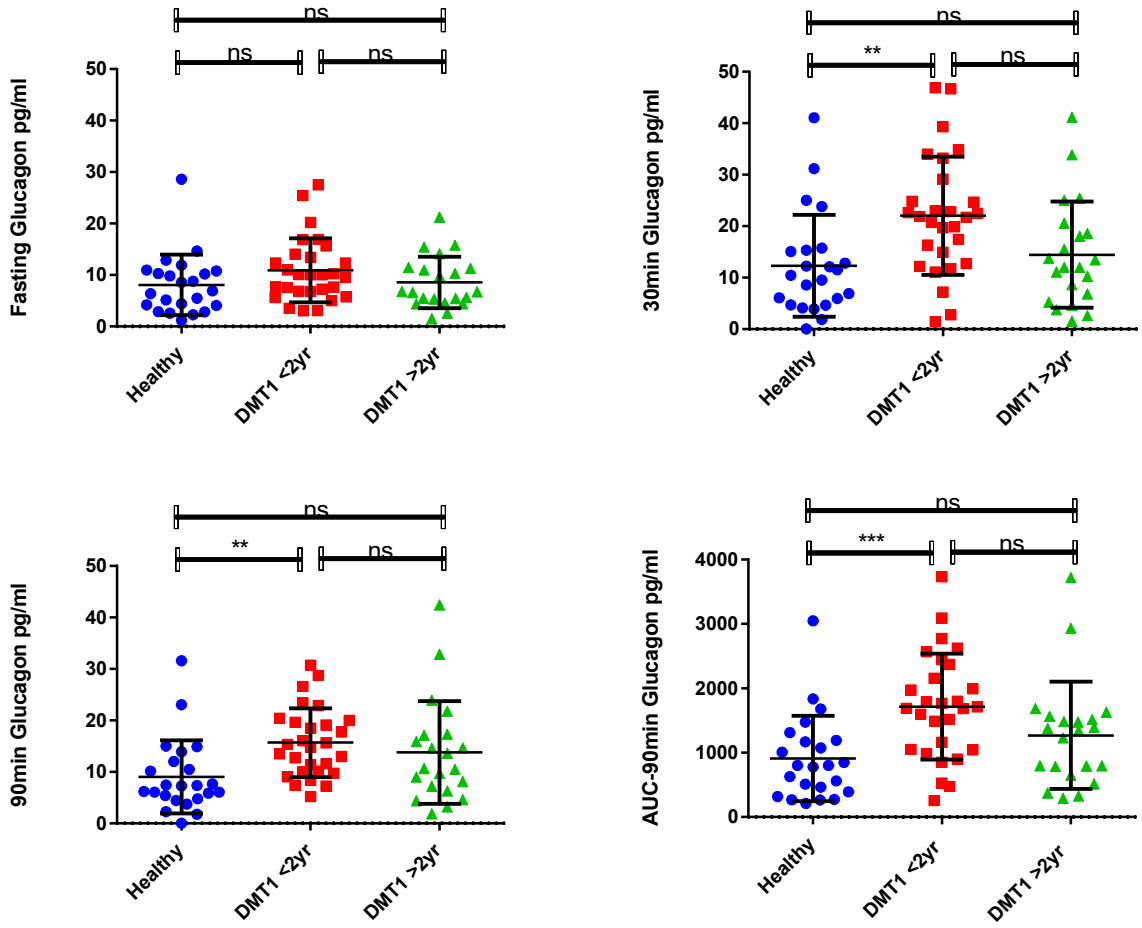


Figure 4.10: Glucagon mean and standard deviations for the three groups [healthy controls, subjects with duration of type 1 diabetes <2yrs, subjects with duration of type 1 diabetes >2yrs] (a) fasting, (b) 30 minutes, (c) 90 minutes, (d) Area Under Curve 0-90minutes. Statistical analysis with Kruskal–Wallis was utilised to find significant

differences [legend: (ns ($p>0.05$), * ($p<0.05$), **($p<0.01$), ***($p<0.001$)].

These results suggest that there is a statistically higher level of glucagon in those individuals with type 1 diabetes for <2 years compared to healthy controls at 30min, 90min and AUC 0-90min, but not for 0min. Also, in line with other published data, our results show that paradoxically glucagon levels increase rapidly during the first 30 minutes of a meal stimulated test in both healthy controls and those individuals with type 1 diabetes. Only at the 90 minute timepoint do levels start to significantly decline to that seen in the fasted state. This is counter intuitive as in the fed state there is less chance of hypoglycaemia then in the fasting state. It was therefore hypothesised that other gut hormones may be influencing this change in glucagon levels with the most likely candidates being either a glucose dependent hormone (i.e. insulin) or an incretin.

4.4.8.1 Method used to explore the initial rise in glucagon.

The method used involved calculating the initial 30 minute change in level (Δ 30min level) for each gut hormone and comparing it to the Δ 30min level of glucagon. This was done using Graphpad software. As insulin was a potential candidate hormone, the cohort was split into healthy controls, those individuals with positive C-peptide type 1 diabetes, and those individuals with negative C-peptide type 1 diabetes (as defined by the widely accepted standard of a 90 minute stimulated C-peptide level of <200pg/ml) [159].

4.4.8.2 Results from exploring the paradoxical rise in glucagon

Below are the results of statistical analysis, comparing the change in glucagon levels over the initial 30 minutes (Δ30 min) of the meal stimulated test to changes in levels of gut hormones over the same time period for healthy controls, individuals with positive C-peptide type 1 diabetes and individuals with negative C-peptide type 1 diabetes.

	Δ30 min glucagon in healthy controls	*Δ30 min glucagon in positive c-peptide type 1 diabetes*	*Δ30 min glucagon in negative c-peptide type 1 diabetes*	*Δ30 min glucagon in combined cohort of healthy controls and type 1 diabetes*
Δ30 min Glucose	p=0.24	n/a	p=0.47	p=0.21
Δ30 min C peptide	p=0.74	p=0.20	n/a	p=0.21
Δ30 min GLP-1	p=0.91	p=0.001 r=0.57	p=0.70	p=0.10
Δ30 min Ghrelin	p=0.036 r=0.44	p=0.26	p=0.65	p=0.011 r=0.3
Δ30 min GIP	p=0.23	p=0.29	p=0.45	p=0.23
Δ30 min Leptin	p=0.71	p=0.12	p=0.52	p=0.21
Δ30 min PP	p=0.76	p=0.011 r=0.45	p=0.27	p=0.25
Δ30 min PYY	p=0.70	p=0.16	p=0.36	p=0.56

Table 4.4 Pearsons correlation coefficient (r) and significance value (p) for change in glucagon levels over initial 30 minutes (Δ30 min) versus change in levels of gut hormones over initial 30 minutes (Δ30 min) for healthy controls, individuals with positive C-peptide type 1 diabetes, individuals with negative C-peptide type 1 diabetes and combined cohort of healthy controls and those with type 1 diabetes. Legend: ns means p>0.05.

There were three statistically significant correlations found between the Δ30 min levels of glucagon and Δ30 min levels of other gut hormones. All of the correlations were of moderate strength and no identical correlations occurred in all three cohorts. In the healthy controls a moderately strong correlation was found for active ghrelin (r=0.44 p=0.04). In the individuals with positive C-peptide type 1 diabetes a moderate

correlation was found for active-GLP-1 (r=0.57 p=0.001) as well as for Pancreatic Polypeptide (r=0.45 p=0.011). No correlations were found in the negative C-peptide cohort with type 1 diabetes. When the cohort of healthy controls, positive and negative c-peptide diabetes were combined then active ghrelin remained significantly correlated to Δ30 min levels of glucagon, but only weakly.

4.4.8.3 Discussion on exploring the initial rise in glucagon

Unfortunately, no single unifying hormone was found to be significantly correlated in all individual groups to explain the initial 30 minute rise of glucagon. In the combined cohort there was a weakly significant correlation between active ghrelin and the initial 30 minute rise of glucagon. This suggests that it is unlikely that one of the hormones explored in this research is solely responsible for the paradoxical Δ 30min rise in glucagon concentrations. This does not exclude the possibility that multiple hormones may be involved, or that there is another hormone that we haven't yet explored. However, our results did reveal one interesting negative finding which was that there was no correlation between the Δ 30min levels of glucagon for either glucose or C-peptide. This suggests that the mechanism is probably neither glucose nor insulin dependent, but could indicate that either the presence of food in the gut or even taste might have a role in stimulating the rise in glucagon. This proposed mechanism would also fit with the observed correlation with active-ghrelin.

In order to localise the stimuli for glucagon secretion in the gut then an *in vivo* model that enables different parts of the gut to be cannulated would be required. Then the various levels of the gut (e.g. mouth, stomach, first part of the duodenum and ileum)

could be independently stimulated with a mixed meal to determine the level that provides the most significant increase in Δ 30min level of glucagon. The results may have significant implications for individuals with type 1 diabetes who undergo metabolic surgery (e.g. gastric band, gastric bypass, duodenal switch, etc) as the operation could be potentially tailored to induce the greatest glycaemic benefit.

4.5 Discussion

The cohort of patients studied in this research were all recruited from a single centre, but from two separate studies (a lifestyle intervention study - ExTOD and an observational study – CDRD). These studies were exploring different aspects of the pathogenesis of type 1 diabetes. The Exercise in Type One Diabetes (ExTOD) study was recruiting individuals with new onset type 1 diabetes and motivating them to take more exercise to see if it would preserve beta cell function. The Chronic Disease Research into Diabetes study was recruiting individuals with all types of diabetes as well as a separate cohort of healthy controls, to explore the chronic disease burden of diabetes. The combined cohort of healthy controls and individuals with type 1 diabetes from both studies underwent a meal stimulated test to explore their gut hormone levels. Thus, the samples analysed here were from two separate populations which were not actively matched to ensure similar demographics. We acknowledge that without adequate matching we could introduce confounding factors into our data analysis.

The subjects with type 1 diabetes from the two cohorts were split into two groups in order to explore the effect that duration of diabetes has on gut hormone levels:

Group 1) individuals with diabetes for less than 2 years

Group 2) individuals with diabetes for more than 2 years

In fact the mean duration of diabetes in the group with a duration of diabetes <2yrs was 0.5 years and in the group with a duration of diabetes >2 years was 30yrs. Thus, the grouping could be misleading to the casual reader if they were to imagine a clear separation between the groups at 2 years. In essence, it is important to interpret the data as from a group with acute onset type 1 diabetes and a group with chronic duration of type 1 diabetes. If the opportunity were to arise whereby this research could be undertaken with a larger population of matched participants, it would be better to compare gut peptide levels according to a more evenly distributed range of diabetes duration.

As exogenous insulin is essential for individuals with type 1 diabetes, then serum insulin levels do not always correlate with the endogenous insulin secretion of beta cells. To determine endogenous insulin secretion, and thus beta cell function, it is necessary to measure the levels of the co-secreted peptide - 'C-peptide'. Results from our analysis of C-peptide show that beta cell function is highest in healthy controls and lowest in those with chronic type 1 diabetes. These results are in line with those of the DCCT trial, confirming both fasting and 90minute stimulated C-peptide levels decline with duration of type 1 diabetes [185]. It is worth noting that currently both fasting and 90 minute stimulated C-peptide levels are used in clinical practice. However, a 30 minute stimulated C-peptide level is not currently used. Our results demonstrate that a 30 minute stimulated C-peptide is as specific and of similar sensitivity to that of a 90 minute C-peptide level for aiding the diagnosis of type 1 diabetes (30min c-peptide <1600pg/ml (95% sensitivity, 55% specificity); 90min c-peptide <2960pg/ml (95%

sensitivity, 65% specificity)). However, even at these levels, 5% of cases of type 1 diabetes may be missed with wrongly classified type 1 diabetes occurring in 35% of the 90min c-peptide samples and 45% of the 30min c-peptide samples.

Further research is needed to determine which test has the best sensitivity and specificity in differentiating between new onset type 2 diabetes and type 1 diabetes. Not least considering the low numbers used in this research sample. This is especially important when considering that the phenotypical distinction between type 1 and type 2 diabetes is becoming increasingly blurred. The classical phenotype of new onset type 1 diabetes is of a thin individual, whilst in contrast the clinical phenotype of type 2 diabetes is that of an obese individual. This distinction is being complicated by the obesity epidemic. The increasing prevalence of obesity has resulted in ~20% of under 18 year olds now being classed as obese, resulting in an increasing prevalence of young type 2 diabetes [186]. In ambiguous cases clinicians are frequently turning to anti-islet antibody levels to help differentiate between an obese individual with new onset type 1 or type 2 diabetes.

The clinical utility of a same day combined fasting and 30minute stimulated C-peptide test should not be underestimated. It could provide a time and cost efficient means of increasing diagnostic accuracy over current clinical diagnostic skills, either alone or in combination with other biomarkers ie. anti-islet antibody levels. To determine the utility of a 30 minute C-peptide, a new prospective study would be required. This would need to be performed with a 'real world' population, specifically targeting ambiguous cases of early onset type 2 diabetes and type 1 diabetes, as our study population was small and unrepresentative of the population of cases that clinicians would face. However, data

from our and other studies could be used for power calculations to determine the appropriate study sample size. Only after this has been completed could the utility, sensitivity and specificity of a 30 minute stimulated C-peptide be determined. If its accuracy is confirmed then it could have a key place in the early implementation of immuno-modulating therapy to delay the progression of early onset type 1 diabetes. However, it is important to note that whilst it may help distinguish between type 1 and type 2 diabetes, it may not have a use in detecting other forms of diabetes (e.g. maturity onset diabetes of the young (MODY), gestational diabetes, latent autoimmune diabetes of adulthood (LADA), drug induced diabetes, etc).

The secretion of incretins occurs following the consumption of food. As food enters the gut, incretins stimulate insulin secretion from beta cells. If incretin homeostasis were to be adversely affected in type 1 diabetes then there could be a role for exogenous incretin supplementation. However, our results found that both active-GLP-1 and GIP levels are not significantly different in individuals with type 1 diabetes compared to healthy controls. This suggests that physiological incretin hormone secretion is independent of beta cell function and unaffected by their decline. Although physiological incretin supplementation is unlikely to benefit individuals with type 1 diabetes, it does not exclude the possibility that supra-physiological levels may benefit insulin resistant individuals with type 1 diabetes.

As the consumption of food causes a rise and subsequent decline in incretin levels, then this could potentially be used as a measure of gut transit time. Our results suggest that this normally occurs within a 90 minute time frame. Current means of measuring gut transit time include swallowing radio-opaque contrast medium before performing timed

intervals of x-rays to observe the transit of the contrast through the gut, thus exposing an individual to multiple doses of radiation. If a future study could demonstrate a method of accurately measuring gut transit time through incretin levels, then this could provide a method that does not expose individuals to radiation. This would facilitate investigations of conditions such as gastroparesis or gastric dumping syndrome.

Leptin acts as an appetite stimulant and is released from adipose tissue. Levels of leptin slowly decline following the ingestion of food. However, high levels of leptin are seen in insulin resistance but its importance in type 1 diabetes is unknown. Leptin analogue drugs are in development and are aimed at improving insulin sensitivity and aiding in weight loss. The results of our analysis confirm that leptin still declines in individuals with type 1 diabetes following ingestion of the meal. Also, there was no statistical difference in Leptin levels between the three groups suggesting that duration of type 1 diabetes does not affect secretion of leptin. As the homeostasis of leptin does not appear to be affected by the pathogenesis of type 1 diabetes, then leptin analogues may have a niche role in insulin resistant individuals with type 1 diabetes. As the prevalence of obese individuals with type 1 diabetes increases, leptin may become an important marker for insulin resistance in individuals with type 1 diabetes.

Histopathological analysis of pancreatic tissue from individuals with new onset type 1 diabetes has revealed an infiltrative cellular and inflammatory process that leads to beta cell death [187]. As beta cells share a common developmental cell linage with neighbouring endocrine cells, then this process could affect other islet cells. As pancreatic polypeptide secreting cells are co-localised within the islet then they may also be at risk of being damaged. If this were to occur we would expect to see a

progressive decline in PP secretion with duration of type 1 diabetes. Our results show no statistical difference in circulating levels of PP between the groups. This suggests that either PP cells are protected from the inflammatory process or that PP secretion occurs outside of the Islets of Langerhans. It is most likely that PP cells are protected, but to help determine this then either PP levels would need to be measured in humans (or animals) that had undergone a total pancreatic resection or alternatively the number of PP secreting cells would need to be measured in islets from samples taken from humans (or animal models) with varying durations of type 1 diabetes.

