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Biomarkers associated with glycaemic control, comorbidity and response to GLP-1 analogue therapy

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Abstract

Glycaemic dysregulation drives type II diabetes (T2D) pathogenesis, perpetuating multimorbidity that is thought to be linked to mood and cognitive decline. Glucagon-like peptide-1 receptor agonists (GLP-1Ras) are an effective insulin sensitising and weight loss medication. Preclinical studies have shown them to have positive effects on cognition. Whether such effects are seen in humans is unknown. The aims of this PhD were to investigate potential disease mechanisms and assess current and novel biomarker panels, clinically, that relate to T2D, cognitive decline and GLP-1Ra response. qPCR arrays using 3 *in-vivo* models showed leptin and insulin related genes were differentially affected by treatment duration in brain, and these changes may be evident peripherally. Clinically, ECR records of 500 T2D patents indicated diabetes is poorly managed, and that cardiovascular disease (CVD) and hyperlipidaemia management appear to be prioritised. Current biochemical measures of diabetes (HbA_{1c}, BMI and plasma lipid profile) did not correlate strongly with any aspect of T2D, while C-peptide, currently not routinely measured, may be a valuable addition to the clinic. Multiplex proteomics of 374 T2D participants and 20 controls, screened 368 proteins across 12 variables; previously shown to be central in T2D. Unique protein panels were identified for each variable, and vascular endothelial dysfunction was a commonalty between all. Genetic supplementation of the proteomic markers was also achieved via SNP genotyping of 20 insulin and leptin-related genes. One hundred and five SNPs differentiated T2D and controls and 25 were indicative of GLP-1Ra response. GLP-1Ra non-responders were found to have the greatest level of comorbidity and highest scores on the Beck depression and anxiety inventories. This translated to lower cognitive domain scores in, memory and perception. These data have identified prescribing and comorbidity patterns in a Northern Irish T2D population, and demonstrated that current measures do not adequately correlate with disease progression or comorbidity onset. Novel markers identified may allow for higher predictive capability, and may reduce the impact of cognitive decline in T2D.

Abbreviations

A&E	Accident and Emergency
AC	Adenylate Cyclase
ACCORD	Action To Control Cardiovascular Risk In Diabetes
ACE	Angiotensin Converting Enzyme
ACE2	Angiotensin Converting Enzyme 2
AD	Alzheimer's Disease
ADA	American Diabetes Association
ADMA	Asymmetric Dimethylarginine
ADVANCE	Action In Diabetes And Vascular Disease
AGEs	Advanced Glycation End-Products
AKT	Protein Kinase B
ARB	Angiotensin Receptor Blockers
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
Aβ	Amyloid Beta
BACE1	Beta secretase-1
BBB	Blood Brain Barrier
BcL2	B-Cell Lymphoma 2
BMI	Body Mass Index
BP	Blood Pressure
CA5A	Carbonic Anhydrase 5A
cAMP	Cyclic Adenosine Monophosphate
CASP1	Caspase 1
CCL11	CC Motif Chemokine 11
CCL25	CC Motif Chemokine 25
CDCP1	CUB Domain-Containing Protein 1
cDNA	Complementary DNA
CKD	Chronic Kidney Disease
CLEC4C	C Type Lectin Domain Family 4 Member C
CNS	Central Nervous System

CNTN1	Contactin 1
CPA1	Carboxypeptidase A1
CPB1	Carboxypeptidase B1
CRE	cAMP Response Elements
CREB1	cAMP Response Element-Binding Protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTRC	Chymotrypsin C
CTSD	Cathepsin D
CVD	Cardio Vascular Disease
CXCL10	C-X-C Motif Chemokine 10
DC	Day Care
DCCT	Diabetes Control And Complications Trial
DCN	Decorin
DDP-IVi	Dipeptidyl Peptidase IV Inhibitors
EASD	European Association For The Study Of Diabetes
ECG	Electrocardiogram
ECR	Electronic Care Records
ELISA	Enzyme-Linked Immunosorbent Assays
EPAC2	Exchange Protein Directly Activated By Camp 2
EpCAM	Epithelial Cell Adhesion Molecule
FABP2	Fatty Acid-Binding Protein 2
FABP4	Fatty Acid Binding Protein 4
FBC	Full Blood Count
FFA	Free Fatty Acids
FGF-5	Fibroblast Growth Factor 5
Flt3L	Fms-Related Tyrosine Kinase 3 Ligand
FOXO1	Forkhead Box Protein O1
FPG	Fasting Plasma Glucose
Gal-4	Regulatory Protein GAL4
GAL-4	Galactose 4
GDF15	Growth Differentiation Factor 15
GERD	Gastroesophageal Reflux Disease

GH	Growth Hormone
GIP	Glucose-Dependent Insulinotropic Polypeptide
GLO1	Glyoxalase I
GLP-1	Glucagon-Like Peptide-1
GLP-1Ra	Glucagon-Like Peptide-1 Receptor Agonists
GPCRs	G Protein-Coupled Receptors
GSK3	Glycogen Synthase Kinase 3
GWAS	Genome-Wide Association Studies
HAOX1	Hydroxyacid Oxidase 1
HbA_{1c}	Glycated Haemoglobin
HDL	High-Density Lipoproteins
HGF	Hepatocyte Growth Factor
HIF	Hypoxia-Inducible Factor
HOMA	Homeostatic Model Assessment
hOSCAR	Osteoclast-Associated Immunoglobulin-Like Receptor
HSD11B1	Hydroxysteroid 11-Beta Dehydrogenase 1
HSP 27	Heat Shock Protein 27
ICD-9	International Statistical Classification Of Diseases And Related Health Problems 9
ICH	Intracerebral Haemorrhage
IDF	International Diabetes Federation
IGF	Insulin Growth Factors
IGF-1R	IGF Receptor Type-1
IGFBP-2	Insulin-Like Growth Factor Binding Protein 2
IgG4	Immunoglobulin G4
IL-1 ra	Interleukin-1 Receptor Antagonist
IL10	Interleukin 10
IL-17C	Interleukin 17C
IL-17RA	Interleukin 17 Receptor A
IL-18	Interleukin-18
IL-18BP	Interleukin-18-Binding Protein
IL-1RT1	Interleukin-1 Receptor Type 1

IL4	Interleukin 4
IL6	Interleukin 6
IMI	Innovative Medicine Initiative
IP_3R	Inositol Trisphosphate Receptors
IRS	Insulin Receptor Substrate
ITGA11	Integrin Alpha-11
ITGB8	Integrin Subunit Beta 8
JAK2	Janus Kinase 2
KIM1	Kidney Injury Molecule-1
LADA	Latent Adult Onset Diabetes
LAG3	Lymphocyte-Activation Protein 3
LDL	Low-Density Lipoproteins
LEP-R	Leptin Receptor
LM	Logical Memory
LOAD	Late Onset Alzheimer's Disease
LOD	Late Onset Depression
LPL	Lipoprotein Lipase
MAF	Minor Allele Frequency
MAPKs	Mitogen-Activated Protein Kinases
MB	Myoglobin
MCI	Mild Cognitive Impairment
MCP3	Monocyte-Chemotactic Protein 3
MDD	Major Depressive Disorder
MMP3	Matrix Metalloproteinase-3
MMP7	Matrix Metalloproteinase-7
MMSE	Mini Mental State Exam
MSD	Meso Scale Discovery
mTOR	Mammalian Target Of Rapamycin
NF-KB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NFT	Neurofibrillary Tangles
NHANES	National Health And Nutrition Examination Surveys
NHS	Nation Health Service

NICE	National Institute for Health and Care Excellence
NIECR	Northern Ireland Electronic Care Record
NIRKO	Neuron-Specific IR Knockout Mice
NOTCH	Notch Homolog 1
NT-proBNP	Pro-Brain Natriuretic Peptide
OA	Osteoarthritis
OCT1	Organic Cation Transporter 1
OGTT	Oral Glucose Tolerance Test
OS	Oxidative Stress
PAI-1	Plasminogen Activator Inhibitor-1
PCR	Polymerase Chain Reaction
PCSK9	Proprotein Convertase Subtilisin/Kexin Type 9
PD-L2	Programmed Cell Death Ligand 2
PEA	Proximity Extension Assay
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
PKA	Protein Kinase A
POMC	Pro-Opiomelanocortin
PON3	Paraoxonase 3
PPARy	Peroxisome Proliferator-Activated Receptor Gamma Receptor
PPIs	Proton Pump Inhibitors
PYY	Peptide YY
PRDX1	Peroxiredoxin-1
PRMT1	Protein Arginine Methyltransfetase-1
PRSS27	Marapsin
PRSS8	Prostasin
PTP1B	Tyrosine-Protein Phosphatase Non-Receptor Type 1
QC	Quality Control
qMCI	Quick Mild Cognitive Impairment Screen
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red Blood Cells
REN	Renin
ROS	Reactive Oxygen Species

RT	Reverse Transcriptase
SCF	Stem Cell Factor
SERPINA12	Serpin Family A Member 12
SGLT2i	Sodium-Glucose Cotransporter-2 Inhibitors
SH2B1	Src Homology 2B 1
SLAM	Signalling Lymphocyte Activation Molecule
SLCO1B1	Solute Carrier Organic Anion Transporter Family Member 1b1
SMMSE	Standardized Mini-Mental State Examination
SNPs	Single Nucleotide Polymorphisms
SOCS3	Suppressor Of Cytokine Signalling 3
SORT	Sortilin
STAT3	Signal Transducer And Activator Of Transcriptome 3
SU	Sulphonylureas
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TBF	Total Body Fat
TBI	Traumatic Brain Injury
TNFRSF10A	TNF Receptor Superfamily Member 10a
TNFRSF13B	Tumor Necrosis Factor Receptor Superfamily Member 13b
TNFSF14	Tumor Necrosis Factor Superfamily Member 14
TNFα	Tumor Necrosis Factor Alpha
TR	Thioredoxin Reductase
TfR1	Transferrin Receptor Protein 1
TRAF2	TNF Receptor-Associated Factor 2
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TZDs	Thiazolidinediones
UK	United Kingdom
UKPDS	UK Prospective Diabetes Study
uPA	Urokinase-Type Plasminogen Activator
USA	United States of America
VADT	Veterans Affairs Diabetes
VEGF	Vascular Endothelial Growth Factor

VF	Verbal Fluency
VM	Verbal Memory
VSIG2	V-Set And Immunoglobulin Domain Containing 2
WBC	White Blood Cells
WMH	White Matter Hypertensities
WT	Wild-Type
ZBTB16	Zinc Finger And BTB Domain Containing Protein 16

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Chapter I

Introduction

Diabetes mellitus is characterised by high blood glucose due to either the pancreas' inability to produce enough insulin or the bodies inability to effectively use the insulin it produces (Chamberlain et al., 2017b, WHO, 1999). Early symptoms include, excessive thirst (polydipsia), increased passage of urine (polyuria), blurred vision, constant hunger and fatigue (Diabetes UK, 2017b).

Type I diabetes (T1D) accounts for 5-10% of all cases. It is caused by the auto-immune destruction of insulin producing pancreatic β-cells, resulting in significant insulin deficiency (Maahs et al., 2010). The disease is thought to have a short prodome with a rapid onset. It is widely thought that clinical symptoms do not manifest until 90% of β-cells are destroyed (Veijola et al., 2016). Onset of T1D is thought to be linked to acute stress and increased insulin resistance, which accentuate the effects of an underlying insulin deficiency (Wang et al., 2017). T1D predominantly occurs at a younger age and can be triggered by infection, illness or physiological changes related to insulin (Akirav et al., 2008). In 1993 another form of T1D was introduced, latent adult onset diabetes (LADA). Contrasting classic Type 1 (Type Ia) characteristics, this disease presents in adult life with a slow onset and no initial dependence for insulin therapy (Thivolet, 2001).

Type II diabetes (T2D) is the most common form, accounting for 90% of all cases (Maahs et al., 2010). It is directly linked to obesity, with 80% of individuals with T2D being classed as overweight (WHO, 1999). The WHO classifies obesity as having a total body fat percentage (TBF) of >35% in women and >25% in men and advise that obese individuals are 80 times more likely to develop T2D (WHO, 1999).

Studies have shown that abdominal fat initiates an inflammatory response causing the body to become less responsive to insulin (Patel and Abate, 2013). There is also a significant dysregulation of glucose dynamics in the liver. Obesity and diabetes impair insulin-induced suppression of gluconeogenesis and glycogenolysis and impairments have been shown to correlate with plasma free fatty acid concentrations (Basu et al., 2005).

It is well established that age, lifestyle, diet and obesity have a substantial impact on the development of T2D but it is still not fully understood why some people develop pre-diabetes and then T2D and others do not (Tuomilehto and Bahijri, 2016). Genetic risk factors are now thought to be increasingly important.

There are a number of genetic variants implicated in the pathogenesis of the disease in genes associated with insulin secretion, insulin sensitivity and obesity. These include ABCC8 and TCF7L2 which both negatively affect insulin secretion (Jäger et al., 2017). It has also recently been shown that hyperglycaemia, due to reduced insulin secretion, seems in part to be genetically determined (Park, 2011), with a 4 fold increased risk of developing the condition if it is present within the immediate family (Meigs et al., 2000).

1.1 Demographics of diabetes

Recent figures estimate the diagnosed global prevalence of diabetes to be 422 million (World Health Organisation, 2016), and 46% of individuals with diabetes are thought to be undiagnosed. Prevalence has been projected to rise to 642 million by 2040 (World Health Organisation, 2016). In the United Kingdom (UK) 3.5 million people have been diagnosed with diabetes with an estimated 550,000 individuals undiagnosed, representing 6% of the UK population. This number is projected to rise to 5 million by 2025 (Diabetes UK, 2016).

Diabetes is already having a profound impact on the National Health Service (NHS). It was estimated in 2012 that diabetes and its complications cost the NHS £14 billion; the treatment of complications represents the majority of that spend, with a reported £9 billion (66%) spent on inpatient care and £3.2 billion (22%) on pharmacological treatment (Hex et al., 2012).

1.2 Diagnosis of diabetes

Glycated haemoglobin (HbA_{1c}) is the current preferred diagnostic measure for diabetes (Edge et al., 2010). It is formed when haemoglobin in red blood cells (RBCs) binds to glucose and provides an average blood sugar level over the lifespan of RBCs, which is approximately 8-12 weeks in humans (Argento et al., 2014).

Currently a diabetes diagnosis is established if a patient presents with an HbA_{1c} of >48 mmol/mol (6.5%) on two occasions. Pre-diabetes is diagnosed when HbA_{1c} is between 42 (6%) - 47 (6.4%) mmol/mol., while a normal HbA_{1c} level is below 42 mmol/mol (6%) (Kowall and Rathmann, 2013). Diagnostically, HbA_{1c} has several

limitations (Lippi and Targher, 2010). Readings can be affected by genetic variants, chemically altered by-products of haemoglobin (Soranzo, 2011) and various conditions that affect RBCs and RBC survival such as pregnancy and anaemia (English et al., 2015). Other diagnostic measures include; Fasting Plasma Glucose (FPG) and oral glucose tolerance tests (OGTT), however both require a minimum of 8 h fasting and are therefore not as convenient as HbA_{1c}. A glucose reading of 43 mmol/mol (6.1%) or above is indicative of diabetes in the FPG test (Yun et al., 2010), while a glucose concentration of 48 mmol/mol (6.5%) 2 hours after consumption of 75g of glucose in the OGTT warrants a diabetes diagnosis (Hutchinson et al., 2012).

1.3.1 Non-pharmacological treatment

T2D may be prevented or treated by lifestyle changes alone. Upon diagnosis it is recommended that patients receive education and ongoing nutritional and dietary support to provide them with the knowledge and skills to effectively manage their disease outside of primary and secondary care (National Institute for Health and Care Excellence, 2017). The major environmental risk factors for T2D are an unbalanced diet and a sedentary lifestyle (Cai et al., 2017). Both factors are more prevalent in urbanised areas but are now equally relevant across both hemispheres (Bhupathiraju and Hu, 2016). Dietary advice can have a profound impact on the management of diabetes (NICE, 2017). Simple carbohydrates rapidly increase blood glucose levels requiring a large, rapid insulin response (Marsh et al., 2011). It is recommended that those with T2D avoid simple carbohydrates and focus on complex carbohydrates which result in a slow sustained release of glucose providing lasting energy and fullness (NICE, 2017). Physical activity reduces the risk of T2D by up to 50%; just 30 minutes of moderate exercise a day significantly reduces the risk of CVD by improving hepatic insulin resistance, reducing dyslipidaemia and ultimately reducing body weight (Cai et al., 2017). The American Diabetes Association (ADA) states that exercise induced improvements in glucose management, blood fats and body weight will result in patients experiencing more energy, better sleep, reduced depression and a longer life (ADA, 2016).

1.3.2 Pharmacological treatment

Pharmacological management of blood glucose and achieving a healthy HbA_{1c} is directly related to a reduced risk of heart disease, kidney damage and other microvascular complications. Diabetes is not simply a disease about glucose control but a close interaction between glucose, lipids and blood pressure (BP) (Skrha et al., 2016). The Action to Control Cardiovascular Risk in Diabetes (ACCORD) (Ismail-Beigi et al., 2010), Action in Diabetes and Vascular Disease (ADVANCE) (Group et al., 2008) and Veterans Affairs Diabetes (VADT) (Duckworth et al., 2009) randomised clinical trials indicated that aggressive glycaemic control significantly reduced cardiovascular complications. Other trials such as the UK Prospective Diabetes Study (UKPDS) (Scheen et al., 2008) and Diabetes Control and Complications Trial (DCCT) (American Association of Diabetes, 2002) suggested that such intensive treatment creates a "legacy effect" and benefits to the patient are not apparent until years later. Consideration of the benefit of insulin over oral therapy must be assessed carefully as the more aggressive the glycaemic control the greater the risk of weight gain, hypoglycaemia and drug interactions (Gale, 2008).

1.3.3 Non-insulin Diabetes treatments

There are currently seven classes of non-insulin antidiabetic drugs available; biguanides, sulphonylureas, meglitinides, thiazolidinediones, sodium-glucose Cotransporter-2 inhibitors (SGLT2i), dipeptidyl peptidase IV inhibitors (DDP-IVi), and glucagon-like peptide-1 receptor agonists (GLP-1Ra). Each class has a specific mode of action and adverse event profile, and all, except GLP-1Ra, are oral medications and have a more favourable risk profile than insulin (NICE, 2017). NICE (National Institute for Health and Care Excellence) have published guidance for clinicians and recommend prescription strategies for each class of anti-diabetic drugs which aims to minimise adverse events, drug reactions and cost, while achieving adequate control (NICE, 2017). The definition of "adequate control" varies depending on the severity of diabetes. If diabetes can be managed by monotherapy the target HbA_{1c} is 48 mmol/mol (6.5%), if HbA_{1c} rises above 58 mmol/mol (7.5%) it is recommended that drug treatment is intensified and a new target of 53 mmol/mol (7.0%) set.

Diabetes treatment regimens are intensified until a 4th line treatment is prescribed, with insulin introduced as a last resort if adequate control cannot be achieved (NICE, 2015).

1.3.3.1 Monotherapy

Metformin is the preferred first line therapy. It reduces hepatic glucose output and improves insulin sensitivity (DeFronzo and Goodman, 1995). Doses are titrated from 500-2000 mg over several weeks, as necessary, provided gastrointestinal side effects are not limiting (NICE, 2017). The major risk associated with metformin is lactic acidosis (Salpeter et al., 2010), which results in a low blood pH, sickness, and in severe cases death (Connelly et al., 2017). Biguanides are cleared via the kidneys therefore not suitable in patients suffering from chronic kidney disease (CKD).

1.3.3.2 Dual Therapy

If 2000 mg/day of metformin does not initiate a satisfactory HbA_{1c} response, NICE guidelines recommend intensification of treatment. This involves the addition of either sulphonylurea, thiazolidinedione, SGLT2i or a DDP-IVi (NICE, 2017).

Sulphonylureas (SU) regulate glycaemia by increasing insulin release from β-cells. They block potassium (K^+) channels, inhibiting the influx of K^+ thus opening calcium (Ca^{2+}) channels triggering insulin release (Linden and Brooker, 1978). They also sensitise β-cells to glucose, limit glucose production in the liver and decrease lipolysis (Kuhn, 1988). Adverse events include hypoglycaemia and weight gain. Meglitinides, also induce insulin secretion, differing only in their affinity and faster dissociation from the sulphonylurea receptor (SUR1) (Guardado-Mendoza et al., 2013). Functioning β-cells are required by both SU and meglitinides, therefore it is advised that patients with low C-peptide are not initiated on either therapy (Linden and Brooker, 1978).