PYY is released from enteroendocrine cells in the gut in response to ingestion of food. As yet no role has been found for its control of glucose homeostasis in type 1 diabetes. The results from this study are challenging to interpret, as statistical differences between the groups were detected. Firstly, fasting levels were higher in those with a diabetes duration of <2yrs compared to healthy controls or those with longer duration of type 1 diabetes. This difference disappears on ingestion of the mixed meal. However, the statistically lower levels of PYY in the participants with >2yrs duration of type 1 diabetes compared to <2yrs duration persists. These results are not in line with what was anticipated. One possible explanation is that those with a duration of type 1 diabetes of <2yrs were statistically younger than those from the other cohorts, potentially making PYY a marker for ageing or activity. This has been loosely proposed in other studies, but would require further research before we can confidently say that this conclusion can be drawn [128].

Ghrelin is also secreted by enteroendocrine cells in the stomach, but unlike other gut hormones the stretching of the stomach by food inhibits its secretion. There is no known

mechanism that links beta cell function to ghrelin levels. However, our results revealed significantly lower levels of active ghrelin in individuals with type 1 diabetes compared to healthy controls at both fasting and 90 minutes. It is tempting to propose a new mechanism that links active ghrelin secretion to the pathogenesis of type 1 diabetes but for the fact that active ghrelin levels are influenced by food types [188]. High protein diets tend to lead to lower ghrelin levels than standard diets. At diagnosis of new onset type 1 diabetes, our patients are warned against eating excess carbohydrates to optimise their glycaemic control. Thus individuals with type 1 diabetes may tend to choose meals lower in carbohydrates and higher in protein and fibre. If dietary changes had occurred in this cohort, then this could potentially explain differences in active ghrelin levels. However, one key reason for using a standard meal for all participants in this study was to eliminate the differences in nutrient proportions in the meal used to stimulate the gut hormones. Unfortunately, as we did not control all meals prior to the test, then these could possibly have introduced a confounding factor that influenced active ghrelin levels. In order to determine the degree that diet influences ghrelin levels, then a similar study would be needed whereby individuals with both type 1 diabetes and healthy controls are matched for dietary habits prior to the test. If dietary influences could be excluded, then an explanation for our results could be that the pathogenesis of type 1 diabetes directly influences levels of active ghrelin.

Glucagon is secreted by alpha cells in the islets of Langerhans. In the traditional 'bihormonal' model it is secreted in response to hypoglycaemia, but subsequent research has suggested it is also secreted in response to food consumption [135], [189]. As alpha cells share a common embryological origin to beta cells, then the inflammatory process may also affect them and their ability to secrete glucagon. The analysis of glucagon

found that fasting glucagon levels were no different in all three groups, but stimulated levels (30minutes and 90 minutes) were significantly higher in those with diabetes duration <2yrs compared to either the healthy controls or duration >2yrs. This is in line with some published data and could be explained by a potential compensatory alpha cell hyperplasia occurring shortly after the onset of type 1 diabetes [80], [190]. In order to investigate this hypothesis, further pancreatic samples from individuals with type 1 diabetes would need to be histopathologically examined. Alternatively, a non invasive means to measure alpha cell mass would need to be developed. An international project run by the Juvenile Diabetes Research Foundation has set up a database of pancreatic slides from organ donors with type 1 diabetes that we requested permission to access in order to explore this hypothesis further (see Chapter 6).

5. EXPLORE THE EFFECTS OF MARKERS OF HEALTHY LIFESTYLE ON GUT HORMONES IN TYPE 1 DIABETES.

5.1 Introduction

The pathogenesis of type 2 diabetes is strongly influenced by lifestyle factors such as obesity, exercise and age that can affect the homeostasis of gut hormones [191]–[193]. The role of lifestyle modifying factors has not been extensively explored in individuals with type 1 diabetes. One of the aims of the CDRD study was to explore anthropometric and lifestyle data as potential disease markers. This data could then be used to compare gut hormone levels to lifestyle factors and thus increase our understanding of how lifestyle modifying factors can affect the homeostasis of gut hormones in type 1 diabetes.

Lifestyle data was collected from CDRD study participants by means of validated closed answer questionnaires. This method of data collection was utilised due to the low cost and its high reproducibility. Variable aspects of lifestyle behaviour were considered for study including eating behaviour, alcohol consumption, physical activity, physical functional ability, cognition, psychological wellness and fatigue. As the final questionnaire needed to be an acceptable length for our participants to complete, it was decided to focus on three key lifestyle behaviours: eating behaviour, physical activity and psychological wellness.

When considering which validated eating behaviour questionnaire to use it became obvious that both food diaries and food frequency questionnaires were not appropriate

due to their length. Food diary and food frequency questionnaires also provide a descriptive result that is better suited for large epidemiology studies rather than a small observational study. The “Eating Attitudes Test - 26” was also considered due to its brevity with 26 closed answer questions. The test was free to use as long as permission was granted. The Eating Attitudes Test is a validated self administered questionnaire that provides a score that is well correlated to an individuals concern about their diet, body weight or problematic eating behaviour. Finally, the Three Factors Eating Questionnaire R18 (TFEQ-18) was considered due to it containing 6 closed answer questions for three separate eating behaviour domains (ie cognitive restraint, uncontrolled eating and emotional eating) [184]. The “cognitive restraint” domain considers any conscious restriction of the consumption of food instead of acting on internal cues such as hunger and satiety. The “uncontrolled eating” domain measures the inability to control those internal cues (ie satiety) that lead to overeating. The “emotional eating” domain measures the likelihood of choosing to eat in response to negative emotions. The TFEQ-18 could provide an ordinal measure of the interplay between internal physiological cues, cognitive and emotional behaviour. As many gut peptides influence the internal cues of satiety and hunger I decided to select the validated TFEQ-18 as the questionnaire to explore eating behaviour.

A participant's psychological wellness focuses on the experiences of anxiety and depression of which depression is considered the more clinically important. Three commonly used questionnaires were considered; the Hospital Anxiety and Depression Scale, Becks Depression Inventory and Patient Health Questionnaire-9. The Hospital Anxiety and Depression Scale is a 14 item self reported questionnaire that provides an ordinal value for the level of anxiety, depression and overall emotional distress a

participant is experiencing. However, it does not strongly correlate to clinical diagnosis of anxiety or depression. “Beck's Depression Inventory” is a 13 item self reported questionnaire that provides an ordinal value for the level of depression. It is strongly correlated to diagnostic criteria for clinical depression, but was not selected due to the payable fee for using it. Finally the Patient Health Questionnaire - 9 (PHQ-9) was considered as it is a 9 item self reported questionnaire that is free to use and provides a value that strongly correlates to the severity of clinical depression [183], [194]. Therefore the PHQ-9 was selected for its ability to detect the clinical impact on psychological wellness.

Physical activity can be measured through means of questionnaires and wearable devices (eg. pedometers, heart rate monitors and GPS tracking accelerometers). Due to cost of these devices, a questionnaire approach was chosen. The ideal questionnaire would cover both physical activity and inactivity. Activity diaries and logs were considered, but it was felt that prospectively they would be unacceptably time intensive for our participants and if used retrospectively then they could be open to significant recall error. The “International Physical Activity Questionnaires - short” and “Global Physical Activity Questionnaire” are both validated and relatively short questionnaires that covered aspects of both physical activity and inactivity. Both are validated means of calculating physical activity as metabolic equivalents (amount of energy per kg of body mass expended per hour) and physical inactivity (total amount of awake sedentary time). The International Physical Activity Questionnaire – short version consists of 7 closed questions and the Global Physical Activity Questionnaire consists of 16 closed questions. The International Physical Activity Questionnaire – short version was selected for its brevity and ability to measure both physical activity and inactivity.

It had been decided that an acceptable length for an educated participant to complete the combined questionnaires would be under 15 minutes. A volunteer from our laboratory was timed at completing all three questionnaires (ie Three Factors Eating Questionnaire R18, Patient Health Questionnaire - 9 and International Physical Activity Questionnaire – short version). This took them ~19 minutes to complete and they reported difficultly in accurately answering the physical activity part of the International Physical Activity Questionnaire. Therefore, the physical activity part of the International Physical Activity Questionnaire was removed and the physical inactivity part was kept. This enabled the questionnaire to be completed in under 15 minutes.

5.2 Methods used to explore the influence of lifestyle factor's on gut hormone levels.

The measurements of body mass index (BMI), sedentary time, depression, age and eating behaviour were accessed from the CDRD database for those participants that had undergone a meal stimulated test. In order to exclude the influence of residual endogenous insulin secretion, the cohort of patients was split into two groups:

1. Healthy controls
2. Individuals with negative c-peptide levels type 1 diabetes (less than 200pg/ml) as determined by the 90 minute stimulated time point for c-peptide.

The results from the lifestyle questionnaires from each group were compared to ensure there was no statistical difference. Then the results obtained for gut hormone level analysis for 90 minute stimulated area under the curve (AUC 0-90 minutes) were compared to lifestyle factors (age, sedentary time, BMI, depression and eating

behaviour) using Graphpad software.

5.3 Results of the analysis of lifestyle factors influence on gut hormone levels

Comparisons of the lifestyle questionnaire responses between the healthy controls and those with negative c-peptide type 1 diabetes demonstrated no statistical difference (see Table 5.1).

	Healthy	Negative c-peptide DMT1	Statistical difference
n	23	17	p>0.05
Mean age [SD]	42.5 [13.6]	50.7 [15.7]	p>0.05
Mean sedentary time (min) [SD]	173 [113]	203 [153]	p>0.05
Mean BMI (kg/m2) [SD]	24.3 [4.9]	27.5 [6.7]	p>0.05
Mean PHQ-9 score [SD]	2.2 [2.3]	5.8 [4.5]	p>0.05
Mean TFEQ – cognitive score [SD]	12.1[2.6]	14.1 [3.7]	p>0.05
Mean TFEQ – uncontrolled score [SD]	17.7 [5.3]	15.2 [4.5]	p>0.05
Mean TFEQ – emotional score [SD]	5.3 [2.0]	5.4 [2.1]	p>0.05

Table 5.1: Mean and standard deviations for the group of healthy participants and negative c-peptide type 1 diabetes participants. Statistical analysis with Kruskal-Wallis was utilised to find significant differences.

In order to determine which lifestyle factors influence gut hormone levels in non-diabetic conditions, the lifestyle questionnaire results from the healthy participants were compared to their 90 minute area under the curve gut peptide results (see Figures 5.1-5.8). Then the lifestyle questionnaire results from the negative c-peptide type 1 diabetes participants were compared to their 90 minute area under the curve gut peptide results

(see Figures 5.9-5.16).

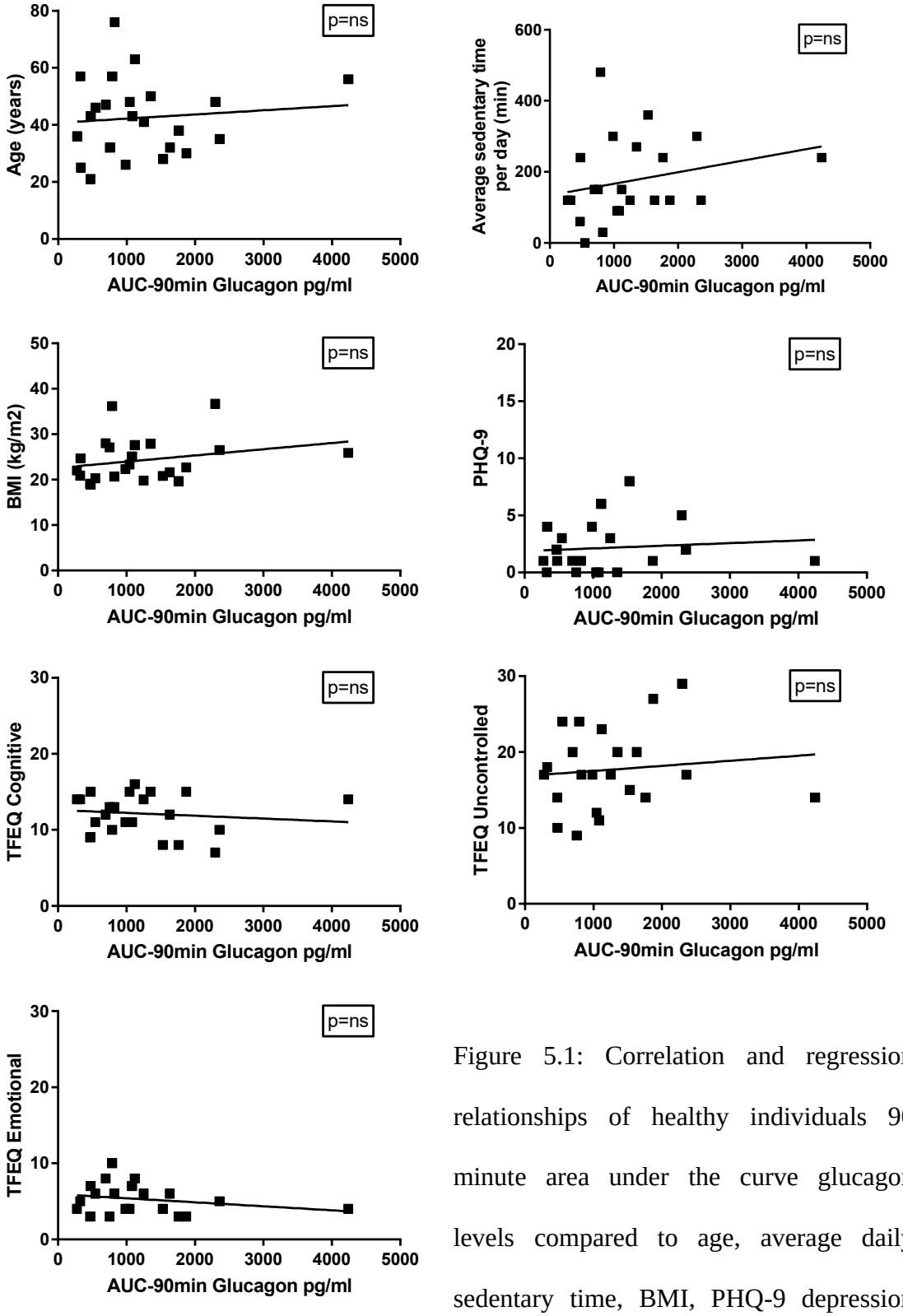


Figure 5.1: Correlation and regression relationships of healthy individuals 90 minute area under the curve glucagon levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

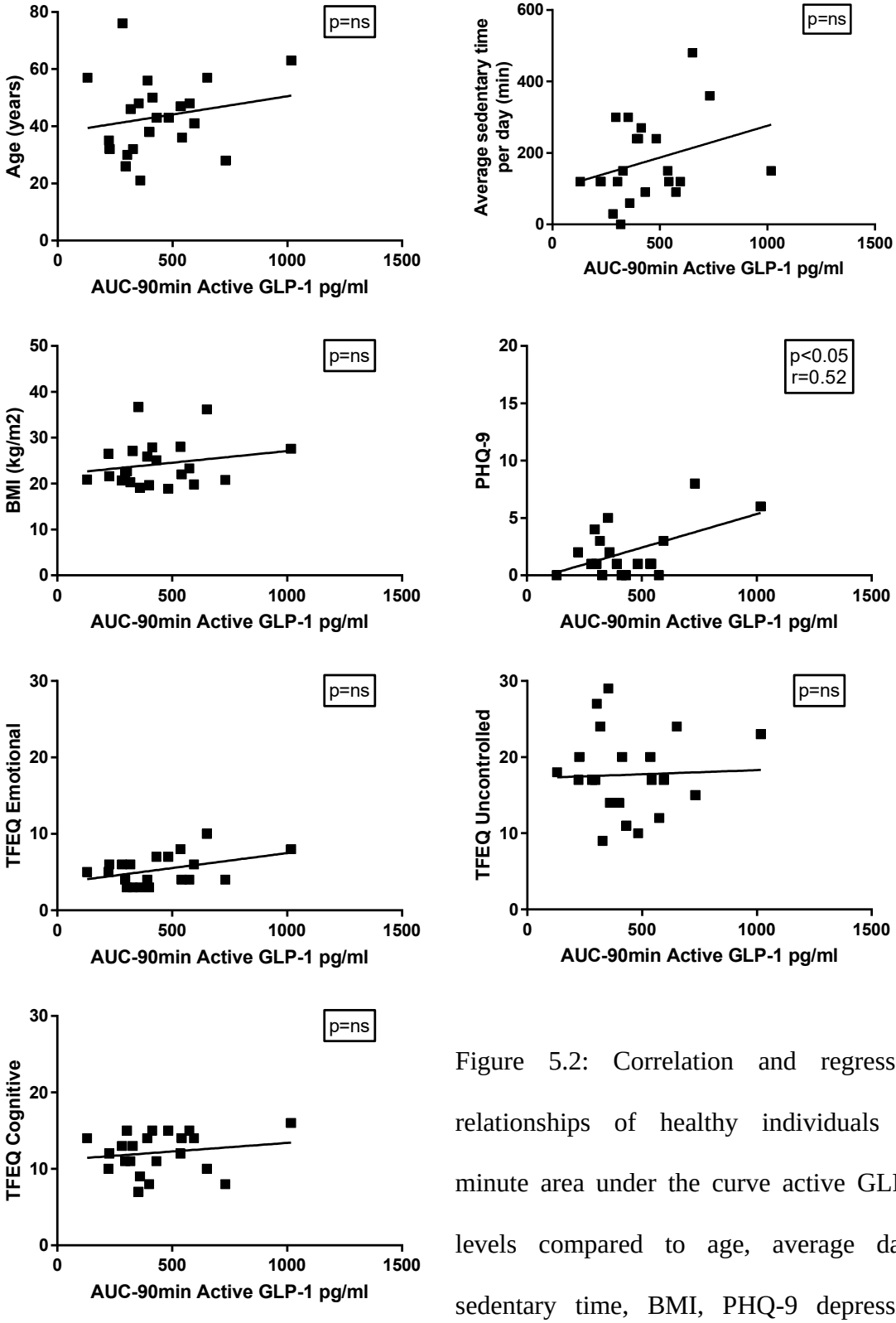


Figure 5.2: Correlation and regression relationships of healthy individuals 90 minute area under the curve active GLP-1 levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