Thiazolidinediones (TZDs) reduce the bodies resistance to insulin by targeting lipid metabolism. These drugs activate the peroxisome proliferator-activated receptor gamma receptor (PPARγ), which is found abundantly on adipocytes (Flemmer and Scott, 2001). Treatment results in lower plasma fatty acid levels due to increased

storage of free fatty acids (FFA) by adipocytes (Flemmer and Scott, 2001). This causes cells to oxidise blood glucose for cellular processes thereby reducing plasma glucose (Sengupta et al., 2012). TZDs also lower BP, increase high-density lipoproteins (HDL), lower LDL and have shown promise in the treatment of CVD (Griffis et al., 2004). TZDs today (pioglitazone) are generally well tolerated but are associated with weight gain and oedema of the lower extremities. This is thought to occur in 5% of patients and the exact cause is not known (Sengupta et al., 2012).

SGLT2 inhibitors prevent reabsorption of glucose in the kidney and increase glucose excretion in the urine (Steen and Goldenberg, 2017). This has been shown to increase insulin sensitivity, reduce gluconeogenesis and improve first phase insulin release (Reed, 2016). The mode of action is independent of β-cell function and insulin resistance (Kawasoe et al., 2017). There is no loss of potency as β-cell function declines, and no interference with native glucose transport or insulin release which results in increased glycaemic efficacy, reduced body weight and a positive effect on blood pressure (BP) (Kadokura et al., 2014). Due to the high output of glucose in urine some patients, particularly women, are at risk of urinary tract infection. Diabetic ketoacidosis is also reported as a risk (NICE, 2017). Patients suffering with CKD are not suitable for this medication (Farahani, 2017).

Dipeptidyl peptidase IV inhibitors (DDP-IVi) block the action of the DDP-IV enzyme which metabolises the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Kazafeos, 2011). GLP-1 has been implicated in having a number of positive effects on glycaemia including; increasing insulin secretion (only during hyperglycaemia), delaying gastric emptying and suppressing glucagon secretion (Astrup et al., 2012). DDP-IVi have been shown to significantly increase GLP-1 levels and have a mild adverse event profile, with most incidences being gastrointestinal (Brunton, 2014). DDP-IVi are metabolised via renal and hepatic systems and have been assessed in patients with various degrees of renal impairment (Ramirez et al., 2013). NICE advise that they are safe to be prescribed with advanced CKD, although dosing is to be modified according to CKD severity (NICE, 2017).

1.3.3.3 Triple and Quad therapy

Triple therapy is recommended if HbA_{1c} targets have not been achieved after 6 months dual therapy. NICE guidelines recommend third line therapies consist of metformin and sulphonylurea plus either, DDP-IVi, SGLT2i, or a thiazolidinedione. The patient must be trialled on a triple medication for 6 months with regular renal assessments and HbA_{1c} and weight recordings upon completion. Only upon inadequate response can 4^{th} line therapy or initiation of GLP-1R agonists be considered (NICE, 2017).

GLP-1R agonists are an injectable therapy and are modified versions of the native hormone GLP-1. GLP-1 potentiates the release of insulin after feeding, however the native peptide has a very short half-life due to degradation by DDP-IV (Kazafeos, 2011). GLP-1Ra are resistant to DDP-IV degradation and therefore have a sustained effect on insulin release, glucagon activity and gastric emptying, with no chance of hypoglycaemia unless used in combination (Kazafeos, 2011). Liraglutide has been shown to have a positive impact on weight loss and has been licensed as an obesity treatment in the USA (Nuffer and Trujillo, 2015). Side effects are common but tend to be mild gastrointestinal effects (Buse et al., 2011).

NICE have stipulated specific guidelines for the prescription of GLP-1Ras. The patient must have a body mass index (BMI) of 35 kg/m^2 and another medical problem associated with obesity. The only time it is recommended to prescribe this drug to an individual with a BMI less than 35 kg/m^2 is if insulin therapy would have a significant occupational implication or if weight loss would benefit other co-morbidities (NICE, 2017). As with other therapies patients are trialled on a GLP-1Ra for 6 months. Response is defined as a reduction in HbA_{1c} of 11 mmol/mol (1%) and a 3% reduction in body weight, if these criteria are not met, the therapy must be discontinued and it is recommended that insulin should be introduced to achieve target HbA_{1c} (NICE, 2015).

1.3.4 Insulin therapy

Insulins are classed as rapid, intermediate, long or ultralong based on initiation phase, maximum peak duration and total duration (Figure 1). All can be injected

subcutaneously or intravenously, and have various delivery mechanisms including needle and syringe and cartridge systems. On initial prescription dose is calculated dependent on weight, height and sensitivity (Kakou, 2002). The initiation of insulin therapy is accompanied with significant risks and side effects, the most common of which are hypoglycaemia and hypertrophy of the injection site (Honegger and Spinas, 1997).

Insulin	Initiation of effect	Time to peak	Duration of therapeutic effect
Rapid acting (Humalog®)	10 min	1 hour	2 h
Intermediate (Neutral proatamine)	1 hour	5 h	18 h
Long acting (Lantus®)	8 h	No peak	24 h
Ultra-Long acting (Tresiba®)	8 h	No peak	48 h

Table 1.1 – **Current clinically available types of insulin for the treatment of type II diabetes.** Rapid acting insulins (Humalog, Eli Lilly) active ingredient is insulin lipro and typically initiates response after 10 min. It most commonly prescribed in T1D but is used to treat T2D (Home, 2012). Intermediate insulin (Neutral proatamine Hagedorn, NPH) is commonly prescribed with short acting insulin but risks of night time hypoglycaemia are reported, and it has a 1 hour activation lag. Long insulin (Lantus, Sanofi-Aventis) and ultralong insulins (Tresiba, Novo Nordisk) are often favoured by the NHS as they have a long initiation period of 8 h and no peak action, therefore a lower risk of hypoglycaemia (Lepore et al., 2000).

1.3.5 Metabolic surgery for T2D

Pharmaceutical approaches aimed at restoring glucose homeostasis in diabetes are effective and can reduce long term complications and mortality but adherence is a significant confounding factor (Ionova et al., 2015). There is emerging evidence that gastrointestinal operations such as vertical sleeve gastrectomy, roux-en-Y gastric bypass, biliopancreatic diversion and laparoscopic adjustable gastric banding can provide significant weight loss, reduce obesity-related complications and reverse insulin resistance, dramatically improving glucose control (Osland et al., 2017). Metabolic surgery causes rapid transport of nutrients to the lower intestine which has been shown to result in increased incretin secretion (Osland et al., 2017). The omission of the proximal intestine is also though to inhibit any anti-incretin signals

by affecting peptide YY (PPY) and ghrelin (Cummings et al., 2007). These unique circumstances enable a sustained incretin effect, which impacts on food intake, weight loss and insulin sensitivity (Alamuddin et al., 2017).

The cost of this type of surgery is £9000 - £15000 (Villamizar and Pryor, 2011). This may be more cost effective than years of pharmacological treatment, and has led to organisations such as the International Diabetes Federation (IDF) taskforce to suggest T2D patients with a BMI >40 kg/m^2 and patients with a BMI between 35 - 39.9 kg/m^2 undergo surgery if HbA_{1c} is inadequately controlled by medical therapy (IDF, 2012).

1.4 Complications associated with T2D

1.4.1 Microvascular complications

T2D patients usually suffer from multiple chronic conditions that arise directly or indirectly from hyperglycaemia (Bhupathiraju and Hu, 2016). The UKPDS and DCCT trials (American Association of Diabetes, 2002) defined a clear link between microvascular disease and glucose control (American Association of Diabetes, 2002). Microvascular disease occurs predominantly in the retina, kidney and vascular endothelium (McElduff, 2016), where glucose uptake is independent of insulin (Duckworth et al., 2009). High levels of glucose directly damage the endothelium, causing oxidative stress (OS), and advanced glycation end-products (AGEs) which alter blood flow and cause significant organ dysfunction (Ettelaie et al., 2008).

Diabetic retinopathy is the most common form of visual loss in the western world (Gale, 2008). It occurs due to microvascular damage in the retina and is associated with basement membrane thickening, increased capillary permeability and micro-aneurysms (Chronopoulos et al., 2011). These changes cause retinal ischaemia which drives the production of neo-vascularisation, poor lymphatic drainage and ultimately macular oedema (Yun and Adelman, 2015). Retinopathy is directly related to duration of diabetes and tends to be more prevalent in long term patients (Mathur et al., 2017).

Nephropathy is caused by elevated blood glucose in combination with hypertensive driven glomerular damage (Tan et al., 2013). Oxidative stress reduces

dilation of blood vessels in the kidney causing endothelial dysfunction and the release of cytokines, exacerbated by atherosclerosis, leading to significant kidney damage (Cade, 2008). This is thought to happen in 40% of T2D patients within 25 years (Tan et al., 2013). Aggressive BP reduction is recommended for the treatment of nephropathy. Angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARB) are recommended by NICE as a first line treatment. If adequate control cannot be achieved pharmacologically then renal replacement therapy is a last resort (NICE, 2017).

Diabetic neuropathy refers to a range of nerve disorders caused by diabetes. The condition is usually persistent and most commonly a distal, symmetrical sensorimotor neuropathy which affects both feet (Greig et al., 2014). Symptoms include tingling, numbness, weakness and pain (Malik, 2014). The risk of developing neuropathy is proportional to the magnitude and duration of hyperglycaemia (Greig et al., 2014). Initial management is achieved through good glycaemic control but once neuropathy is established glucose medications have little effect on the pain and analgesics and opiates are prescribed, dependent on severity (NICE, 2017). Tricyclic antidepressants such as amitriptyline have also shown promise, particularly in the early stages (Max et al., 1992).

Complications associated with neuropathy including ulceration, infection and charcot arthropathy, and are the main driving forces behind most foot amputations (Mascarenhas and Jude, 2014).