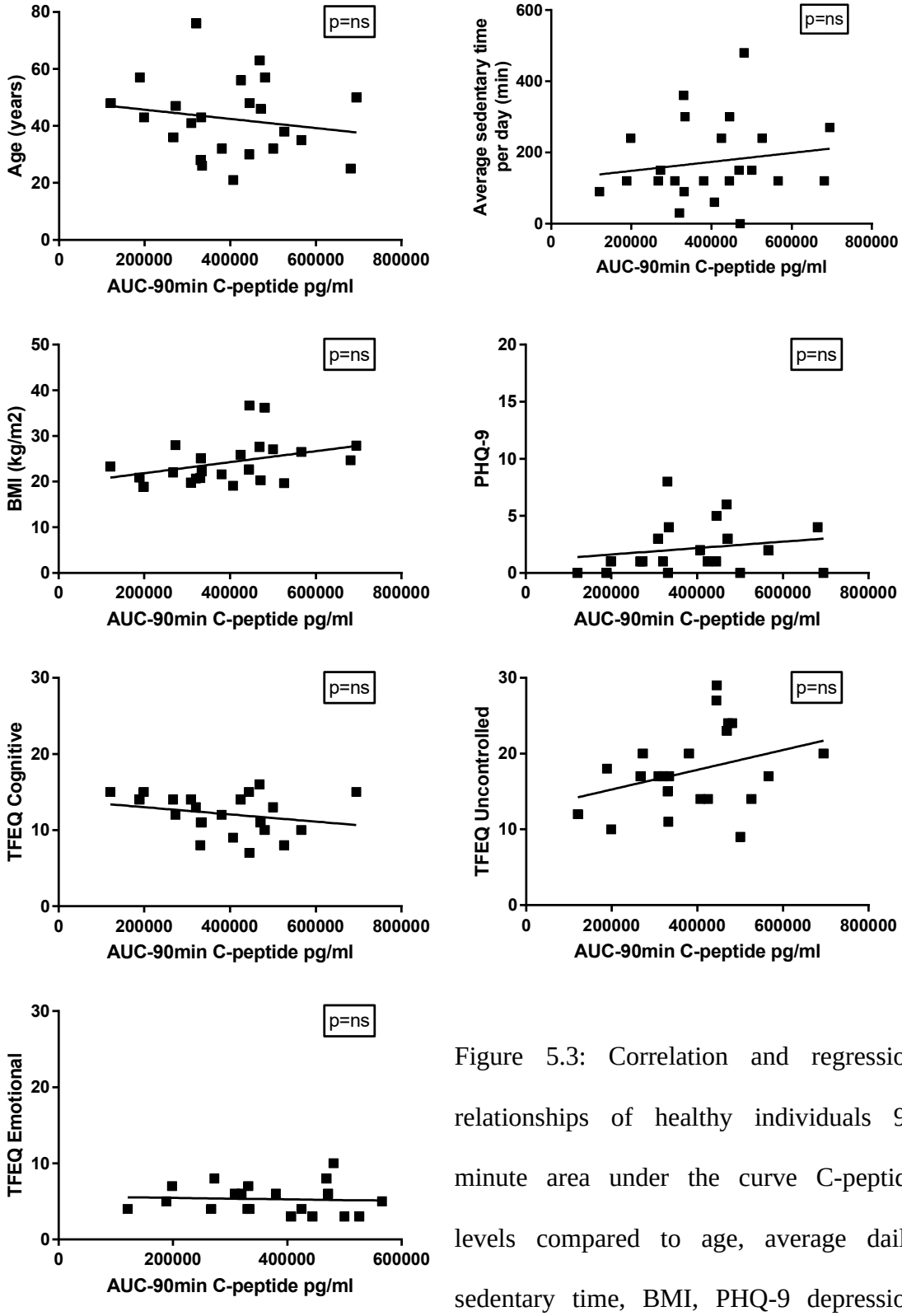
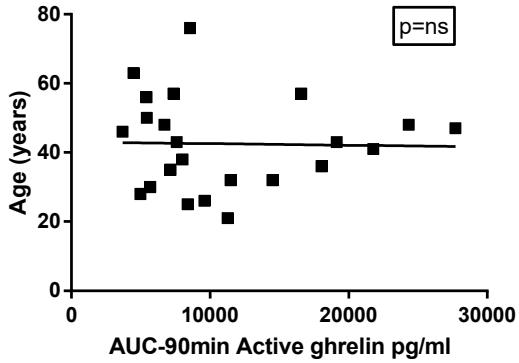
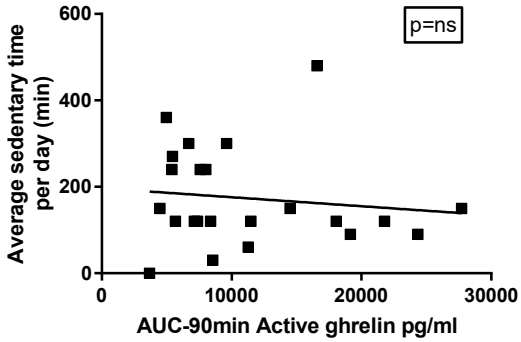
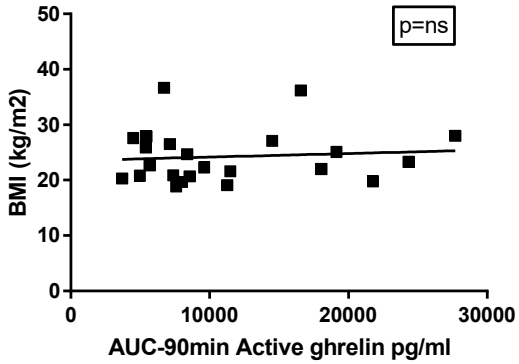
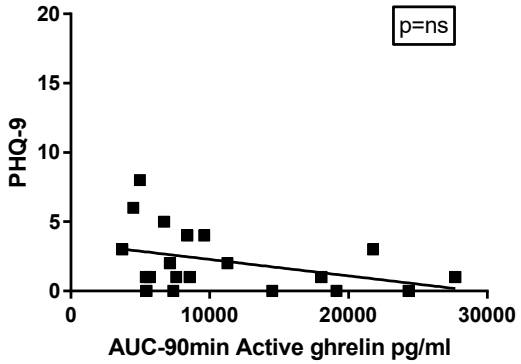


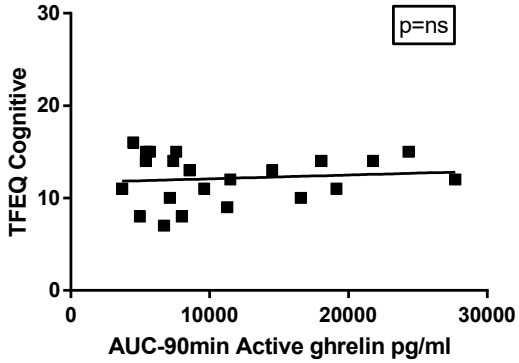
Figure 5.3: Correlation and regression relationships of healthy individuals 90 minute area under the curve C-peptide levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

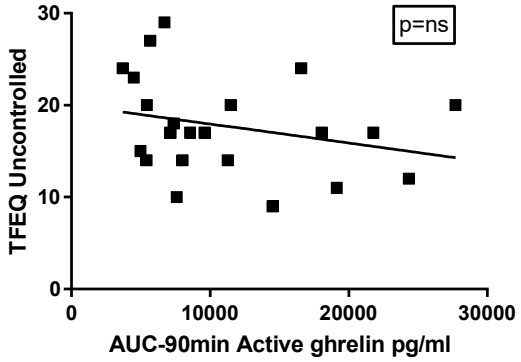












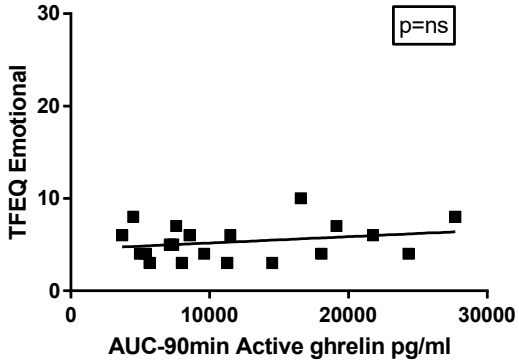


Figure 5.4: Correlation and regression relationships of healthy individuals 90 minute area under the curve active ghrelin levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

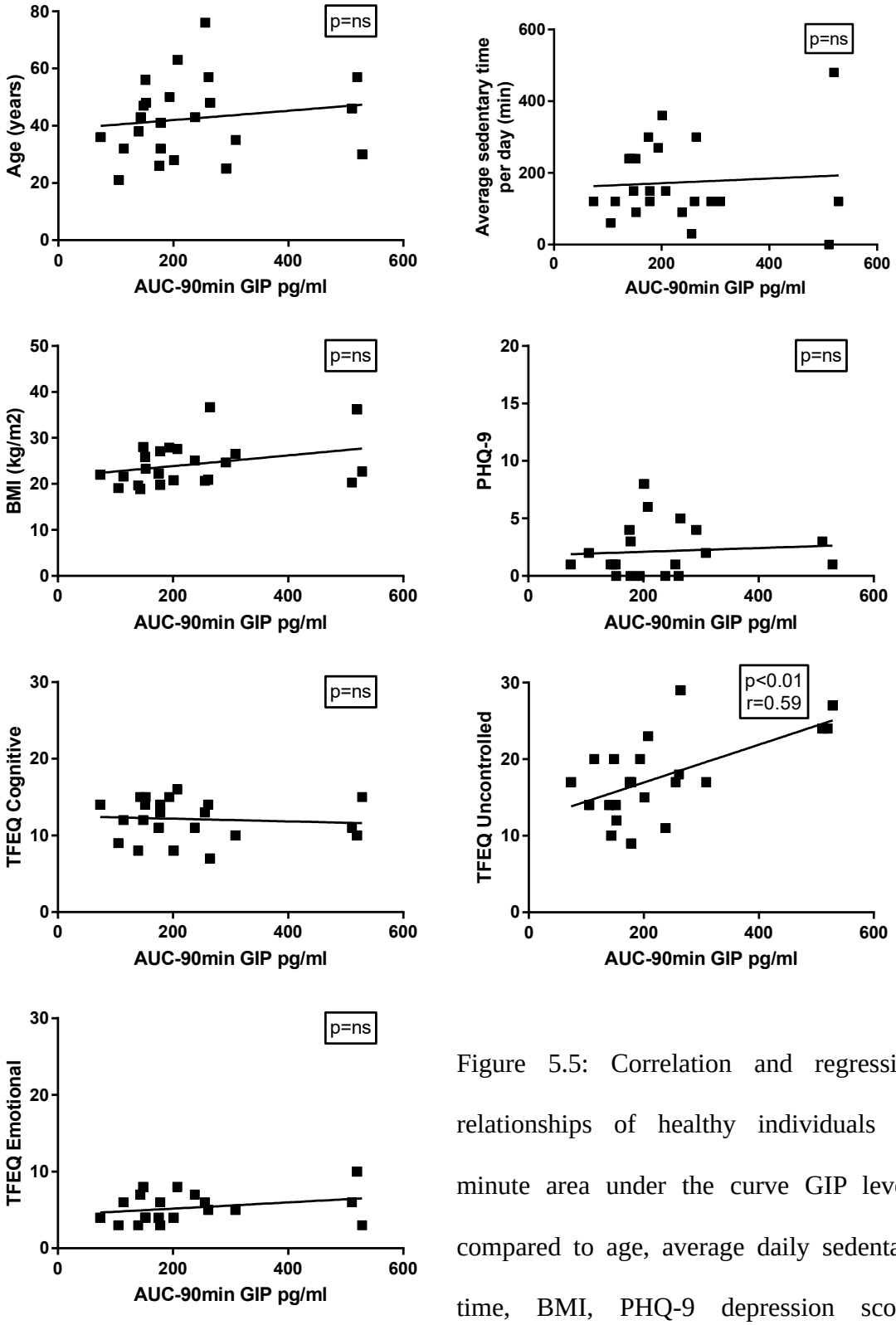


Figure 5.5: Correlation and regression relationships of healthy individuals 90 minute area under the curve GIP levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

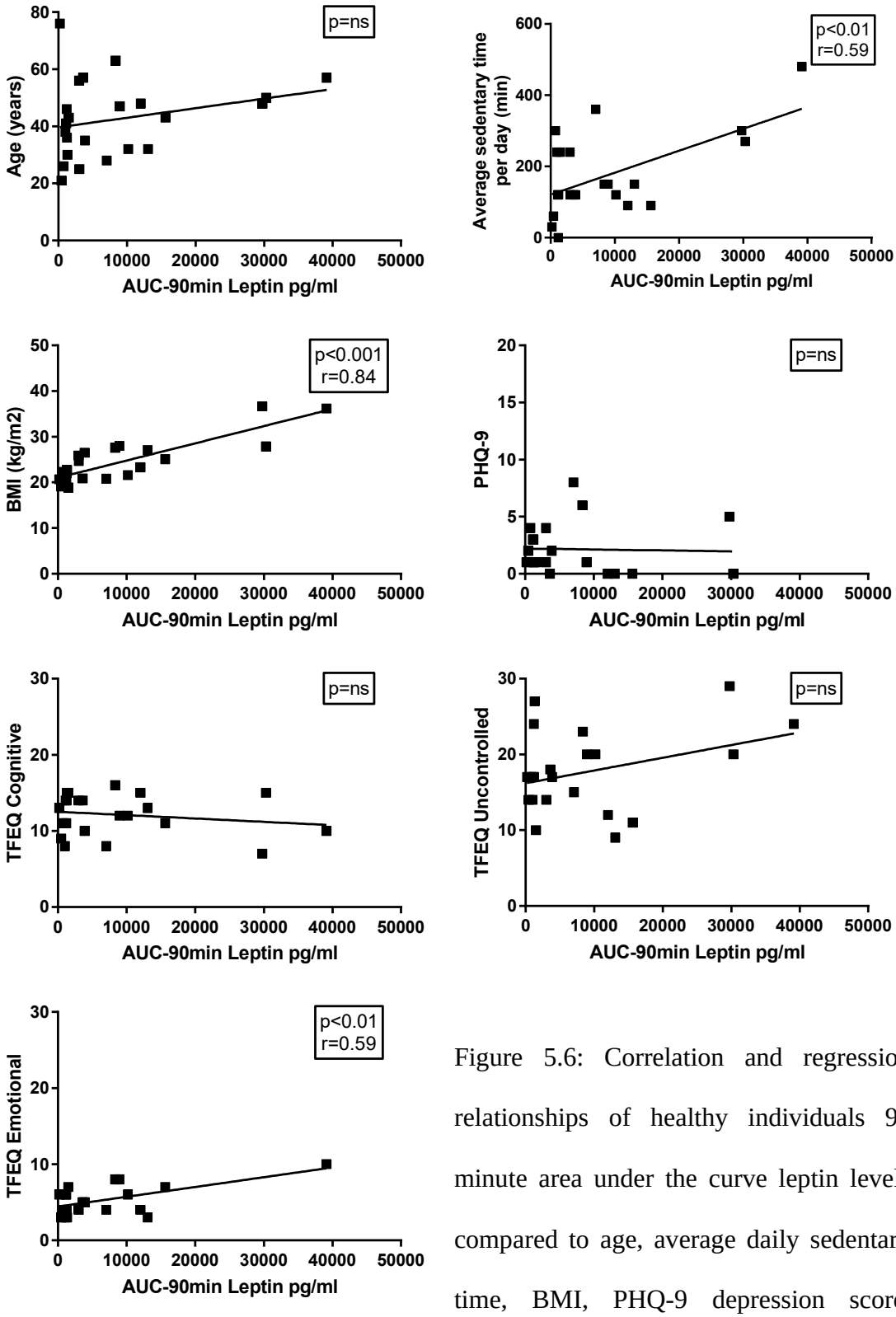


Figure 5.6: Correlation and regression relationships of healthy individuals 90 minute area under the curve leptin levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