1.4.2 Macrovascular complications

Macrovascular complications of T2D are coronary artery disease, peripheral arterial disease and stroke (Fowler, 2008). Pathologically the macrovasculature is at greatest risk from atherosclerotic processes, which lead to the narrowing and eventual blocking of arteries leading to heart attacks or stroke (Bhupathiraju and Hu, 2016). It is thought that atherosclerosis is the result of chronic inflammation and oxidative stress that damages peripheral arterial walls (Dinh et al., 2017). Dyslipidaemia greatly increases the risk of macrovascular conditions and is common in T2D. Dyslipidaemia is characterised with abnormally high levels of LDL (Shishino et al., 2007). These particles penetrate and form strong attachments to arterial walls

before being oxidised. Once oxidised the immune system recognises LDL as “foreign” and results in a cascade resulting in the formation of atherosclerotic plaque (Chiesa et al., 1998). This causes a number of serious complications; angina is one of the most common and occurs if the heart is not receiving enough blood, resulting in pain and discomfort (Yao et al., 2017). Myocardial infarction and cerebral infarction occurs as a result of a blood clot that completely cuts off blood supply to the heart or the brain, both of which are life threatening (Xia et al., 2017). As many as 80% of T2D patients die from cardiovascular related complications (Grimaldi and Heurtier, 1999).

Lipids and blood pressure (BP) are aggressively treated in T2D to reduce CVD risk (NICE, 2016a). Statins are often first line therapy to reduce hyperlipidaemia due to their clinical effectiveness and low cost. A blood pressure target of 140/90 mmHg is set for any individual under 80 years of age (NICE, 2016a). First line therapy for BP control are ACE inhibitors or angiotensin receptor blockers. (NICE, 2016a).

1.4.3 Other associated complications

1.4.3.1 Gastrointestinal complications

Diabetes commonly causes problems with digestion, and results in a condition called gastroparesis. In healthy stomach the vagus nerve stimulates (Homko et al., 2016) contractions that drive food out via the pyloric sphincter and into the small intestine. Prolonged hyperglycaemia damages the vagus nerve and disrupts gastric emptying resulting in symptoms including abdominal pain, nausea, vomiting, heartburn and an over secretion of enzymes and acids (Koch et al., 2016). Gastroparesis can also result in bacterial infections from undigested food (Homko et al., 2016).

Proton pump inhibitors (PPI’s) are a group of medications that cause a long lasting and pronounced reduction in gastric acid production. NICE recommend their use for a number of conditions including; dyspepsia, peptic ulcer, gastroesophageal reflux disease and gastritis (NICE, 2014a). These drugs address a number of symptoms related to gastroparesis but do not treat the underlying damage to the vagus nerve or the delay in gastric emptying (Seol, 2006). It is recommended that strict dieting and lowering HbA_{1c} has the best clinical outcomes over the long term (NICE, 2014a).

1.4.3.2 Respiratory complications

Diabetes has been directly linked to a decline in lung function and numerous respiratory conditions (Bazdyrev et al., 2015). It is thought that those with diabetes are 8% more likely to get asthma, 22% more likely to get chronic obstructive pulmonary disease (COPD), 54% more likely to get pulmonary fibrosis and twice as likely to get pneumonia than the general population (Diabetes UK, 2014). Hyperglycaemic conditions cause systematic inflammation and oxidative stress impairing the immune system (Craig et al., 2009). A low oxygen environment and poor immune response allow for easy colonisation of bacteria such as streptococcus pneumonia (Rao, 2016). High blood glucose has also been shown to be a direct cause of pulmonary fibrosis, which negatively effects gaseous exchange causing shortness of breath and chest pain (Ehrlich et al., 2010).

1.4.3.3 Musculoskeletal disorders

T2D increases the risk of bone and joint disorders due to diabetic neuropathy, vascular disease and obesity. Osteoporotic fractures are most common, and higher in females than males (Xia et al., 2012), attributable to the effect of the menopause on bone mineral density and bone size (Sotornik, 2016). Insulin resistance, obesity, inflammation and the formation AGEs are associated with changes in bone structure and fracture risk in T2D (Xia et al., 2012).

Osteoarthritis (OA) is a chronic joint disease common at older age and can affect the knees, hands and hips (Thijssen et al., 2015). It is caused by the degradation of articular cartilage (Roach, 2008). The high incidence in T2D is likely due to obesity (Thijssen et al., 2015). Obese patients have increased loads on the joints, which breaks down collagen and proteoglycans disrupting cartilage integrity (Dong et al., 2017). Chondrocytes then undergo apoptosis reducing joint space and increasing friction between the bones, causing stiffness, swelling and pain (Thijssen et al., 2015). Diabetes tends to make arthritis worse; the inflammatory state induced by hyperglycaemia has been shown to increase pro-inflammatory cytokines Tumor

necrosis factor alpha (TNFα) and Interleukin 6 (IL-6) in the synovial fluid, increasing cartilage degradation and disease progression (Mobasheri et al., 2002).

Gout is another form of arthritis which most commonly affects men. Gout is associated with an increased risk of T2D (Rho et al., 2016). Gout is diagnosed when urate crystals saturate the serum (6 mg/dl) and is worsened if the patient is suffering from obesity (Vargas-Santos and Neogi, 2017). Uric acid crystallises in the joints causing inflammation and severe pain.

1.4.4 Psychological factors and Diabetes Mellitus

One third of individuals with diabetes are thought to have psychological and/or social problems which impact their ability to manage their condition (Garrett and Doherty, 2014). This is thought to be due to significant changes being required in how they live their lives which impacts relationships, work and finances (Penckofer et al., 2007). The most significant changes are the requirement for multiple medications throughout the day, and regular clinic or hospital visits to monitor response. This is known to have a negative effect on mental health, leaving individuals vulnerable to anxiety disorders, mild and severe depressive disorders and other emotional conditions (Rasmussen et al., 2013). Studies have shown that depression is prevalent in T2D, with patients more likely to suffer a depressive episode than the general population (Nouwen et al., 2010). Depression is a known risk factor for non-compliance in terms of diabetic regimes (Hasan et al., 2015) and many studies highlight links between depression and increased mortality in diabetes (Hofmann et al., 2013).

Treating and managing depression can be difficult. Surveys have indicated only 34% of patients with chronic or recurrent depression believe they receive adequate treatment (Bostwick, 2010). NICE do not offer any prescription guidance in relation to depression subtypes but instead focus on the effect certain types of anti-depressants have on other physical disorders (NICE, 2016b). There are 9 classes, with similar efficacy, but the severity of side effects can be quite different depending on the individual and their underlying conditions (NICE, 2016b). There is growing evidence that supports depression being a significant risk factor for the onset of Alzheimer's disease (AD) (Tsuno and Homma, 2009). Previous history of depression,

up to 10 years prior to AD diagnosis, doubles the risk (Ownby et al., 2006). Neuropathologically patients with a history of depression have been found to have substantially more hippocampal beta amyloid plaque and neurofibrillary tangle formation when compared to patients without a history of depression; pathology characteristic of AD (Rapp et al., 2006). This damage is thought to be due to sustained elevations in cortisol associated with a depressive state (Swaab et al., 2005). Some suggest that depression represents a pre-dementia syndrome as many patients with late onset depression (LOD) go on to develop AD within a couple of years (Chertkow et al., 2013).

1.4.4.1 Increased risk of Alzheimer's disease in T2D

Alzheimer's disease (AD) is the most common type of Dementia (Dening and Sandilyan, 2015) and T2D is a risk factor for development of AD (Biessels et al., 2006) with patients having a 60% increased risk of developing the condition if they are suffering from hyperglycaemia (Peila et al., 2002). Clinically most cases (60%) of AD are classified as late onset (LOAD) and characterised by a progressive memory loss and gradual cognitive decline (Luchsinger, 2010). AD is a progressive neurodegenerative disease, recognised by various neuropathologies including; neurofibrillary tangles (NFT), senile plaques and significant neuron and synapse loss in the cerebral cortex and other subcortical regions (Haroutunian et al., 2008). Substantial epidemiological evidence links T2D to AD (Ramirez et al., 2015). Both are characterised by various signalling mechanisms that are involved in insulin resistance, inflammation and oxidative stress (Malkki, 2015).

A model for cognitive decline in obesity is illustrated in Diagram 1. T2D and obesity are associated with poor cognitive function (Nameni et al., 2017). High BMI has been found to positively correlate with cognitive deficits and is consistent irrespective of age (Benito-Leon et al., 2013). HbA_{1c} is a significant risk factor for cognitive decline; elevations have been shown to correlate with increased incidence of all cause dementia and AD (Ramirez et al., 2015). Poor glycaemic control decreases brain integrity and is linked to reduced hippocampal volume (Ho et al., 2010). This is thought to be due to adverse brain changes caused by glucolipotoxity (Pugazhenthi et al., 2017). High glucose and FFA reduce cerebral perfusion and alter

hemodynamics (Alosco et al., 2013). This is known to affect brain regions essential for reasoning, attention, processing speed and verbal memory (Alosco et al., 2012). Macrovascular complications are also linked to cognitive decline (Ezzati et al., 2017). Hypertension, atherosclerosis and hyperlipidaemia have been shown to increase circulating inflammatory markers (Kasashima et al., 2017). Advanced glycation end products (AGE) are associated with elevated glucose and are implicated in cognitive decline associated with T2D, obesity and AD (Du Yan et al., 1996, Münch et al., 1998). Impaired insulin action and chronic hyperglycaemia increases the rate of cognitive decline (Jakobsen et al., 1990) and are associated with reduced amyloid beta (Aβ) degradation (Ravona-Springer et al., 2012). Diabetes patients have brain changes analogous to those in AD, such as reduced total brain volume, white matter hypertensities (WMH) and altered brain structure (Schmidt et al., 2004). This is thought to be due to tau and amyloid beta (Aβ) protein abundance (Luchsinger, 2012), high levels of Reactive Oxygen Species (ROS) and pro-inflammatory cytokines; key markers of insulin resistance in obesity, T2D and of deteriorating cognitive function (Block and Hong, 2005). A number of hormones and neuropeptides including leptin and ghrelin are also disrupted in T2D and obesity (Akieda-Asai et al., 2014), and leptin, in particular, has been shown to be neuroprotective, and important in cognitive function (Greco et al., 2008).

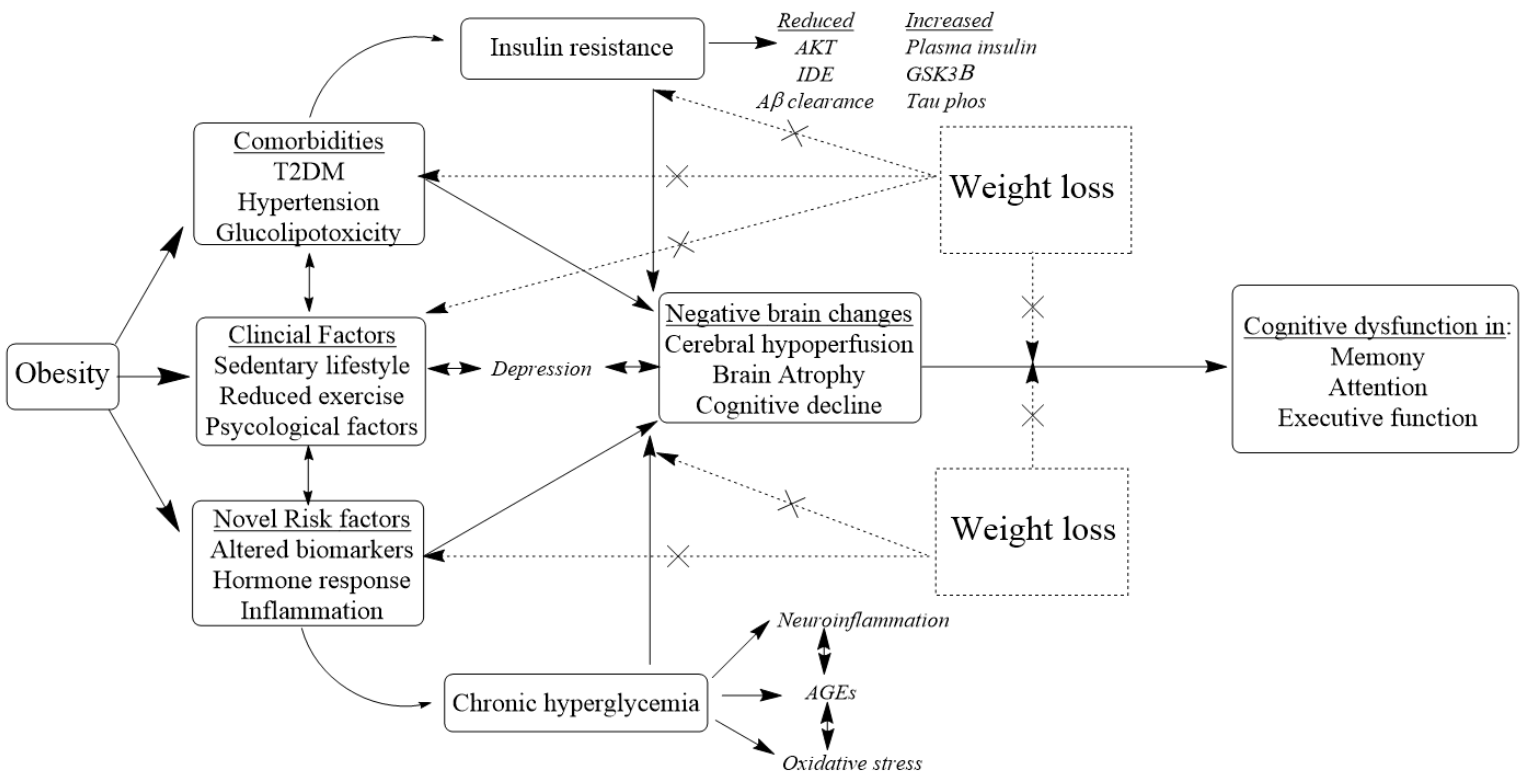


Diagram 1.1 – **Model for cognitive decline in obesity**. Obesity has a number of associated comorbidities, clinical features and novel risk factors (Weng et al., 2017). Insulin resistance and chronic hyperglycaemia are key pathologies in the development of adverse brain changes (Naderali et al., 2009) and have been implicated in the development of AD even in the absence of diabetes (Talbot et al., 2012a). Reduced insulin signalling and hyperinsulinemia results in hyperphosphorylation of GSK3β and tau, decreasing GSK3 enzymatic activity and increasing NFT formation respectively (Wick et al., 2003). Reduced PI3K/AKT signalling reduces IDE, ultimately increasing Aβ neuronal protein levels (Vekrellis et al., 2000). Chronic hyperglycaemia affects AD development via multiple mechanisms. High LDL and glucose increase the production of Advanced Glycation End products (AGE) that mediate RAGE (the receptor for advanced glycation end products) induced neuronal inflammation and oxidative stress (Slowik et al., 2012). Weight loss and eating a diet low in saturated fats has been found to have positive effects on cognition (Hardman et al., 2016)

In brain, insulin and insulin growth factors (IGFs) control synaptic maintenance and have neuroprotective qualities (Freude et al., 2009). The insulin receptor and IGF receptor type-1 (IGF-1R) are functionally and structurally similar allowing insulin and IGF-1 to activate both receptors (Laviola et al., 2007), to trigger auto phosphorylation of the insulin receptor substrate (IRS) (Gasparini and Xu, 2003). IRS is essential for the activation of various insulin signalling cascades including Protein kinase B (AKT), the mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3 (GSK3). Disruption of this substrate has been shown to result in severe insulin resistance (Wick et al., 2003).

Inflammation is a characteristic of T2D, obesity and cognitive decline and perpetuates insulin resistance (Ndumele et al., 2006). Mitochondrial injury induced by hyperglycemia can trigger the formation of proinflammatory protein complexes called inflammasomes, which cause the activation of Caspase 1 (CASP1) and neuronal secretion of IL-18 and IL-1β (Singhal et al., 2014). Another inflammatory pathway triggered by hyperglycaemia is the Mitogen-Activated Protein Kinase (MAPK) pathway, which contributes to cognitive decline (Chen et al., 2017) by regulating neuronal apoptosis and phosphorylating APP and tau (Blurton-Jones and LaFerla, 2006).

1.5 Implications for treatment of cognitive decline with current T2D medications

1.5.1 Metformin and Thiazolidinediones

It is well established that improved insulin action in neurons improves cognitive function (Yu et al., 2015). Metformin increases insulin sensitivity and reduces gluconeogenesis in liver (Tokubuchi et al., 2017). Preclinical data has indicated metformin can cross the BBB (Moreira, 2014). Hyperinsulinemic neuronal cells lines, when treated with metformin, can re-establish normal insulin signalling (El-Mir et al., 2008). Hippocampal neurons treated with metformin are protected against MAPK induced apoptosis and beta amyloid (Aβ) induced cytotoxicity (Chen et al., 2016). These protective effects are supported *in-vivo* in tau transgenic mice (Kickstein et al., 2010). Clinical studies have raised questions regarding the safety of metformin in AD.

One study assessed long term usage of SUs, TZDs, insulin and metformin on AD progression and indicated metformin was associated with a decline in cognitive function via an increase in beta secretase-1 (BACE1) (Imfeld et al., 2012).

TZDs regulate plasma FFAs via the PPARγ receptor (Kelly et al., 1999), which was thought to have a significant impact on cognition after it was found to be highly expressed in the temporal cortex of AD patients (Sato et al., 2011). Pre-clinical work demonstrates PPARγ agonists reduce pro-inflammatory genes and decrease Aβ plaques (Cramer et al., 2012), however clinical trial outcomes have been variable (Risner et al., 2006). There is currently a large phase III clinical trial underway assessing the effect of 24 months treatment with pioglitazone on mild cognitive impairment (MCI) in individuals with different apolipoprotein (APOE) status (Tomorrow trial, NCT1931566).

1.5.2 Glucagon-like peptide-1 receptor agonists (GLP-1Ra)

In brain GLP-1R activation has neuroprotective effects (Dunphy et al., 1998). *In vitro* GLP-1 has been shown to reduce apoptotic cell death and induce outgrowth of neuronal processes (Kimura et al., 2009). Studies with Val(8)GLP-1 (Gengler et al., 2012) and liraglutide in the APP(*swe*)/PS1(ΔE9) (APP/PS1) mouse model (McClean and Holscher, 2014) of AD have demonstrated significant improvements in learning and memory and reductions in AD pathologies. A clinical trial in early Alzheimer's disease is ongoing, Evaluating Liraglutide in Alzheimer's disease (ELAD, NCT01843075).

1.5.2.1 GLP-1 analogues currently licenced for the treatment of T2D

GLP-1Ras are designed to mimic the effects of native hormone GLP-1. This peptide is synthesised in intestinal endocrine cells and is one of two incretin hormones GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) (Drucker, 2001). GLP-1's main effect is known as the incretin effect, characterised by increased insulin secretion due to an oral glucose load, when compared to the same load administered intravenously (Nauck et al., 1986). This effect is thought to account for 60% of insulin secreted after a meal and has be shown to be impaired in T2D (Nauck and Meier, 2016). GLP-1 also has a number of other effects including delaying gastric

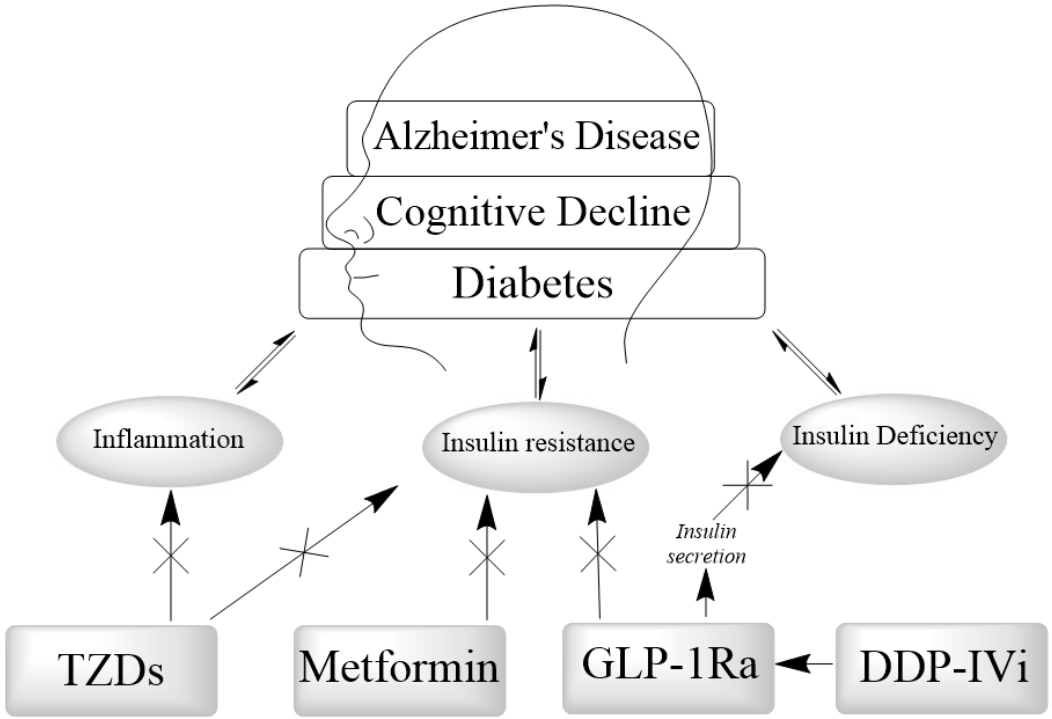


Diagram 1.2 –**T2D therapeutics for the treatment of cognitive decline and AD**. Inflammation, insulin resistance and insulin deficiency are characteristics of T2D and AD. TZDs such as pioglitazone have been shown to reduce fatty acid mediated inflammation and as a secondary effect improve insulin resistance (Kelly et al., 1999). Metformin has also been shown to improve insulin resistance. It is thought it achieve this by increasing insulin receptor activity, enhancing glycogen synthesis and augmenting GLUT4 translocation, all of which are compromised in AD (El-Mir et al., 2008). Stimulation of the incretin effect is a third mechanism by which cognitive deficits and AD may be targeted with T2D drugs. DPP-IVi and GLP-1a have been shown to increase insulin secretion and sensitivity as well as having a significant effect on appetite and weight loss (Nuffer and Trujillo, 2015). GLP-1Ras are capable of crossing the BBB and activating neuronal GLP-1Rs (Astrup et al., 2012). GLP-1R activation has various effects on insulin sensitivity, and insulin secretion via the activation of the cAMP signaling cascade, and leptin pathways (Kazafeos, 2011).