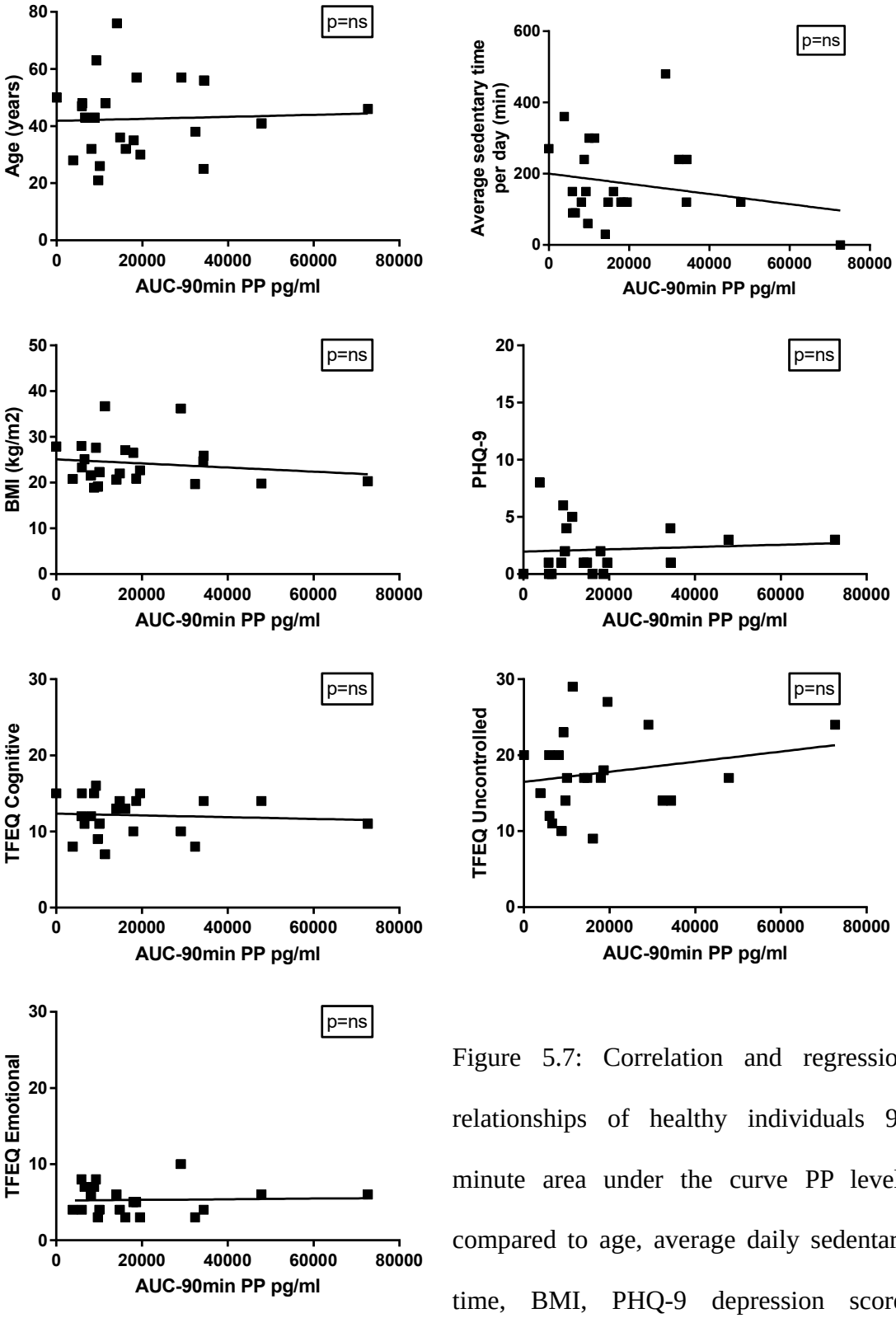


Figure 5.7: Correlation and regression relationships of healthy individuals 90 minute area under the curve PP levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

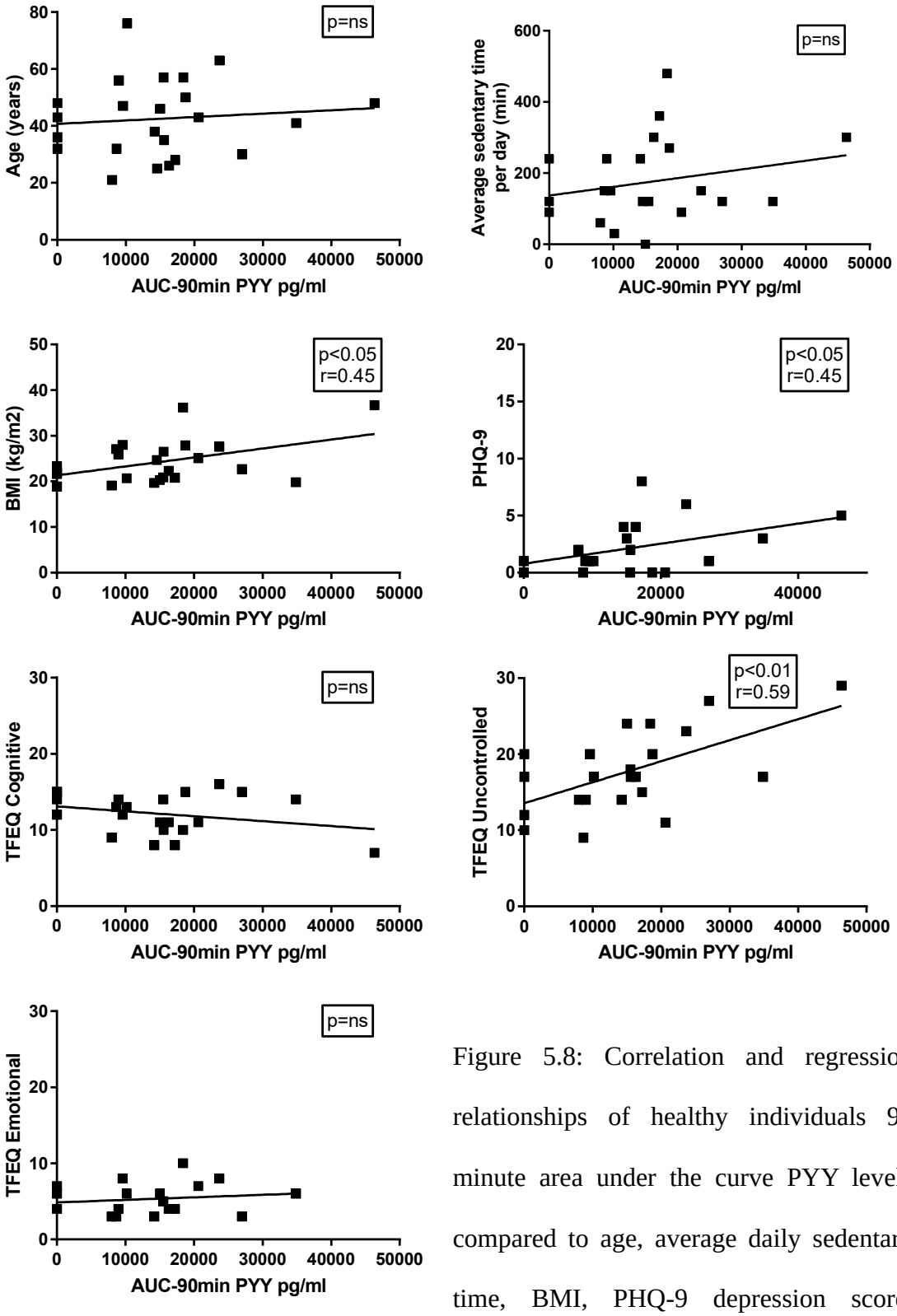


Figure 5.8: Correlation and regression relationships of healthy individuals 90 minute area under the curve PYY levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

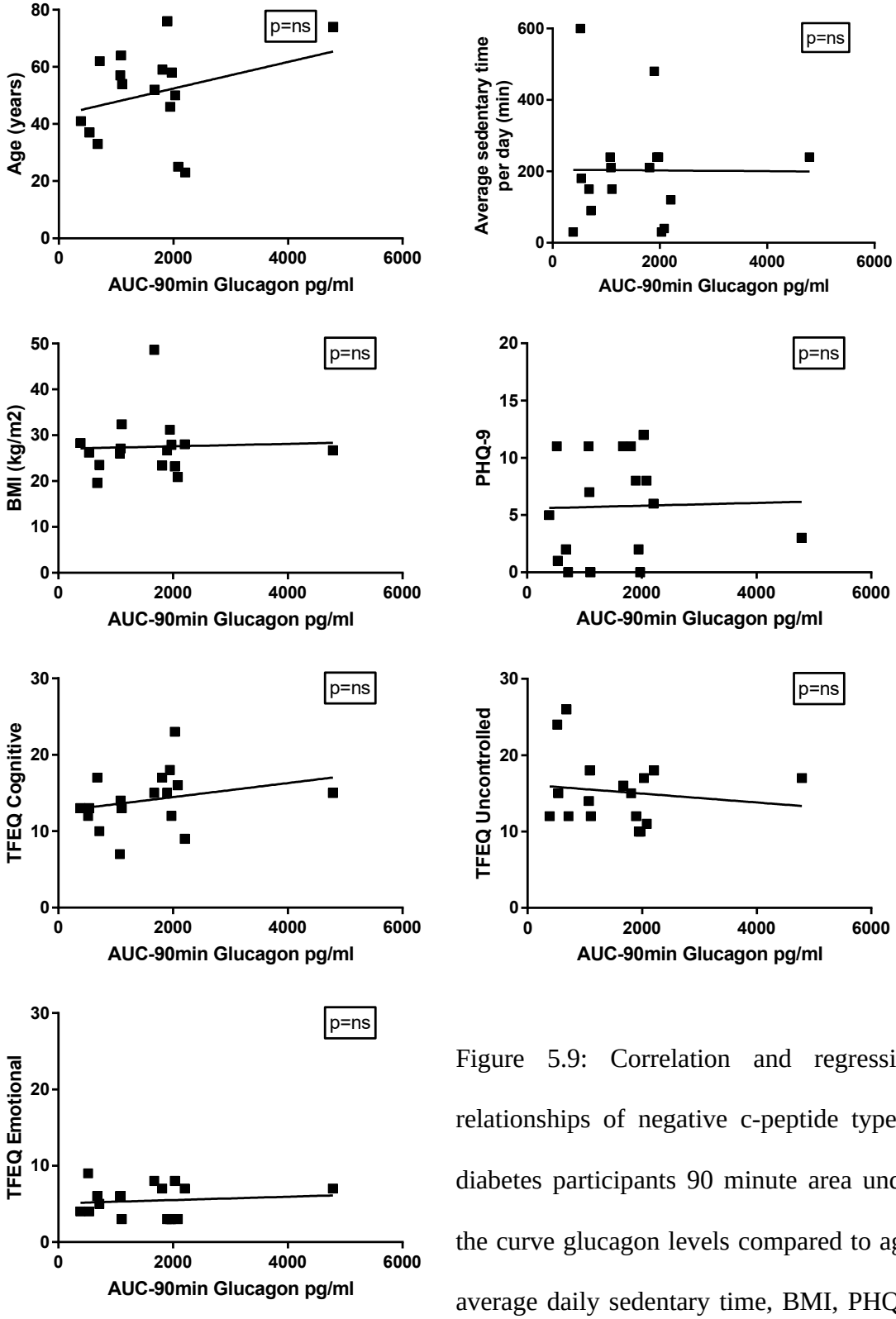


Figure 5.9: Correlation and regression relationships of negative c-peptide type 1 diabetes participants 90 minute area under the curve glucagon levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

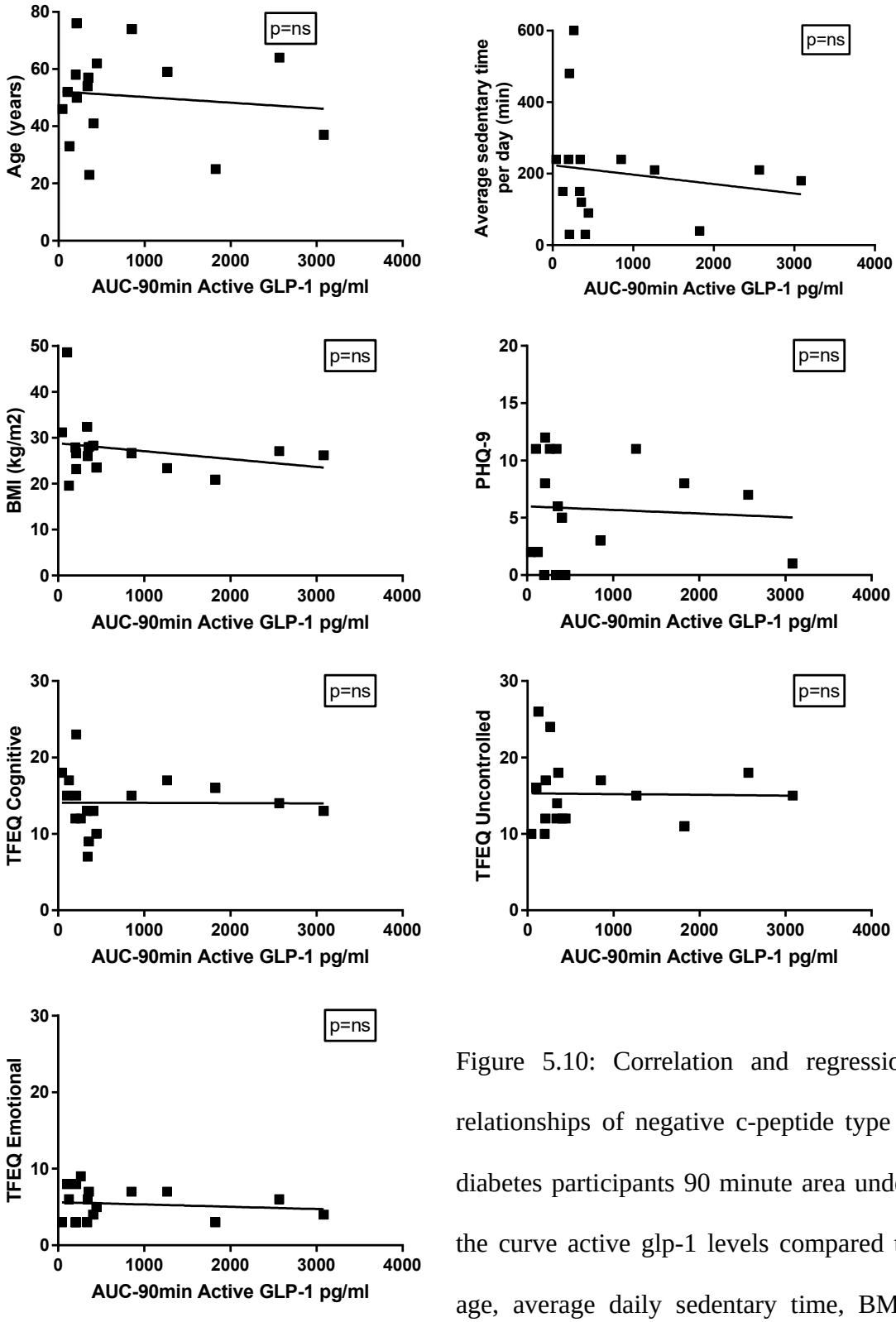


Figure 5.10: Correlation and regression relationships of negative c-peptide type 1 diabetes participants 90 minute area under the curve active glp-1 levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