emptying, inhibiting glucagon secretion and regulating food intake (Astrup et al., 2012). Native GLP-1 is rapidly degraded within 2 min by DDP-IV (Kazafeos, 2011). Various enzyme resistant analogues have been developed to prolong its half-Life (Kalra, 2013). There are currently five GLP-1 analogues in use, albiglutide (Tanzeum©), dulaglutide (Trulicity©), Exenatide (Byetta© + Bydureon©), lixisenatide (Lyxumia©) and liraglutide (Victoza©), each with different pharmacokinetics and pharmacodynamics (Table 2) and adverse event profiles (Table 3).

Albiglutide is a recombinant peptide made up of two 30 amino acid sequences of altered human GLP-1 which is fused to human albumin (Bronden et al., 2017). It is administered once weekly and in clinical trials has been shown to lower HbA_{1c} by 11 mmol/mol (1%) (Rendell, 2017). It is considered less effective than liraglutide (1.8 mg) when used as add on therapy and does not have an effect on weight loss (Nauck et al., 2016). Injection site reactions are common but nausea is significantly less frequent than with other GLP-1R agonists (Rendell, 2017). Dulaglutide is also a once weekly analogue, it is covalently bonded to an Fc section of human immunoglobulin G4 (IgG4) and has been shown to reduce HbA_{1c} by 11 mmol/mol (Pozzilli et al., 2017). Interestingly, it is the only other GLP-1Ra that has not been shown to be inferior to liraglutide with respect to glycaemic control and weight loss (Morey-Vargas and Shah, 2014). The main reason it is not as widely used as liraglutide is its adverse advent profile. Along with the classical gastrointestinal side-effects it is also associated with various digestive system problems including gastrointestinal reflux disease, anaphylaxis, and pancreatitis (Dungan et al., 2016). Exenatide (Byetta©) was the first GLP-1Ra approved. It is a synthetic version of exendin-4, a hormone first isolated from Gila monster venom, and has 50% amino acid homology with native GLP-1 (Bhavsar et al., 2013). It is suitable for twice daily (Byetta©) and once weekly injections (Bydureon©). Byetta has the most severe adverse event profile out of all GLP-1 analogues. The most common are the classic incretin effects (nausea, vomiting and diarrhoea) but it also poses a significant risk of hypoglycaemia when used in combination with a SU (Simo et al., 2015). Injection site reactions, headaches, dizziness, anaphylaxis and pancreatitis have been reported (Diamant et al., 2014).

Drug	Pharmacokinetics									Pharmacodynamics				
	Reference	Injection Frequency	PPC	Bio availability	Cmax	Tmax	V.O.D	Excretion Rate	Excretion Method	Insulin Secretion	Glucagon secretion	β-Cell function	Gastric Emptying	Licenced Weight loss
Albiglutide 30 mg (Tanzeum©)	*(Bronden et al., 2017)*	1 weekly	5 days	/	/	/	11L	67ml/Hr	Ubiquitous protease	↑	↓	↑	↓	⊘
Dulaglutide 1.5 mg (Trulicity©)	*(Geiser et al., 2016)*	1 weekly	2 days	47%	114,000 pg/ml	36hrs	18L	100ml/Hr	Ubiquitous protease	↑	↓	↑	↓	⊘
Exenatide 2 mg (Byetta©)	*(Shi et al., 2012)*	2 Daily	2 hrs	25%	211 pg/mL	4hrs	28.3L	9.1L/Hr	Kidneys	↑	↓	↑	↓	⊘
Lixisenatide 10 mcg (Lyxumia©)	*(Werner et al., 2010)*	1 Daily	2.5 hrs	55%	84 pg/ml	3.5hrs	100L	35L/hr	Kidneys	↑	↓	↑	↓	⊘
Liraglutide 1.8 mg (Victoza©)	*(Astrup et al., 2012)*	1 Daily	11 hrs	55%	34 nmol/l	12hrs	25L	1.2L/hr	Ubiquitous protease	↑	↓	↑	↓	✓

Table 1.2 – **Pharmacokinetic and pharmacodynamic profile of currently available GLP-1Ras.** Clinical trial results have indicated that each GLP-1a analogue has different tissue specific effects (Kalra, 2013). The pharmacokinetic profile of each drug is different. Injection frequency varies from twice daily to weekly. Dependent on the frequency of injection the time at peak plasma concentration (PPC) is variable (Bronden et al., 2017). Bioavailability tends to be approximately 50%, with the exception of Exenatide. C_{max} is the maximum serum concentration the drug can achieve and T_{max} is the amount of time is takes to reach C_{max}. The volume of distribution (V.O.D) is the amount of liquid the drug needs to be distributed into be equal concentration to that of plasma. The excretion rate is how quickly the drug is removed from the body and excretion method describes the mechanism that may involve filtration by the kidneys or ubiquitous protease degradation (Geiser et al., 2016). Each analogue induces insulin secretion, reduces glucagon secretion, improves β-cell function and reduces gastric emptying. To date, only liraglutide has been licenced for weight loss (Morey-Vargas and Shah, 2014).

Drug	Adverse Events (Phase III clinical trials)		
	Common	Uncommon	Rare
Albiglutide 30 mg (Tanzeum©) (Nauck et al., 2016)	Injection site reactions, diarrhoea, nausea, hypoglycaemia when used in combination with insulin, pneumonia	Atrial fibrillation, hypoglycaemia when used in combination with metformin, gastro reflux	Pancreatitis, hypersensitivity reaction
Dulaglutide 1.5 mg (Trulicity©) (Pozzilli et al., 2017)	Nausea, vomiting, diarrhoea, hypoglycaemia when used in combination with insulin or SU	Fatigue, various digestion problems, injection site reaction	Anaphylactic reaction, acute pancreatitis
Exenatide 2 mg (Byetta©) (Shi et al., 2012)	Nausea, vomiting, diarrhoea, hypoglycaemia when used in combination with SU	Various injection site reactions, headache, dizziness, various digestion problems	Anaphylactic reaction, acute pancreatitis, ocular disorders
Lixisenatide 10 mcg (Lyxumia©) (Meier et al., 2015)	Nausea, vomiting, diarrhoea, hypoglycaemia when used in combination with SU	Hypoglycaemia when used in combination with metformin, dizziness, dyspepsia, injection site pruritus	Anaphylactic reaction
Liraglutide 1.8 mg (Victoza©) (Russell-Jones, 2009)	Nausea, Vomiting, Diarrhoea	Gastritis, abdominal pain, gastro reflux, injection site reaction	Renal impairment, pancreatitis

Table 1.3 – **Adverse events associated with currently available GLP-1Ras from phase III clinical trials.** Studies have indicated that GLP-1 analogues are commonly associated with gastrointestinal adverse events and some pose a risk of hypoglycaemia if used as a combination therapy (Danne et al., 2017). Each analogue has a different adverse event profile and affects individuals in different ways. Discontinuation of therapy can often be because of adverse events and not because patients have not responded to the medication. Exenatide is considered as having the most severe adverse event profile (Shi et al., 2012) while liraglutide has the mildest (Danne et al., 2017). It is important to recognise the different risk factors with each drug and take into account comorbidity when prescribing. Albiglutide, for example, is less associated with nausea when compared to liraglutide but is thought to pose a risk to CVD patients (Bronden et al., 2015).

Lixisenatide and liraglutide are more commonly prescribed in the clinic (Werner et al., 2010). Both drugs have been shown to have a similar pharmacodynamic profile but liraglutide has consistently demonstrated superior efficacy in head to head clinical trials (Meier et al., 2015). This is likely due to the formulation and structure of the peptides. Lixisenatide is a 44 amino acid peptide, derived from the first 39 amino acids of Exenatide (Werner et al., 2010). Liraglutide is 97% homologous to the native GLP-1 with one lysine to arginine substitution at position 34 and the addition of a C16 acyl group to lysine 26 (Neumiller, 2009). It tends to have a more sustained impact on HbA_{1c} and a greater propensity to induce weight loss (Davies et al., 2015). Real world evidence in the form of electronic care records (ECR) is now emerging and supporting the findings of clinical trials highlighting liraglutide as being the most effective GLP-1Ra available in the clinic today (Nyeland et al., 2015). Liraglutide also has the most favourable adverse event profile of the GLP-1Ras (Kaku et al., 2011).

1.5.3 Identification of potential genes and proteins that may be important in response to GLP-1 therapy derived from mechanism of action

1.5.3.1 Glucagon-like peptide-1 receptor signalling in the pancreas

The GLP-1R is a seven transmembrane domain protein and a member of the glucagon receptor family (Yang et al., 2016). It is wildly expressed in pancreatic islets, brain, gastrointestinal tract, heart and kidney (Dunphy et al., 1998). In the pancreas GLP-1 increases insulin gene transcription (Buteau et al., 1999) and replenishes β-cell stores (Xu et al., 1999). GLP-1R activation causes PDX1 protein levels to increase causing it to translocate to the nucleus and increase insulin gene promoter activity (Wang et al., 2005a). This activity results in phosphorylation of AKT and Forkhead box protein O1 (FOXO1). Upon activation FOXO1 is excluded from the nucleus which results in further disinhibition of PDX1 activity (Elghazi et al., 2006). GLP-1R activation also increases cAMP response element-binding protein (CREB). This transcription factor binds to cAMP response elements (CRE) on the insulin promoter gene increasing its transcription (Jhala et al., 2003).

GLP-1 is an effective stimulator of glucose-induced insulin secretion. Adenylate cyclase (AC) is positively coupled to its receptor (Bos, 2006). When

activated this catalyses the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) leading to the activation protein kinase A (PKA) and exchange protein directly activated by cAMP 2 (EPAC2) (Shigeto et al., 2017). Calcium entry carries Inositol trisphosphate receptors (IP_3R) and ryanodine (RYR) inside the cell which are activated by PKA and EPAC2, respectively. PKA then phosphorylates the K_{ATP} channel facilitating its closure ultimately regulating the docking and fusion of insulin granules to the plasma membrane (Seino and Shibasaki, 2005). It has been shown *in vivo* that G_s alpha subunit (G_{sa}) and the PI3K/AKT complex are the key proteins involved in GLP-1's β-cell protective effects. In a G_{sa} deficient mouse model β-cell proliferation is severely compromised resulting in reduced insulin secretion and β-cell mass (Xie et al., 2007).

A relatively new anti-apoptotic target of GLP-1 is the enzyme protein arginine methyltransferase-1 (PRMT-1) (Hashimoto et al., 2016). Studies in the diabetic kidney found that GLP-1R activation resulted in significant reductions in AGE-induced RAGE expression and reduced the generation of ROS (Ojima et al., 2013). GLP-1R activation was found to directly increase mRNA levels of PRMT-1 which negatively regulates asymmetric dimethylarginine (ADMA) which is essential in AGE induced ROS generation, and is elevated by oxidised LDL (Böger et al., 1998). Interestingly ADMA is also increased in AD (Selley, 2003) and is associated with vascular pathology and increased inflammation (Zhu et al., 2007). In kidney, in addition to its anti-inflammatory properties, PRMT1 has been shown to methylate FOXO1, critically blocking AKTs phosphorylation, allowing FOXO1 to translocate into the nucleus (Yamagata et al., 2008). It is well established that this process increases genes involved in cell death (Yamagata et al., 2008). Recent studies have identified PRMT1 as being widely expressed in the CNS and essential for healthy brain function. PRMT1 KO mice have significant brain abnormalities and die within two weeks of birth, due to substantial loss of oligodendrocytes (Hashimoto et al., 2016). Accordingly the effects of GLP-1 analogues on this enzyme are of interest.

1.5.3.2 Glucagon-like peptide-1 receptor signalling in the brain

The effect that insulin has in the brain is well documented (Vilsbøll et al., 2008). Acute action in the central nervous system, affects whole body metabolic function

(Obici et al., 2002b, Obici et al., 2002a), and emerging data has implicated it in memory and cognition (Benedict et al., 2007). GLP-1R activation is also known to regulate appetite and induce weight loss via an interaction with leptin (Goldstone et al., 1997b). Insulin and leptin are closely linked and both capable of crossing the blood brain barrier (BBB) and acting as adiposity feedback signals (Baskin et al., 1999). In obesity and T2D this mechanism is severely impaired, resulting in excessive weight gain (Kahn et al., 2006).

The relationship between the following factors is illustrated in Diagram 3. After Leptin binds to its receptor (LEP-R) it forms a complex with Janus kinase 2 (JAK2) (Agrawal et al., 2011). Under 'normal' conditions this causes the phosphorylation of signal transducer and activator of transcriptome 3 (STAT3), which results in its translocation into the nucleus and appetite reduction via Pro-opiomelanocortin (POMC) expression and neuropeptide Y (NPY) inhibition (Bates et al., 2003). The LEP-R / JAK2 complex is now thought to provide a link between appetite regulatory mechanisms and insulin signalling pathways (Morris et al., 2010). Src homology 2B 1 (SH2B1) protein has been found to enhance leptin's anti-obesity action by recruiting to the LEP-R/JAK2 complex and amplifying STAT3 activity; this recruitment has now also been shown to also allow for the binding and activation of IRS1 (Ren et al., 2007).

When IRS1 is activated via the IR it phosphorylates PI3K at its p85 subunit but when it is recruited to the LEP-R/JAK2/SH2B1 complex it activates PI3K at its second subunit (p110) (Duan et al., 2004). This indirect action allows for dual phosphorylation of PI3K and increased activity of its downstream target AKT (Cantley, 2002). This process increases glucose translocation, and FOXO1 phosphorylation allowing for increased CREB (insulin gene transcription) and POMC expression (appetite reduction) (Morris et al., 2010). The expression of SH2B1 is thought to be critical in the maintenance of leptin and insulin signalling; studies have found SH2B1 deficiency or various single nucleotide polymorphisms (SNPs) of the gene to result in severe leptin resistance, energy imbalance, obesity, and T2D (Jamshidi et al., 2007).

Tight regulation of STAT3 is essential for appetite regulation. High nucleic levels have been shown to increase the expression of suppressor of cytokine

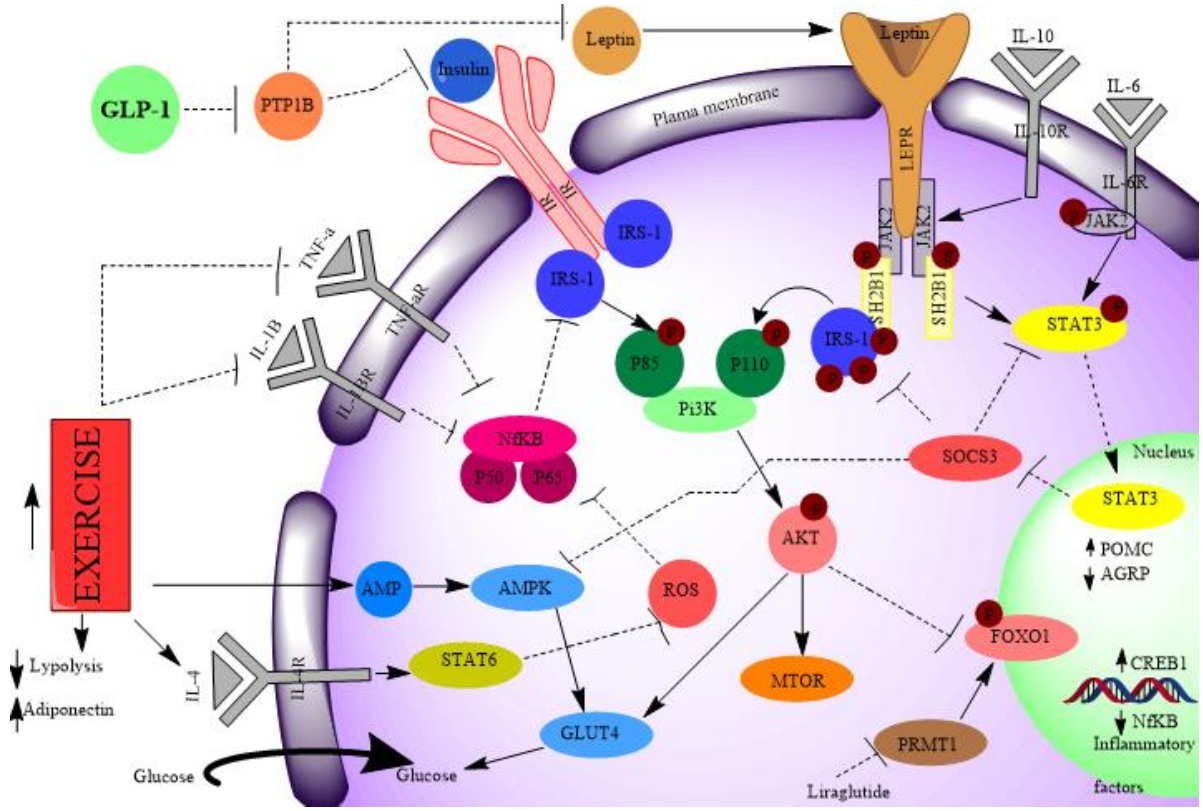


Diagram 1.3 – **Mechanism by which liraglutide treatment in brain affects insulin and leptin signalling improving glucose sensitivity, appetite and inflammation.** GLP-1 regulates appetite via the activation of the leptin signalling pathway. The binding of leptin to the LEP-R results in auto-phosphorylation of JAK2 and the recruitment of SH2B1. This triple complex has profound effects on both insulin and leptin signalling. It allows for increased phosphorylation and translocation of STAT3 into the nucleus to regulate POMC expression and appetite (Bates et al., 2003), but also permits IRS1 binding. IRS bound to the LEP-R/JAK2/SH2B1 complex can phosphorylate PI3K at its P110 subunit rather than its P85 subunit which has a positive effect on downstream AKT, inhibiting FOXO1 nuclear localisation, thereby increasing CREB activity and increasing glucose transport across the plasma membrane via GLUT4 (Ren et al., 2007). From an anti-inflammatory perspective treatment is thought to increase energy expenditure and movement, increasing adiponectin and decreasing lipolysis. This has been shown to result in reductions in IL-1β and TNFα and an increase IL-4. This expression pattern negatively regulates NFKB reducing its inhibitory effect on IRS1 signalling (Bjørbæk et al., 2000).

signalling 3 (SOCS3) (Bjørbæk et al., 2000) (Diagram 3). SOCS3 negatively regulates key aspects of both leptin and insulin signaling via suppression of STAT3 and IRS1 (Yang et al., 2012).

1.5.4 GLP-1's effect on leptin signalling

Both GLP-1 analogues and leptin therapies have been shown to have weight loss, neuroprotective and insulin sensitising effects (Ronveaux et al., 2015b). Recently attention has been drawn to potential combination therapies. Pre-clinical work in rats has shown that hypothalamic and hindbrain neurons are critical for GLP-1R and LEP-R interaction and that combined activation of the receptors in brain results in a more pronounced effect on weight loss than either receptor alone (Scott et al., 2011b). A follow up study showed that leptin increased pSTAT3, and this effect was increased when co-administrated with liraglutide (Kanoski et al., 2015). Tyrosine-protein phosphatase non-receptor type 1 (PTP1B) is a well-established negative regulator of JAK2, causing it to de-phosphorylate which inhibits STAT3 activation (Zabolotny et al., 2002b). GLP-1R activation has been shown to directly reduce PTP1B at the protein level, directly augmenting leptin activity (Kanoski et al., 2015).