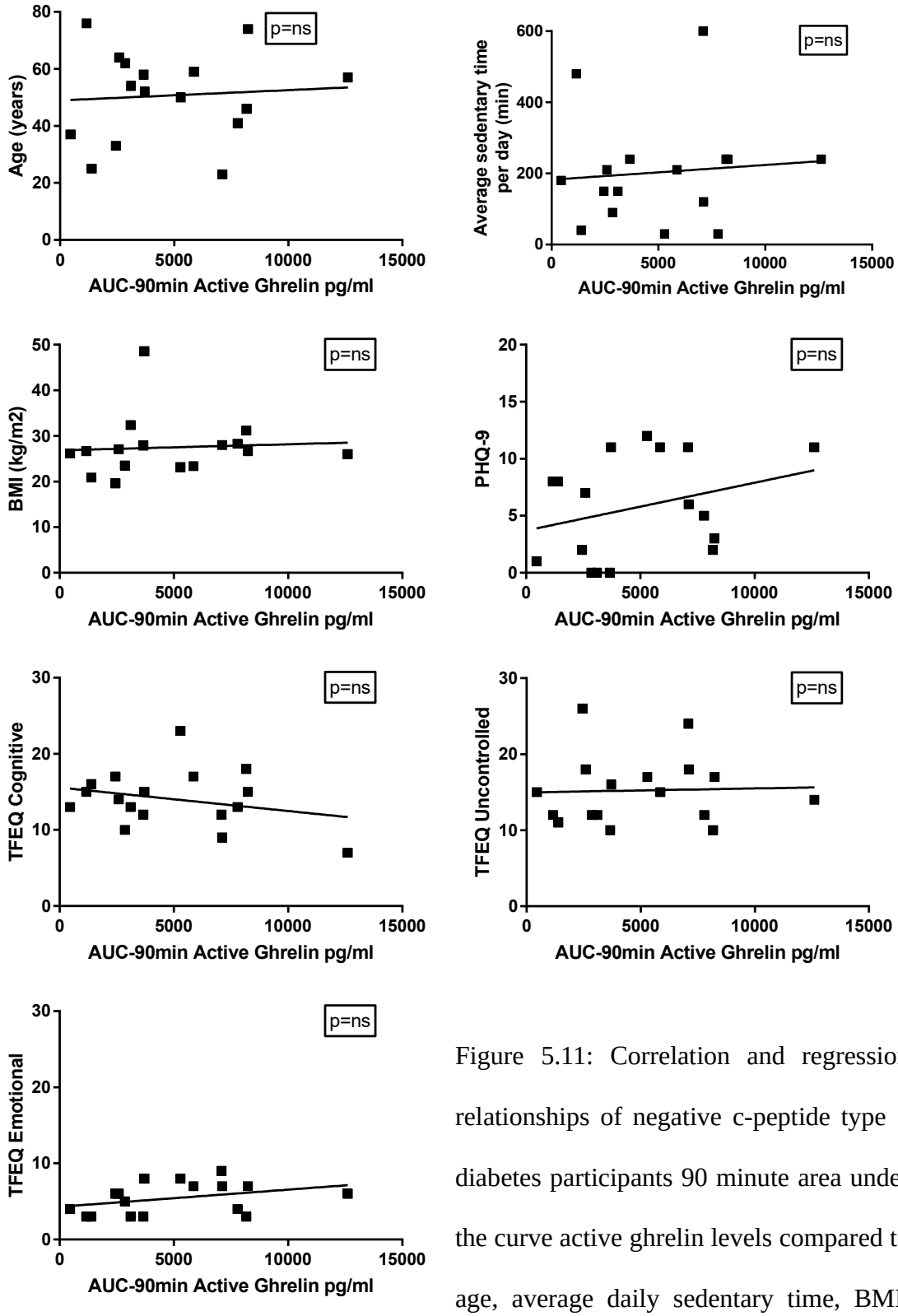


Figure 5.11: Correlation and regression relationships of negative c-peptide type 1 diabetes participants 90 minute area under the curve active ghrelin levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

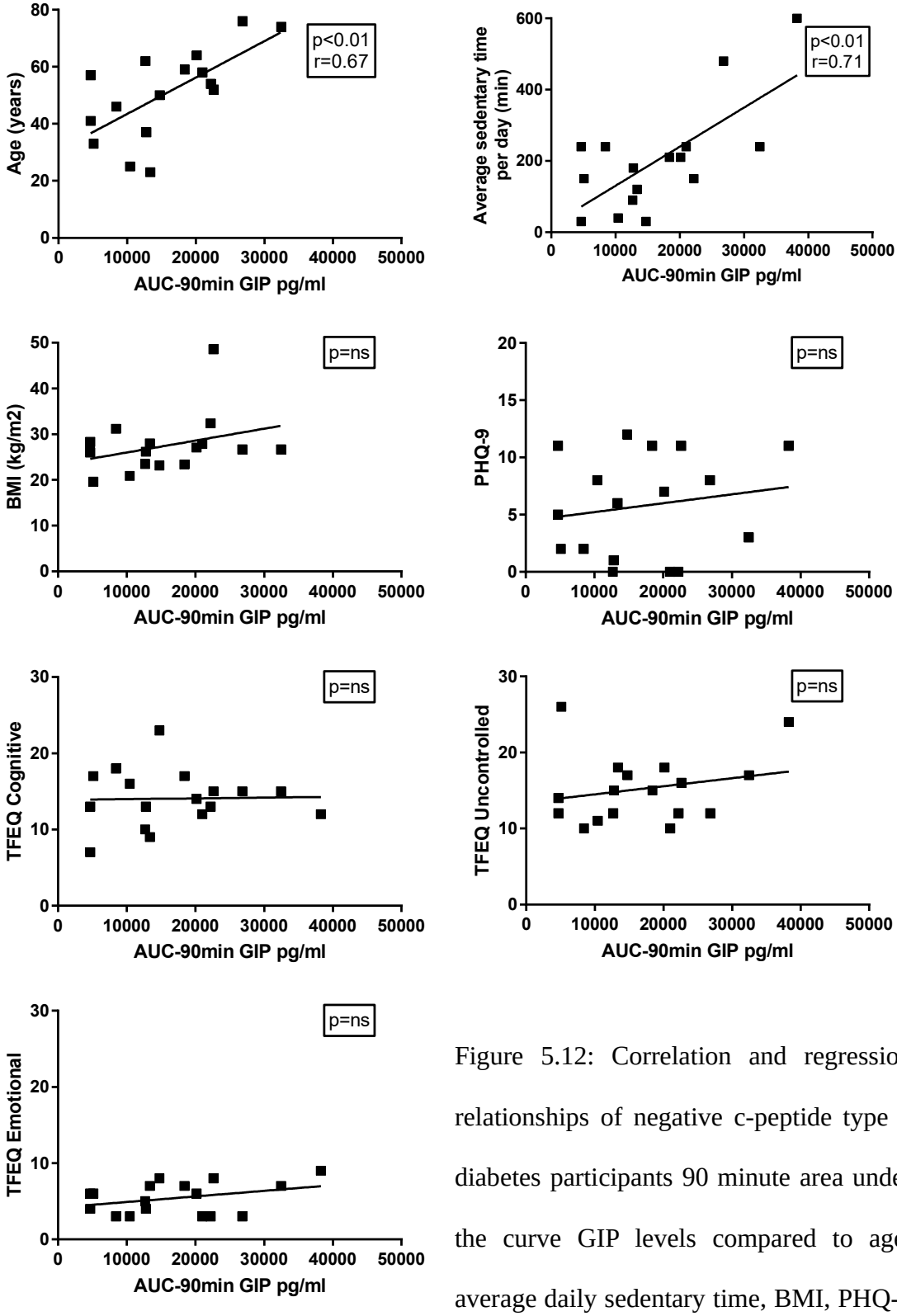


Figure 5.12: Correlation and regression relationships of negative c-peptide type 1 diabetes participants 90 minute area under the curve GIP levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

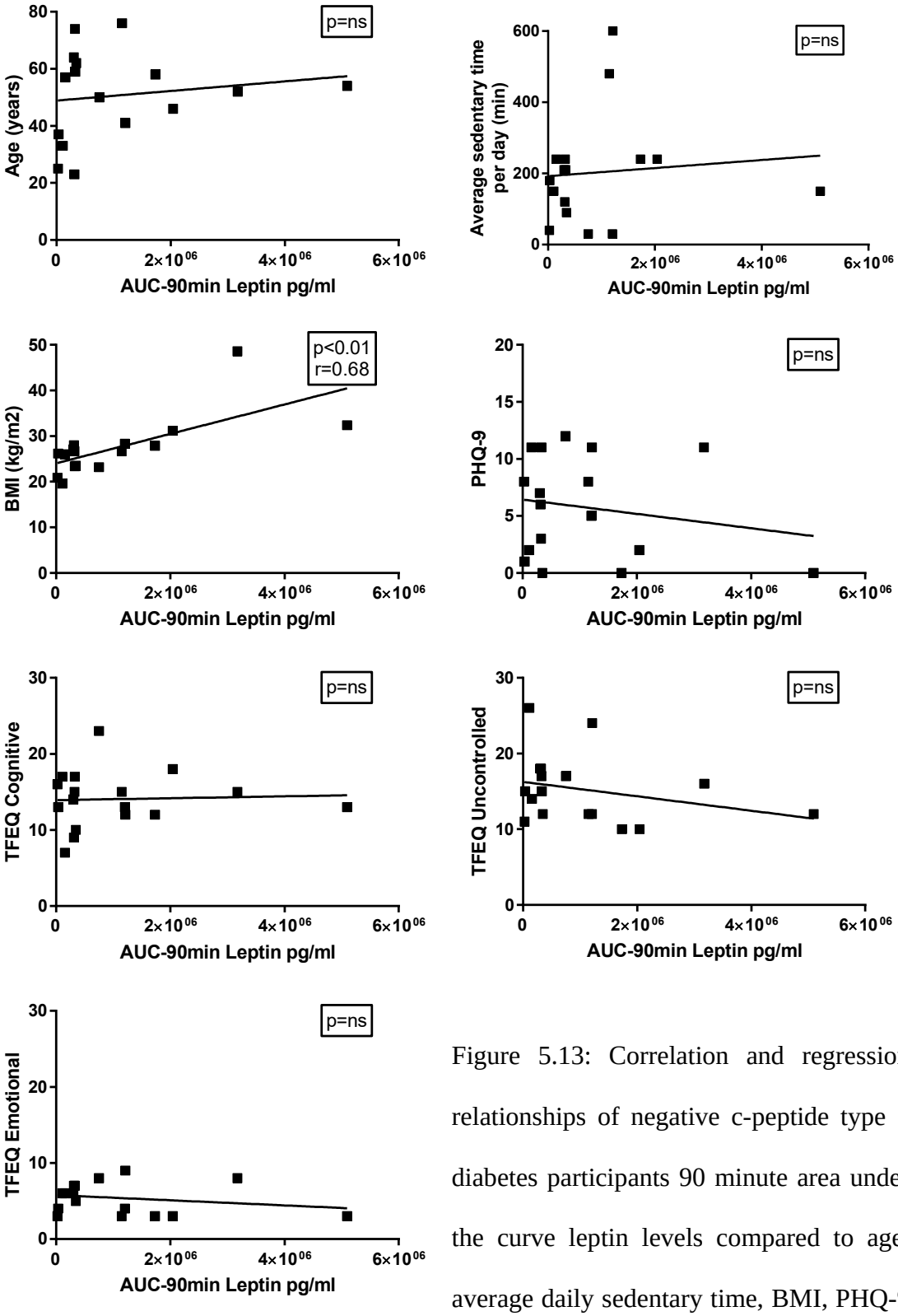


Figure 5.13: Correlation and regression relationships of negative c-peptide type 1 diabetes participants 90 minute area under the curve leptin levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

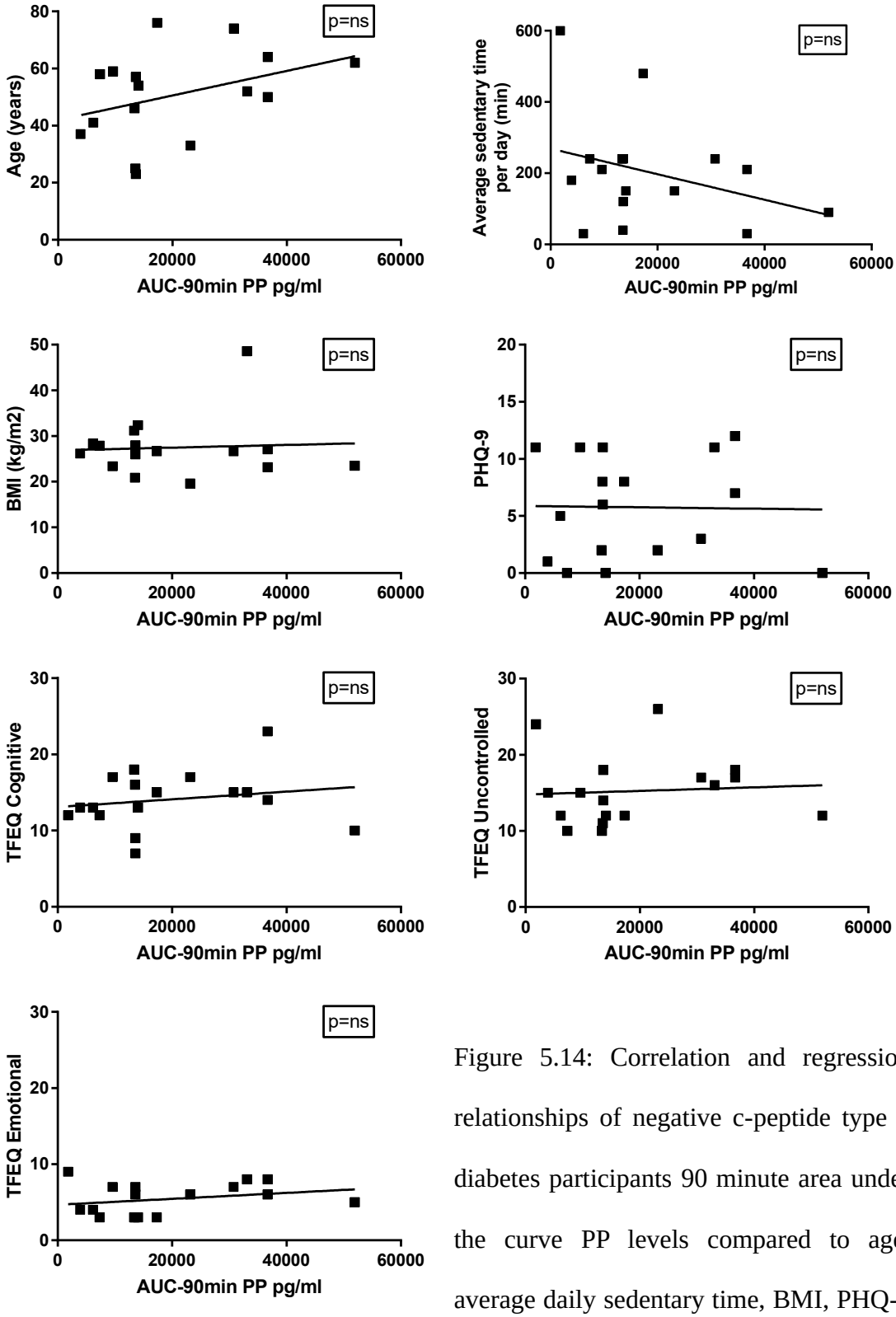


Figure 5.14: Correlation and regression relationships of negative c-peptide type 1 diabetes participants 90 minute area under the curve PP levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

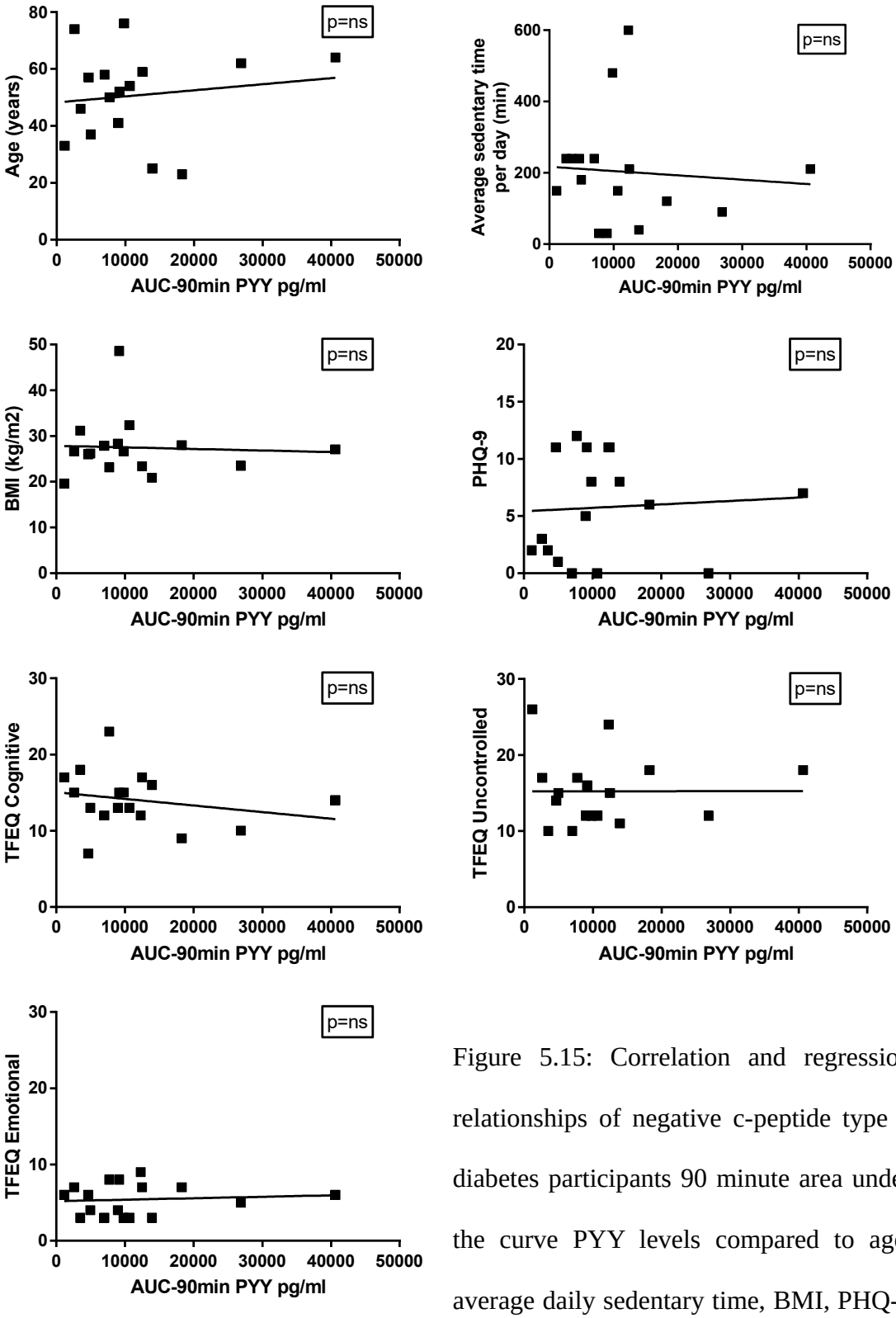


Figure 5.15: Correlation and regression relationships of negative c-peptide type 1 diabetes participants 90 minute area under the curve PYY levels compared to age, average daily sedentary time, BMI, PHQ-9 depression score, TFEQ-cognitive score, TFEQ-uncontrolled score and TFEQ-emotional score. Statistical analysis with significance values (p) and Pearsons correlation coefficient (r) for all statistically significant parameters.

The results from the correlations between lifestyle questionnaire responses from the healthy participants and their 90 minute area under the curve gut peptide results are summarised in table 5.2.

	Glucagon	*GLP-1*	*C-peptide*	*Ghrelin*	*GIP*	*Leptin*	*PP*	*PYY*
Sedentary Time	p=0.24	p=0.17	p=0.46	p=0.57	p=0.74	p=0.003 r=0.59	p=0.34	p=0.27
Age	p=0.66	p=0.38	p=0.43	p=0.92	p=0.48	p=0.22	p=0.85	p=0.66
BMI	p=0.25	p=0.37	p=0.09	p=0.68	p=0.15	p<0.001 r=0.84	p=0.48	p=0.031 r=0.45
PHQ9	p=0.68	p=0.02 r=0.52	p=0.42	p=0.1	p=0.72	p=0.89	p=0.75	p=0.048 r=0.45
TFEQ – cognitive	p=0.58	p=0.45	p=0.28	p=0.63	p=0.70	p=0.41	p=0.75	p=0.21
TFEQ – uncontrolled	p=0.62	p=0.86	p=0.13	p=0.22	p=0.003 r=0.59	p=0.11	p=0.35	p=0.004 r=0.59
TFEQ – emotional	p=0.30	p=0.07	p=0.81	p=0.30	p=0.23	p=0.007 r=0.59	p=0.87	p=0.51

Table 5.2: Summary of significance values (p) and Pearsons correlation coefficient (r) for gut peptides (Area Under Curve 0-90minutes) vs lifestyle questionnaires in healthy controls.

In the healthy controls there were many significant correlations between lifestyle factors and gut hormones. The only strong correlation was between leptin and BMI ($r=0.84$, $p<0.001$). This has previously been described in papers and was anticipated, as the volume of adipose tissue directly influences the levels of leptin secretion [67]. Other moderately strong correlations found were between leptin and sedentary time ($r=0.59$, $p<0.01$). This again would be expected as sedentary time is closely associated with obesity [195]. Leptin also had a moderately strong correlation with emotional eating ($r=0.59$, $p<0.01$), which has not been reported before, but again would be expected as comfort eating is associated with obesity [196]. There was also a moderately strong correlation between uncontrolled eating and PYY ($r=0.59$, $p<0.01$) and GIP ($r=0.59$,

p<0.01). As PYY is a potent appetite suppressing hormone this could suggest that the behaviour of uncontrolled eating may be partially due to either ineffective PYY signalling or psychological over riding of PYY appetite suppressing mechanisms.

The results from the correlations between lifestyle questionnaire responses from the participants with negative c-peptide type 1 diabetes and their 90 minute area under the curve gut peptide results are summarised in table 5.3.

	Glucagon	*GLP-1*	*Ghrelin*	*GIP*	*Leptin*	*PP*	*PYY*
Sedentary Time	p=0.98	p=0.56	p=0.74	p=0.002 r=0.71	p=0.73	p=0.22	p=0.77
Age	p=0.25	p=0.67	p=0.78	p=0.005 r=0.67	p=0.58	p=0.15	p=0.61
BMI	p=0.88	p=0.36	p=0.80	p=0.22	p=0.004 r=0.68	p=0.82	p=0.85
PHQ9	p=0.91	p=0.81	p=0.23	p=0.52	p=0.47	p=0.94	p=0.80
TFEQ – cognitive	p=0.32	p=0.98	p=0.30	p=0.92	p=0.86	p=0.47	p=0.38
TFEQ – uncontrolled	p=0.61	p=0.94	p=0.89	p=0.39	p=0.27	p=0.78	p=0.99
TFEQ – emotional	p=0.67	p=0.62	p=0.16	p=0.18	p=0.40	p=0.30	p=0.73

Table 5.3: Summary of significance values (p) and Pearsons correlation coefficient (r) for gut peptides (Area Under Curve 0-90minutes) vs lifestyle questionnaires in subjects with negative C-peptide type 1 diabetes.

Our expectation was that results would probably be very similar if type 1 diabetes did not influence gut hormone levels. In the population of individuals with negative C-peptide levels type 1 diabetes, there was a moderately strong correlation between leptin and BMI (r=0.68, p<0.01). This was similar to that seen in healthy controls. However, unlike the healthy controls there was no correlation between leptin and emotional eating factors or sedentary time. This was surprising as we would have expected similar results for both hormones in the healthy controls and individuals with negative C-peptide type

1 diabetes. A possible explanation could be that the population size was too small to detect this correlation. An alternative explanation could be that eating behaviour and sedentary time do not correlate to leptin levels in individuals with negative C-peptide type 1 diabetes, due to either confounding factors such as exercise and dietary advice or even the disease process.

There were other new significant correlations in the population of individuals with negative c-peptide type 1 diabetes. There was a strong correlation between GIP and sedentary time ($r=0.71$, $p<0.01$). This correlation was unexpected, but there have been reports that GIP levels are influenced by physical activity [197]. Alternatively this correlation with GIP could represent an as of yet unexplored adaptive response to the pathogenesis of type 1 diabetes. To better understand this correlation between GIP levels and sedentary time, a future study may consider comparing markers of physical activity (e.g. actigraphs, pedometer, international physical activity questionnaires) and physical fitness (e.g. Metabolic Equivalents of Task, VO2Max, multi-stage fitness test) with GIP levels in individuals with type 1 diabetes.

The other moderately significant correlation was between GIP and age ($r=0.67$, $p<0.01$). This again, was unexpected and has not been previously reported in type 1 diabetes. This may indicate that GIP could be a potential marker of ageing or alternatively, this could be due to age being an uncontrolled confounding factor that occurred due to inadequate matching of the cohorts. To explore this further age matched individuals with negative C-peptide type 1 diabetes could undergo measurements of known biomarkers of ageing (e.g. advanced glycocylated end products, Insulin Growth Factor-1, hand grip strength) and common age related confounding factors (e.g. dietary intake,

exercise, bone health and adrenal function) to see if statistically robust correlation with GIP levels exist.

5.4 Discussion

The method used to explore the influence of type 1 diabetes on the correlations between lifestyle factors and gut peptides involved multiple statistical comparisons. The use of multiple statistical comparisons increases the likelihood of a statistically significant difference occurring by chance and thus, potentially increase the number of false positive results. In the analysis of these results I have focused only on results with a probability value of <0.01. This is because there are potentially 105 independent statistical comparisons which could potentially produce 6 false positive results with a p value of 0.05, but only 1 with a p value of 0.01. However, larger studies of adequately matched participants would be needed to ensure these results have not arisen by chance or confounding.

The gut hormone level results of samples collected from participants of the CDRD study were compared to the lifestyle questionnaire responses and anthropometric data that was gathered during their meal stimulated test. It was anticipated that lifestyle factors such as sedentary time, BMI, age, depression and eating behaviour may influence gut hormones in type 1 diabetes. In particular we expected to see at least a moderate effect with the hormones related to adiposity and satiety. Results from this study confirmed that the adipose derived leptin hormone is strongly correlated with body mass index in individuals with type 1 diabetes and healthy controls. However, new and unexpected moderately strong correlations were found in the group of individuals

with negative C-peptide type 1 diabetes, such as between sedentary time, age and GIP. These new correlations would require further research to confirm statistical significance before determining the mechanisms of action.

6. EXPLORE THE HISTOPATHOLOGICAL EFFECT OF TYPE 1 DIABETES ON GLUCAGON SECRETING CELLS IN THE PANCREAS.

6.1 Introduction

The chronic autoimmune process of type 1 diabetes is traditionally described as a process of cellular infiltration and inflammation in the islets which leads to beta cell death. The endocrine cells of the islets are derived from a common pluripotent cell line that, during embryological development, undergoes differentiation into alpha, beta, delta, epsilon and PP cells [198]. Stem cell research has enabled us to understand this process of differentiation and has facilitated researchers to reprogram alpha cells into beta cells [199]. We therefore wished to explore the effect of duration of type 1 diabetes on the expression of glucagon stained cells in the islets.

6.2 Method for image analysis of glucagon secreting cells in the pancreas

The Juvenile Diabetes Research Foundation network for Pancreatic Organ donors with Diabetes (JDRF nPOD) is an online searchable database of immunohistochemically stained slide samples taken from donors with diabetes. The database was accessed and searched for slides taken from donors with type 1 diabetes that had been stained for glucagon on 4th January 2014. Each donor slide was visually inspected and islets from each slide were localised. The dimensions of each islet were measured using the JDRF nPOD online software that is provided with the database. A JPEG (Joint Photographic Experts Group) image was then taken of four islets from each slide and then the largest islet by area was selected for analysis (see figure 6.1). The largest islet jpeg image was

then opened and analysed using the free open source software "GNU Image Manipulation Program (GIMP)" version 2.8 (www.gimp.org). Image analysis involved cropping the image to the maximum height and width of the islet. The number of pixels for each image was recorded, before the software digitally subtracted all pixels outside of the islet from the image leaving the islet on a blank background. The number of pixels remaining was recorded in order to calculate the actual islet area. The image was then polarized to differentiate between the blue and brown of the immunohistochemical staining. The blue from the image was digitally reduced and then subtracted from the image. Finally the image was converted to grey scale and the contrast increased to allow calculation of the non white area of the image (see figure 6.2). This area equates to the area originally stained brown, indicating glucagon secreting cells, as well as cell nuclei. (See Appendix 3 for further details.)

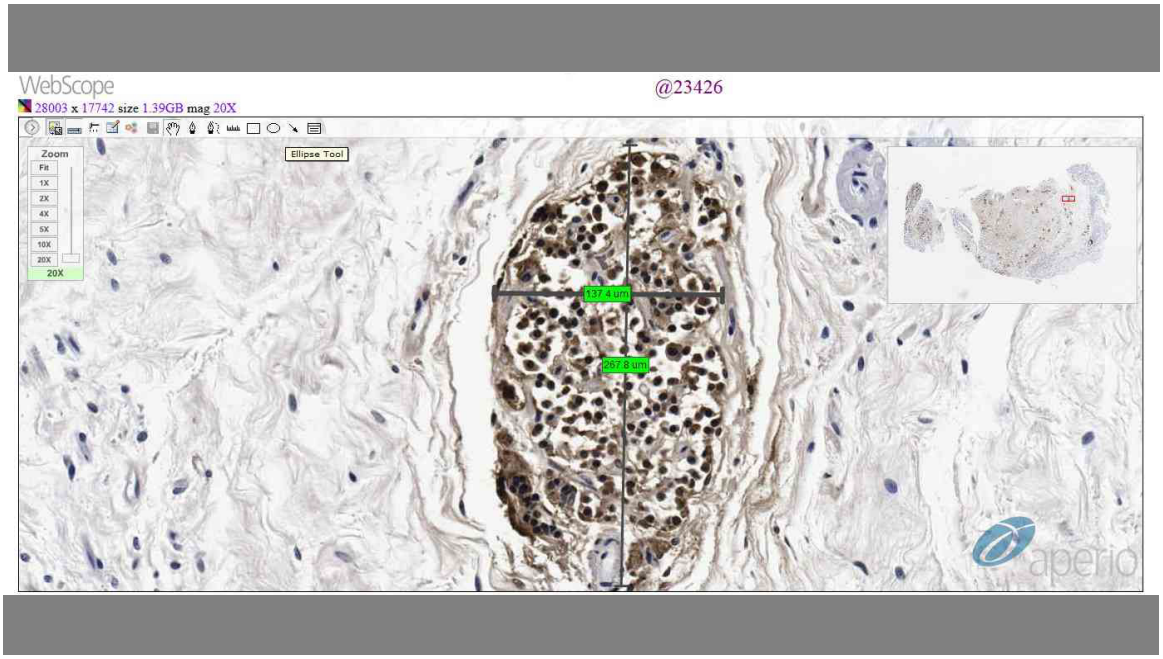


Figure 6.1 Image of a typical islet selected for further analysis from the JDRF nPOD web database.

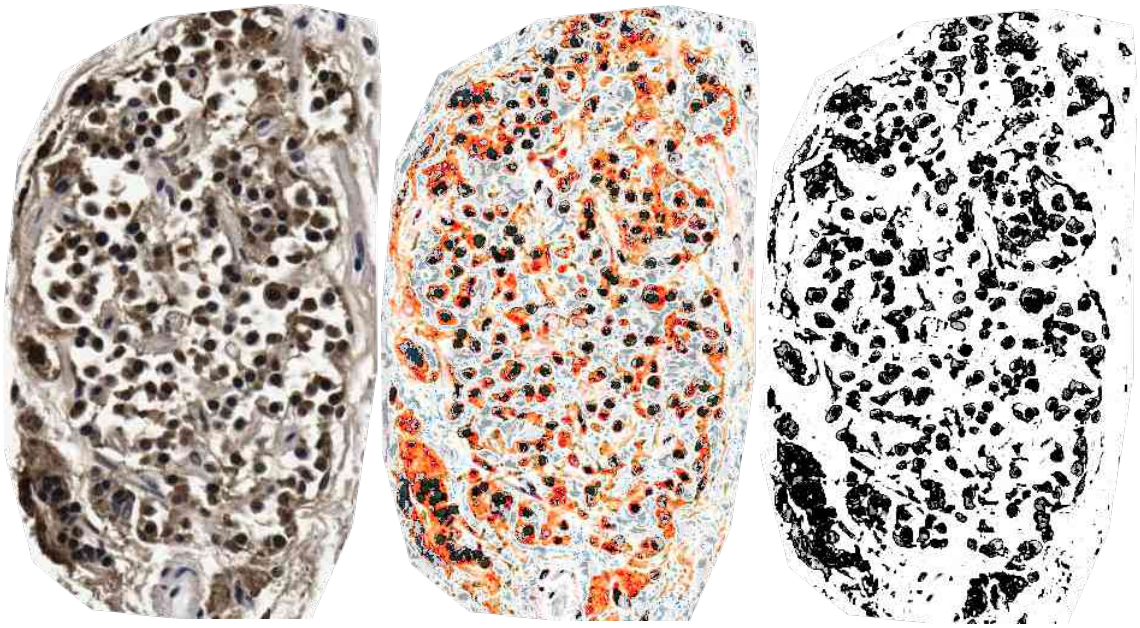


Figure 6.2 Images of a selected islet that has undergone digital manipulation to highlight the area stained for glucagon, before being turned to monochrome whereby the black pixels can be recorded in order to approximate the area of glucagon secreting cells.

6.3 Results of image analysis of glucagon secreting cells in the pancreas

The JDRF nPOD database at the time of access only contained 16 donor slides that had been stained for glucagon. All donors had type 1 diabetes and negative C-peptide levels, suggesting the absence of insulin secreting beta cells. There were no healthy donor slides available in the database that could be used as control samples. Each donors demographic details were recorded (see table 6.1). The results of the image manipulation were compared to age, body mass index (BMI) and duration of diabetes using Graphpad Software. There was one significant correlation detected between age of the donor and the area of glucagon containing cells ($r=0.50$, $P<0.05$).

Case ID	*Diabetes Duration*	*Age*	*Sex*	*BMI*	*Islet Area (μm²)*	*% glucagon*	*Area of glucagon (μm²)*
6036	34	49	f	25	41624	56.3	23434
6038	37	37	f	31	19148	30.3	5802
6035	28	32	m	27	42051	10.3	4331
6032	n/a	33	m	29	9558	37	3536
6033	28	40	m	24	12564	37.9	4762
6031	35	39	m	24	9992	30.5	3048
6026	14	22	m	24	17488	51.7	9041
6025	19	23	m	27	10344	17.1	1769
6128	31	34	f	22	4081	51.5	2102
6113	1	13	f	24	25177	22.4	5640
6071	17	28	f	19	31698	37.7	11950
6046	8	19	f	25	20598	3.1	639
6045	8	26	m	23	24652	17.5	4314
6042	59	60	f	23	35307	28.7	10133
6040	20	50	f	32	23361	44.8	10466
6039	12	29	f	23	6477	50.8	3290

Table 6.1 Demographics of pancreatic donors with type 1 diabetes that underwent immunohistochemical staining for glucagon.

	Diabetes Duration	*Age*	*BMI*
Islet Area (μm²)	ns	ns	ns
% glucagon	ns	ns	ns
Area of glucagon (μm²)	ns	$p<0.05$ $r=0.50$	ns

Table 6.2 Significance value (p) and Pearsons correlation coefficient (r) for islet area and area stained for glucagon vs duration of diabetes, age and BMI for JDRF nPOD donors with type 1 diabetes. Legend: ns means $p>0.05$.

6.4 Discussion of image analysis of glucagon secreting cells

The JDRF nPOD database is an easy to use and important searchable database of donors with type 1 diabetes. The database also enables slides to be downloaded as image files

to undergo image manipulation. Unfortunately, despite its large repository, it only contained 16 donor samples that were stained for glucagon at the time of access. The database also did not contain any healthy controls that had slides stained for glucagon, which limited the statistical power of the database to determine changes in area of glucagon staining cells. Despite these limitations the database is an important expanding international resource that provides researchers with access to pancreatic slides of donors that would not otherwise be readily available.

The method used to manipulate the images in this research was developed by Dr David Hughes (author of this thesis), but is based on techniques used in digital photographic enhancement. The software provided by the website did not allow advanced image manipulation and so other software was required. Image manipulation software such as Microsoft Paint (Redmond, Washington, USA) and ImageJ (Opensource) were explored, but did not have the functional ability to polarize colours to facilitate digital subtraction. The opensource software 'GNU image manipulation program' (GIMP) was freely available and included advanced image manipulating functions that are normally found in more expensive commercial products. The method of image manipulation was refined before reaching the final method used in this research (Appendix 3). The method was not without imperfections as can clearly be demonstrated by its inability to digitally subtract nuclei from the image. This imperfection does introduce a confounding factor in the analysis.

The demographics of the donors in the JDRF nPOD database turned out to be skewed towards those with longer duration of type 1 diabetes, with only one donor having type 1 diabetes for under 2 years. This uneven distribution in demographics limits our ability

to explore the effect of diabetes duration on glucagon secreting cells. The analysis of our results suggests that there is no correlation between the duration of diabetes and the size of the islets or the area stained for glucagon. This could indicate that the area of glucagon stained cells remains constant despite the duration of type 1 diabetes, but due to the limited sample size all conclusions should be made with caution. However, a moderate correlation between area stained for glucagon and age ($r=0.50$; $p<0.05$) was detected. This correlation may indicate that the ageing process in individuals with type 1 diabetes may be associated with an expansion in numbers (or size) of glucagon stained cells. However, due to the limited sample size available it would be prudent to repeat the analysis when a larger cohort becomes available. Hopefully, as the database expands it might be possible to compare glucagon stained areas in healthy controls to those with type 1 diabetes.

7. CONCLUSION

Research into type 1 diabetes has rapidly progressed over the last 100 years leading to important discoveries that have reduced mortality and morbidity rates. Nevertheless, there is still much more work needed in order to bring life expectancy in line with that of the general population. An obesity epidemic has helped by fuelling the investment into type 2 diabetes research which has expanded our understanding of the hormonal networks involved in glucose homeostasis. Whilst these hormones have been extensively researched in type 2 diabetes, there remains a lack of comprehension for their role in type 1 diabetes.

Insulin has been the only effective treatment for type 1 diabetes for nearly a century. For a similar length of time glucagon administration has been known to treat hypoglycaemia by raising blood glucose levels. It is becoming apparent that manipulation of other gut hormones may have a role in treating type 1 diabetes. For instance, our knowledge of glucagon has expanded significantly and it is now known that shortly after the consumption of food there is an initial increase in glucagon stimulation of glycogenolysis and the release of glucose. This new understanding and its clinical impact on glycaemia has led to clinical trials of glucagon suppressing agents such as GLP-1 analogues and DPP-4 inhibitors being used in individuals with type 1 diabetes to control blood glucose levels. The outcomes of these trials have admittedly been mixed and larger trials that are ongoing may open the gateway for novel treatment options.

It is also known that the ingestion of food causes the release of the gut derived incretin hormones (GLP-1 and GIP). These incretin hormones are known to potentiate insulin

release in a glucose dependent manner in healthy individuals. In type 2 diabetes this mechanism of action can be lost and thus drugs are used in type 2 diabetes to reinstate the incretin effect to aid in glycaemic control. Recent large clinical trials of GLP-1 analogues such as the LEADER study have shown that they may have a role to play in reducing the progression of cardiovascular disease in type 2 diabetes [137]. Their cardiovascular protective and glycaemic utility in type 1 diabetes remains largely unknown. GIP is another incretin hormone that has a significant impact in type 2 diabetes with GIP resistance now understood to be a key part of the pathophysiological process leading to insulin resistance. GIP's importance may not be limited to type 2 diabetes, but in nearly all types of diabetes. To dates, studies in to the role GIP plays in the pathophysiology of type 1 diabetes are lacking.

Ongoing research in type 2 diabetes has revealed the importance gut hormones have in the hypothalamic control of appetite and satiety [200], [201]. Studies into appetite have demonstrated that insulin and ghrelin significantly stimulate the sensation of hunger, whilst PP, leptin and PYY can inhibit it. The hypothalamic centre for the control of appetite is also influenced by connections to the limbic system that controls mood, as well as connections from the hind brain that control behaviour. The interplay between mood, eating behaviour, physical activity and gut hormones is considered an essential part of an individuals daily glucose homeostasis. In type 2 diabetes this interplay, along with the effect exogenous insulin has on appetite, is clinically well acknowledged as having a significant impact on an individuals weight. However, the same interplay and effect on weight in type 1 diabetes is relatively unexplored.

Chronic inflammation in the Islets of Langerhans is a hallmark feature of type 1

diabetes that ultimately leads to beta cell death. The chronic inflammatory process is mediated via a variety of monoclonal antibodies that target islet related antigens. The trigger for this process is related to the interaction between an individuals genetic and environmental susceptibility. Currently frontiers of research are expanding the role of immuno-modulating therapy in order to reduce the inflammatory process and preserve functional beta cells. However, these therapies only slow the loss of insulin secreting beta cells and do not result in their regeneration. To reverse type 1 diabetes either a transplant of beta cells must occur or a means of generating new beta cells. Recent research suggests that endocrine islet cells are derived from a common pluripotent cell line [198], [202]. This could mean that there may be a possibility of reprogramming alpha cells into functioning beta cells. If this was to be a success, an ample supply of alpha cells would need to be present in an individual with type 1 diabetes regardless of its duration. To further our understanding of the effects of type 1 diabetes, this thesis has explored the role of gut hormones and attempted to quantify alpha cell numbers. To do this it was important to break our aims down into a series of manageable objectives.

One of the initial key objectives was to develop a means of recruiting participants. It was decided that this would be best achieved through a network of research studies. This was developed by collaborating with researchers from the ExTOD study and through the establishment of the CDRD study. The CDRD study was funded by a research grant from Novo nordisk and was initially set up as a biological banking and questionnaire study. This involved producing a workable protocol and securing appropriate regulatory approval. Subsequently, a substantial protocol amendment was made to facilitate the set up of a gut hormone sub-study to enable the collection and analysis of samples. Finally, the protocol and structure of the study was adapted further

to enable it to be established as a multi-site study. The process from inception to the refinement of the CDRD study at multiple sites was managed by Dr David Hughes (the author of this thesis).

Once a means of recruiting participants was established, it became important to ensure there was uniformity in collecting samples from participants from the both the ExTOD study and CDRD gut hormone sub-study. Therefore the next main objective was to develop valid methods for collecting and measuring gut hormones. It was well recognised that certain gut hormones degrade rapidly if they are not collected with appropriate enzyme inhibitors, but there was a lack of clarity in regards to gut hormone assays. Initially it was decided to only use participants recruited at the Queen Elizabeth Hospital Birmingham site for this subsecton of the study and that they should all undergo the same standardised meal stimulated test. Comparisons were made between collection methods and a variety of assays were compared. Attempts to optimise the assays function came to many frustrating dead ends and the process took longer than was anticipated to complete. However, the final outcome was a reproducible process that we were confident has accurately and reproducibly measured gut hormone levels. In retrospect, I now recognise that with more time and planning a more robust methodology could have been utilised to determine the optimum means of collecting samples and measuring gut hormones. Our chosen sample collection method that yielded the highest concentrations of measurable gut hormones consisted of using two separate gut hormone collection tubes: 1) containing DPP-4 inhibitor and aprotonin for glucagon sample collection and 2) containing DPP-4 inhibitor, AEBSF and protease inhibitor cocktail for all other gut hormone sample collection. Once samples had been collected they were stored at -80°C prior to being processed. Our final choice of assays

used to process these samples were: Millipore's human metabolic hormone panel for active ghrelin, GIP, leptin, PP, PYY and c-peptide; Mercodia's glucagon assay and Millipore's high sensitivity active-GLP-1 assay.

All collected samples were processed on the selected assays by Dr David Hughes (author of this thesis) according to the manufacturers recommended protocol. The analysis of these results revealed that the plasma concentrations of GIP, leptin, PP, PYY and active-GLP-1 were no different in individuals with type 1 diabetes compared to healthy controls. However, the plasma concentrations of glucagon, active ghrelin and PYY were different in type 1 diabetes. Stimulated glucagon levels were found to be significantly higher in new onset type 1 diabetes, but then returned to levels similar to those of healthy individuals as duration of diabetes increased. Elevated stimulated glucagon levels in early type 1 diabetes lends some weight to the hypothesis that in early stages of type 1 diabetes, alpha cells undergo a process of hypertrophy. It could also point to a more focused role for the use of glucagon suppressing pharmacotherapy in the initial years after diagnosis of type 1 diabetes.

Fasting and stimulated active ghrelin plasma concentrations were found to be lower in individuals with type 1 diabetes compared to healthy controls with a trend toward a decrease in levels of ghrelin with the duration of diabetes. There is no current explanation for the lower plasma concentrations of active ghrelin in type 1 diabetes. One could hypothesise that the lower concentrations are due to a isocaloric dietary change that occurs shortly after diagnosis. These changes would need to be isocaloric as the lower active ghrelin concentrations present in patients with type 1 diabetes did not appear to correlate with BMI or eating behaviour.

The fasting PYY plasma levels, but not stimulated PYY levels, were higher within the first 2 yrs duration of type 1 diabetes compared to healthy individuals. These elevated levels then declined to levels similar to healthy individuals as duration of diabetes increased. These elevated fasting PYY levels seen in early onset type 1 diabetes could merely have occurred through chance or possibly by confounding influences on our data (e.g. age). The data from healthy individuals suggest that PYY correlates to ageing and uncontrolled eating behaviour. As eating behaviour data was not collected from ExTOD study participants we are unable to determine the impact this has on recent onset type 1 diabetes. However, data from individuals with negative c-peptide type 1 diabetes showed no significant correlations between PYY and either ageing or eating behaviour.

Results from other studies have shown that oral glucose stimulates an initial 20 minute paradoxical rise in plasma glucagon concentrations, which is not present when the same quantity of glucose is give intravenously. We hypothesised that this paradoxical rise in glucagon levels could be mediated by other gut hormones. However, the analysis of the initial 30 minute change (Δ30 min) in glucagon levels did not identify a sole gut hormone that significantly correlated across each individual cohort population. Moderate correlations for GLP-1, PP and ghrelin were identified, but in different participant groups. When the cohorts were combined then only ghrelin significantly correlated with Δ30 min in glucagon level but with a weak strength (r=0.3). The lack of correlation between glucose and c-peptide with Δ30 min in glucagon suggest that glucagon may initially be controlled through a non-glucose, non-insulin dependent mechanism, possibly originating from within the gut. Altogether, these results pose the hypothesis that the initial 30 minutes paradoxical rise in glucagon may be due to

glucagon being released from a sub-population of glucagon secreting enteroendocrine cells located in close proximity to the ghrelin secreting cells in the stomach.

The next key objective was to explore the effect of the loss of endogenous insulin in type 1 diabetes on correlations between factors that influence appetite and gut hormone concentrations. Results from ours and other studies confirm the significance of leptin as a biomarker of sedentary lifestyle, body mass index and emotional eating in the general population. Our results also show that leptin is an important biomarker of body mass index in individuals with type 1 diabetes. Also new correlations between uncontrolled eating behaviour and stimulated GIP and PYY were found in healthy volunteers, but not in those with type 1 diabetes, although GIP did correlate with sedentary lifestyle and ageing in type 1 diabetes. Together these results suggest that there may be a role for GIP as a biomarker for physical inactivity and uncontrolled eating behaviour. Whilst elevated GIP is associated with insulin resistance in type 2 diabetes, it may also have a function linking lifestyle to the pathogenesis of type 1 diabetes. To better understand this relationship between GIP and modifiable lifestyle factors in type 1 diabetes then future research should also consider GIP's role in glycaemic control.

The final objective was to develop a method to explore the histopathological changes of alpha cells in type 1 diabetes. This was only going to be achieved through the use of samples collected through the 'Juvenile Diabetes Research Foundation network for Pancreatic Organ donors with Diabetes' database. Initial searches revealed that the number of available pancreatic samples stained for glucagon taken from donors with type 1 diabetes was lower than first expected. This, along with the lack of appropriate online image manipulation software, posed a significant obstacle in determining

whether the duration of type 1 diabetes affected the quantity of functional alpha cells. In order to overcome the software problem, methods more commonly used for digital photographic enhancement were refined and utilised. In total sixteen donor slides from the database were analysed. No correlation was found between duration of type 1 diabetes and area of glucagon staining, but a moderate correlation was detected between age and area of glucagon staining. This suggests that the area of glucagon stained alpha cells maybe a marker of ageing and not of duration of type 1 diabetes. What it is not possible to deduce is whether an individuals age, BMI or duration of type 1 diabetes can be used to determine the number of alpha cells present for any future gene manipulation therapy to regenerate beta cells from alpha cells. However, due to the small sample size in this analysis, it would be prudent to remain cautious about conclusions until either the database expands or other acceptable means of quantifying alpha cell numbers can be found.

Apart from the key findings already discussed the results from this research have opened up new interesting avenues for future research. Firstly, the analysis of c-peptide levels in those with diabetes duration under 2 years raises the possibility of a 30 minute stimulated c-peptide level being used to aid in the diagnosis of type 1 diabetes in those clinically ambiguous cases involving obese young individuals. Secondary, the incretin analysis brought to light the possibility that stimulated incretin levels could potentially be used as a marker of gut transit time, which would reduce the need for xray exposure. Thirdly, the findings of elevated stimulated glucagon or reduced active ghrelin in those with less than two years duration of type 1 diabetes suggest a possible role, in combination with other biomarkers, in the early diagnosis of type 1 diabetes potentially prior to the onset of hyperglycaemic symptoms.

In summary, this research has furthered our understanding of the relationship of gut hormones and alpha cells in the pathophysiology of type 1 diabetes. It has shown that type 1 diabetes may cause significant changes to plasma concentrations of some gut hormones (i.e. active ghrelin, glucagon and PYY) and that lifestyle factors can impact on their levels (i.e. GIP and leptin). It has also highlighted the need for further research into understanding the interplay between gut hormones, lifestyle factors and the homoeostatic control of glucose in type 1 diabetes.

Finally, whilst this thesis has focused on the role gut hormones play in the arena of glucose homeostasis in type 1 diabetes, there are areas of interest outside of this domain which are relatively devoid of research. Important questions remain unanswered, including, but not limited to the impact of gut hormones on inflammation, mental health and child development in type 1 diabetes.

8. Appendix 4 – Publications and Presentation Abstracts

Publications & Presentations related to this thesis.

British Journal of Diabetes and Vascular Disease 2014;14:45-51
Title: Alpha cell function in type 1 diabetes
Author: D. Hughes & P. Narendran
From: Centre of Endocrinology, Diabetes and Metabolism, Institute of Biomedical Research, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

Abstract
Our understanding of the pathogenesis of Type 1 Diabetes (T1DM) has traditionally revolved around the insulin deficiency that follows pancreatic beta cell loss. However, there is an increasing appreciation of defect in other gluco-regulatory cells in T1DM. Over secretion of glucagon from pancreatic alpha cells is characteristic of T1DM, and modulating these glucagon levels reduces hyperglycaemia. This article reviews alpha cell function in T1DM. We examine how its function is controlled and compromised, and review studies that target alpha cell function. Finally, we explore potential approaches to modulating alpha cell function in T1DM.

Presentation Abstract - Diabetes UK Conference April 2015
Poster: 171
Title: Physiological levels of gut peptides are different in individuals with type 1 diabetes compared to healthy volunteers.
Author: D. Hughes, P. Narendran
From: Centre of Endocrinology, Diabetes and Metabolism, Institute of Biomedical Research, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

Introduction
A wave of novel drugs that modulate gut peptides are being developed for the treatment of type 2 diabetes and obesity. The modulation of these peptides can alter insulin resistance, insulin secretion and satiety. However, these drugs may have a role in treating type 1 diabetes. However, the physiology of these peptides may be different in type 1 diabetes. This study set out to determine whether the physiological levels of gut peptide levels in individuals with type 1 diabetes is different to healthy volunteers.

Methods
Participants were recruited from the CDR-Diabetes study (REC:12/WM/0089) and ExTOD study (REC:10/H1206/4). Participants underwent a two hour mixed meal test. Analysis of Leptin, Ghrelin, PP, PYY, C-Peptide & GIP were performed using Luminex xMAP. Active-GLP1 & Glucagon analysis was performed through ELISA.

Results
A total of 24 healthy volunteers samples were analysed and 48 participants with type 1 diabetes. Area under the curve were analysed between healthy controls and those with type 1 diabetes. Unpaired T-test analysis was performed to determine if there were a significant difference between the groups: C-peptide ($p<0.001$), Glucagon ($p<0.01$), active GLP-1 ($p=0.15$), GIP ($p=0.23$), Active-Ghrelin ($p<0.001$), Leptin ($p=0.73$), PP ($p=0.46$) & PYY ($p=0.95$).

Conclusions
C-peptide and ghrelin levels are significantly lower in type 1 diabetes. Glucagon levels were significantly increased in type 1 diabetes. Levels of GLP-1, GIP, Leptin, PP & PYY were not significantly different. These differences in physiological gut peptide levels will need further research to determine their potential as therapeutic targets.

Publications & Presentations not-related to this thesis, but research related:

British Journal of Diabetes and Vascular Disease 2016 – In Press
Title: Elevated serum free light chains predict cardiovascular risk in type 1 diabetes mellitus.
Author: Lauren Marie Quinn, Sheridan McWilliam, John P Campbell, Yan Wang, David Hughes, Mark T Drayson & Parth Narendran.
From: University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

Abstract
Aims: Increased serum polyclonal combined immunoglobulin free light chain (cFLC = FLCκ+FLCλ) predicts cardiovascular (CV) events in South-Asians with type 2 diabetes mellitus. We examined whether cFLC also predicted CV risk in unselected patients with type 1 diabetes mellitus.

Methods: cFLC was estimated in the serum of 55 adults with type 1 diabetes mellitus. CV risk was measured through two validated risk engines–Q-Risk and PROCAM. Statistical association was tested using the parametic Pearson's or the Spearmans'-Rank-Correlation-Coefficient test, student t-test or Mann-Whitney-U test and Kruskall-Wallis test for parametric or non-parametrically distributed data accordingly.

Results: cFLC was associated with CV risk. This association was significant when estimated through either risk engine (PROCAM p=0.003, Q-Risk p=0.012). cFLC was associated with diabetes mellitus duration(p=0.003), age(p=0.006), and history of cardiac disease(p=0.042).

Conclusions: These findings indicate that cFLC is a marker of CV risk in people with type 1 diabetes mellitus. Moreover, it supports emerging data demonstrating cFLC as a prognostic indicator for mortality.

Presentation Abstract - ABCD Spring Meeting 2013
Poster number: 2
Title: Risk of Obstructive Sleep Apnoea (OSA) is increased in type 1 diabetes (DMT1): Preliminary results from the Chronic Disease Research in Diabetes Study
Author: Dr Neeral Patel, Dr David Hughes, Jayne Robbie & Dr Parth Narendran
From: Centre of Endocrinology, Diabetes and Metabolism, Institute of Biomedical Research, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

Introduction
The Chronic Disease Research in Diabetes Study commenced in November 2012. It aims to develop a database of clinical information paired with biological samples to explore the natural history of diabetes and its complications. Here we present early data on risk of OSA, depression, functional ability and eating behaviour in DMT1 and type 2 diabetes (DMT2).

Methods
Participants completed the following validated questionnaires at their baseline visit: STOP screening questionnaire for OSA, Patient Health Questionnaire (PHQ-9) for depression, Health Assessment Questionnaire (HAQ-DI) for functional ability, Three Factors Eating Questionnaire (TFEQ-R18) for eating behaviour. Anthropometric measurements including neck circumference were also collected.

Results
30 subjects have been recruited to date. 62% with DMT1 and 38% with DMT2. The STOP score for OSA was higher (p=0.026) for individuals with DMT2 than DMT1, with the overall risk being increased for both conditions. Neck circumference was positively correlated with the STOP risk scores for OSA (p=0.047). No significant difference between DMT1 and DMT2 existed for PHQ-9 depression scores (p=0.072), HAQ-DI functional ability scores (p=0.113) or any domains for TFEQ-R18 eating behaviour questionnaire: cognitive restraint (p=0.730), uncontrolled eating (p=0.601) and emotional eating (p=0.797).

Conclusions
We present preliminary data related to co-morbidities in individuals with diabetes. Early results suggest a yet unexplored risk of OSA in individuals with DMT1, and a trend towards increased risk of depression inDMT2 compared to DMT1.

Award: West Midlands Deanery Foundation Year One Trainee Award 2013 (1st Prize)
Title: Should diabetic patients be routinely screened for obstructive sleep apnoea?
Author: Dr Neeral Patel & Dr Samaresh Mazumdar
Supervisors: Dr David Hughes & Dr Parth Narendran
From: Queen Elizabeth Hospital, Birmingham, UK.

ABSTRACT
The Chronic Disease Research in Diabetes Study (CDR-Diabetes) commenced in November 2012. It aims to develop a database of clinical information paired with biological samples to explore the natural history of diabetes and its complications. Here we present early data on risk of obstructive sleep apnoea (OSA) in type 1 (DMT1) and type 2 diabetes (DMT2). Participants completed the STOP-Bang screening questionnaire for OSA. Anthropometric measurements were also collected to allow calculation of visceral adipose tissue (VAT). Thirty-five subjects have been recruited to date. The STOP-Bang score for OSA was higher for individuals with DMT2 (mean STOP-Bang=5.30±1.16) than DMT1 (mean STOP-Bang=3.24±1.72, p=0.0016). Overall risk was increased for both conditions, with mean STOP-Bang scores greater than 3 as the threshold for moderate-to-high risk of OSA. There is strong positive correlation between VAT and STOP-Bang score (Pearson r value=0.844; p<0.001). Preliminary results from this, the first reported use of STOP-Bang questionnaire in the diabetic population suggests an as of yet, unexplored risk of OSA in individuals with DMT1.

Presentation Abstract - Diabetes UK Conference 2014
Poster number: P224
Title: An improved indicator of risk for obstructive sleep apnoea: estimated visceral adiposity compared to body mass index and waist-hip ratio in individuals with diabetes.

Author: M. Samaresh, D. Hughes, P. Narendran
From: Centre of Endocrinology, Diabetes and Metabolism, Institute of Biomedical Research, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

Introduction
Obstructive sleep apnoea (OSA) is strongly associated with central obesity and type 2 diabetes. Central obesity is caused by an increase in visceral adipose tissue. Visceral adiposity can be accurately measured with CT, MRI or DEXA scans. The recent publication of a formula to estimate visceral adiposity (eVA) could provide a new inexpensive means to determine risk of obstructive sleep apnoea.

Methods
Participants with diabetes were recruited to the CDR-Diabetes study (an ongoing, observational, multi-centred study). As part of the study, body mass index (BMI), Waist:Hip ratio and estimated Visceral Adiposity (eVA) were recorded. Also, participants completed the validated STOP-BANG screening tool for OSA. A STOP-BANG score of >3 is considered moderate-high risk of OSA. SPSS statistical software was then used to determine correlation coefficients and statistical probability.

Results
A total of 31 participants were analysed (type 1 diabetes=21, type 2 diabetes=10). The mean STOP-BANG score was 3.88. The correlation coefficient values for STOP-BANG scores were: BMI=0.616 ($p<0.01$), Waist-Hip ratio=0.696 ($p<0.01$) and eVA=0.852 ($p<0.01$). Sub-group analysis of those individuals with type 1 diabetes: eVA=0.809 ($p<0.01$).

Conclusions
BMI, Waist-Hip ratio and eVA all significantly correlate to risk of OSA. eVA correlates stronger to OSA risk than either BMI or Waist-Hip ratio (even in indiviudals with type 1 diabetes). Further research is required to determine whether eVA has a role as a diagnostic tool for obstructive sleep apnoea.

Poster number: P174
Title: Free light Chains are a biomarker for cardiovascular disease in patients with type 1 diabetes
Author: LM Quinn*, S McWilliam, JP Campbell, D Hughes, MT Drayson, P Narendran
From: Centre of Endocrinology, Diabetes and Metabolism, Institute of Biomedical Research, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

Aims: Light chains (kappa and lambda) are integral components of immunoglobulins that are synthesised in excess by plasma cells and filtered by the kidneys. Polyclonal free light chains (FLC) are frequently elevated in patients with renal disease or immuno-stimulatory disorders. Elevated (combined) polyclonal FLC have also been independently associated with morbidity and mortality in the general population, and more recently with cardiovascular disease (CVD) in patients with type 2 diabetes. We aimed to investigate whether FLC associated with CVD risk in patients with type 1 diabetes.

Methods: CVD risk was estimated in a cohort of 55 patients with type 1 diabetes from the West Midlands chronic disease research database (CDRD) using the Procam and Q-

risk CVD risk engines. FLC was determined using a monoclonal-antibody based assay. Non-parametric statistical analyses were performed to determine whether FLC associated with CVD risk.

Results: Our cohort consisted of male and female, predominantly white British patients, mean age 48 years. FLC kappa/lambda ratio was normal for our cohort. Q-risk 10-year score and Procam Myocardial Infarction risk scores were both significantly associated with FLC (p=0.003 and p=0.012 respectively), as well as duration of diabetes (p=0.003), patient age (p=0.006) and medical history of cardiac disease (p=0.042).

Summary: We show that CVD risk is significantly associated with FLC in patients with type 1 diabetes. The mechanisms linking FLC with CVD risk remain unclear. Elevated polyclonal FLC are associated with inflammation, which in turn is associated with CVD risk. Alternatively FLC may be atherogenic and predispose to coronary artery disease.

Presentation Abstract - Diabetes UK Conference 2017
Poster number: P77
Title: Who Volunteers for Research into Type 1 Diabetes? Implications for Exercise studies.
Author: Jeyaraman, M Charlton*, D Hughes, P Narendran
From: Diabetes Research Unit, Queen Elizabeth Hospital, Birmingham, UK

Aim: To describe obstacles to exercise in a group of research volunteers with type 1 diabetes.
Introduction: Recruiting to research into exercise is challenging. Previous work has identified motivation as a barrier to participation. We looked at characteristics of people with type 1 diabetes who are motivated to take part in research and examined their difficulties with exercise.

Methods: The Chronic Disease Research into Diabetes (CDRD) study is open to all people with diabetes attending participating centres. Exclusion criteria are minimal. Baseline data from recruits with type 1 diabetes at one centre are presented.

Results: 71 volunteers for CDRD with type 1 diabetes were identified. 46.5% were female, median age 50 years (IQR 34-58); 91.5% caucasian. 12% obese. 85% had diabetes >10 years' duration. Insulin was 69% basal-bolus, 24% pumps. 27% had HbA1c ≤58 mmol mol-1, 10% ≥ 100. 8.5% currently smoked. 48% were employed or studying. 66.2% reported >2 hours of recreational screen time daily. Exclusion criteria for exercise trials were quantified: foot ulcers 1%; amputation 1%; blood pressure over 180/100 3%; active proliferative retinopathy 8%; beta-blockers and other rate-limiting treatment 11%. 23% had musculoskeletal co-morbidities and 18% respiratory.
Validated questionnaires showed: 35% moderate or extreme anxiety/depression; 29% breathless walking or hurrying; 27% had mobility difficulties and 35% pain sufficient to affect quality of life; 33% had difficulties in activities of daily living.

Conclusions: People with type 1 diabetes who are most motivated to participate in research have significant physical and psychological barriers to exercise.
When designing clinical trials into exercise in type 1 diabetes researchers need to adapt their interventions or recruitment strategies accordingly.

Poster number: P124

Title: Who volunteeers for research into type 1 diabetes?

Author: D Jeyaraman*, M Charlton, P Kempegowda, M Jain, D Hughes, P Narendran
From: Diabetes Research Unit, Queen Elizabeth Hospital, Birmingham, UK

Aim: To describe the characteristics of a group of research volunteers with Type 1 Diabetes and compare them with a clinic population.

Methods: The Chronic Disease Research into Diabetes (CDRD) study is open to all people with diabetes attending participating centres. Exclusion criteria are minimal. Baseline data from recruits with type 1 diabetes at one centre were compared with non-research clinic attenders in order to define characteristics of people who are motivated to take part in research.

Results: Of 153 volunteers in CDRD, 71 had type 1 diabetes. Controls were an internal audit of 71 consecutive clinic attenders with type 1 diabetes.
Gender and ethnicity were similar in both groups as were HbA1c and insulin regimen. Volunteers' median age was 50 years (IQR 34-58) and controls' 38 (27-49). 12% volunteers (19% controls) had BMI > 30. Volunteers' median blood pressure was 133/77 (controls' 133/83). 58% (controls 39%) had diabetes of 20 years' duration or longer.
Comorbidities were predominantly musculoskeletal affecting 22.5% (controls 6%) and respiratory 18.3% (8%); 4% (3%) had cardiac disease. Only 17% volunteers had no pertinent comorbidities but 45% controls. Polypharmacy was common with mean 3.86 non-insulin drugs per volunteer (1.6 per control).

Conclusions: From a clinic population of people with type 1 diabetes, research volunteers were older and had diabetes of longer duration than average. They took more prescription medication and had more comorbidities, particularly musculoskeletal and respiratory.
In designing clinical trials, researchers need to be aware that people motivated to take part may not be representative of the pool from which they are recruiting.

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