In obese mice (*ob/ob*) leptin signalling is severely compromised and they are characterised by hyperleptinaemia (Münzberg et al., 2004). This effect is also documented in obese humans and is a result of leptin resistance (LR) (Caprio et al., 1996), whereby the body is resistant to circulating leptin, resulting in hyperphagia and increased bodyweight (Raposinho et al., 2001).

Recent clinical evidence no longer supports the notion that leptin is an appetite reducing hormone" but that it is an "anti-fasting" signal (Nuttall et al., 2016). This signal is activated as adiposity reduces, which in turn lowers circulating leptin, resulting in increased food seeking behaviour (Nuttall et al., 2016). Clinical evidence shows that liraglutide treatment over short and long term trials results in reductions in appetite and weight loss and reduced plasma leptin (Iepsen et al., 2015a). It has also been shown to have a significant impact on the leptin receptor (LEP-R) (Iepsen et al., 2015a), of which there are three classes; long (Ob-Rb), short (Ob-Ra) and soluble (Ob-Re) (Tartaglia, 1997). The long and soluble receptors have the greatest impact in the brain; the long form is the anchored receptor and

represents the primary signalling form in the brain and intestine (Tartaglia, 1997). Soluble leptin receptors are produced by proteolytic cleavage of anchored receptors and are the main binding protein in blood. Soluble receptors modulate leptin bioavailability (Gorska et al., 2010). Studies have shown a down regulation of anchored receptors and upregulation of soluble receptors during weight loss (Schaab et al., 2012). This expression pattern has been linked to decreased plasma glucose, insulin and PYY, and increased ghrelin supporting the anti-fasting signal hypothesis (Faraj et al., 2003).

Leptin therapy induces weight loss in lean and obese subjects (Heymsfield et al., 1999), and results in significant improvements in insulin resistance and ingestion behaviours (Petersen et al., 2002). Evidence from transgenic animal studies have shown leptin to have a similar effect to insulin on AD pathology (Greco et al., 2011). Human studies have shown that AD patients have particularly low leptin levels (Merlo et al., 2010) and elderly patients with high leptin levels are at a much reduced risk of developing AD compared to those with low levels (Lieb et al., 2009).

1.6 Biomarkers associated with glycaemic control and response to therapy

1.6.1 Biomarkers

Biomarkers can be any piece of information that functions as an indicator of physiological, pharmacological or pathological processes. In modern medicine they are essential for the development of personalised medicine (Falco et al., 2016). Over the past 20 years there has been an influx of new biomarkers published but very few have been accepted into routine healthcare. New markers tend to fail to meet clinical demands (Cramer et al., 2011). They either do not significantly impact patient care (Cramer et al., 2011) or do not affect clinical decision making. As such, T2D is still diagnosed clinically by HbA_{1c} (identified 1975) (Tattersall et al., 1975), FPG (identified 1946) (Hurwitz and Jensen, 1946) and OGTT (identified 1923) (CrawfordT, 1938). Within the Western Health and Social Care Trust (WHSCT) C-peptide is measured at the point of diagnosis, but is not measured routinely at diabetes clinics. This means that currently T2D management relies solely on HbA_{1c} measurement at 6-monthly appointments to assess if current therapies are adequately managing

glycaemia in T2D, leaving patients vulnerable to adverse reactions and development of secondary complications if they do not respond to the drugs they have been prescribed. There is much room for improvement.

Identification of blood based biomarkers (of glycaemic control and response to therapy in this case) is particularly clinically relevant due to minimal invasiveness, accessibility and low cost (Thambisetty and Lovestone, 2010). Blood based biomarker discovery is difficult, particularly in T2D and AD were the connection between peripheral expression levels and tissue specific levels are poorly understood (Glatt et al., 2005). Many of the current developments focus on how small alterations in a genes nucleic sequence can directly affect its function. These alterations are called Single Nucleotide Polymorphisms (SNPs) and can have profound effects on disease progression, drug response and metabolism (Jablonski et al., 2010). Studies have shown mRNA structure and gene expression alterations that occur as a result of SNPs, result in abnormal proteins that influence disease susceptibility (Harold et al., 2009).

1.6.2 Current pathogenic variants that may influence response to GLP-1 analogue therapy

Low cost genome wide arrays have facilitated large scale screening for SNPs (Akpinar et al., 2017). Classification of pathogenic SNPs is a high priority in medical science (Florez, 2017) and Genome-Wide Association Studies (GWAS) have had success in identifying variants associated with traits of disease (Chung et al., 2015). More recently studies have shifted focus to defining genetic variation indicative of drug response, but have had little success (Pollastro et al., 2015).

Targeted and whole genome sequencing has identified a number of leptin and insulin regulatory genes and polymorphisms that are associated with T2D (Table 4) (Ternouth et al., 2011). The GLP-1R gene has multiple variants but only one (rs10305492) has been associated with any significant clinical outcome (Scott et al., 2016). A large genome wide association study identified a non-synonymous variant of the GLP-1R (rs10305492) that was protective and resulted in reductions in fasting

Gene	Evidence	Chromosome	position	HG build	db SNP ID	Frequency %	Effect
GLP-1R	Strong	6	39046794	19	RS10305492	European (2.81%), Hispanic (0.66%), African American (0.22%)	Lowers fasting glucose, protects against CVD
POMC	Strong	2	25384833, 25385638	19	RS6713532, RS7565427	European (20.6%), Hispanic (49.6%), African American (37.8%), East Asian (41.2%)	Increases waist/hip ratio nutrient intake
SH2B1	Strong	16	28867804, 28881202, 28873398	19	RS7498665, RS8055982, RS4788102	European (37.2%), Hispanic (47.2%), African American (22.4%) South Asian (20.7%)	Increases BMI

Table 1.4 – **Pathogenic variants that effect insulin and leptin signalling identified in the type II diabetes knowledge portal.** The GLP-1R has been found to have one protective variant, thought to affect glucose and lipid metabolism (Wessel et al., 2015). The POMC gene has two pathogenic SNPs that negatively affect body weight and food intake (Ternouth et al., 2011). They have also been verified across multiple studies in various ethnicities (Lombard et al., 2012). There are three pathogenic SNPs linked to the SH2B1 gene all negatively affecting BMI, also verified in large clinical studies (Jamshidi et al., 2007). To date other SNPs present on these genes have not been associated with T2D traits or response to therapy (T2D-GENES Consortium, 2017).

glucose, reduced T2D risk and improved insulin secretion (Wessel et al., 2015), which was validated in another study (Scott et al., 2016).

Variants associated with the POMC gene have been shown to affect weight gain and eating behaviour; the two most prevalent and clinically significant are rs6713532 (Ternouth et al., 2011) and rs7565427 (Lombard et al., 2012).

The gene that codes for SH2B1 has become an interesting candidate for targeted genetic studies because of its relationship between insulin and leptin pathways. Many pathogenic variants are linked with this gene, particularly in Europeans (T2D-GENES Consortium, 2017). A Belgian study comparing obese and healthy lean individuals showed a 26% increased risk of obesity if rs7498665 was present (Beckers et al., 2011). Another study identified that this variant altered serum leptin and cholesterol levels (Jamshidi et al., 2007).

1.6.3 Cognitive screening in T2D

Early detection of cognitive dysfunction in T2D is critical for the management and treatment of the disease and its complications, however cognitive screening is not currently part of the care pathway.

The mini mental state exam (MMSE) is generally used in general practice on presentation of memory complaints. The Standardized Mini-Mental State Examination (SMMSE) is a second generation of the MMSE with specific guidelines on scoring, time allowed and administration to reduce intra test variability (-86%, $P<0.003$) (Molloy et al., 1991). It measures 2 domains of cognitive function; orientation and cognition over 12 questions taking approximately 10 minutes and is scored out of 30. Assessment cut off points have been developed to allow for maximum sensitivity and specificity and there is clear guidance on how scores should be adjusted for differing levels of education, disability and any language or cultural differences (Molloy and Standish, 1997). Generally any score above 26 is normal, 20-25 indicative of MCI, 10-19 moderate cognitive impairment and 0-9 indicative of severe impairment or AD.

The Quick mild cognitive impairment screen (qMCI) was designed to identify MCI. It is a short examination with high sensitivity and specificity for early alterations in cognition (O'Caoimh et al., 2013). It has been validated against many tests around

the world including the ABCS 135 in Canada (O'Caoimh et al., 2016), the SMMSE in Holland (Bunt et al., 2015) and the six item cognitive impairment test in Ireland (O'Caoimh, 2014). It is scored out of 100 and has been shown to take approximately 5 minutes to administer and score. From a design perspective there are 6 domains; orientation, registration, clock drawing, verbal memory (VM), verbal fluency (VF) and logical memory (LM). The greatest weight of marks are associated with VM, VF and LM and when this has been compared to other CSIs allowed for a more sensitive differentiation between MCI and normal cognition (O'Caoimh et al., 2013). It has been shown to improve sensitivity by measuring an individual's capacity to retell a story; ability has been shown to be unaffected by education or age. Cut off points for MCI and dementia are <62 and <36 respectively (O'Caoimh et al., 2012). Each instrument will be used to assess cognition in T2D with and without GLP-1 analogue therapy.

1.7 Electronic care records (ECR)

The progression of medicine has increased the need for advanced data management facilities capable of streamlining data capture, data storage and searchability (Adler-Milstein and Bates, 2010). Routinely recorded data on electronic care records (ECRs) include biochemical (HbA_{1c}, Lipids, BP), prescription, co-morbidity and patient contact details. This is recorded longitudinally and often displayed graphically (Menachemi and Collum, 2011). It can provide important insights into local populations and provide opportunities to characterise disease progression, drug efficiency and the effects of various interventions (Herrett et al., 2015). They can also act as "real world" verification of clinical trial outcomes in different sub populations, verifying findings and directly informing on best practice to improve patient care (Herland et al., 2005).

Current biomarkers, if used in conjunction with ECR data, may be capable of predicting future risk and even preventing disease. Research into CVD developed a new measure of cardiometabolic health by using routinely collected biomarkers in ECRs and generating a new measure that is directly proportional to chronic biological stresses, or the 'allosteric load' in CVD (McEwen and Stellar, 1993). This was validated in three cohorts of patients (diabetes, CVD, healthy), demonstrating that

the newly developed measure was just as capable of predicting biological stress levels as the standard, expensive cytokine measures (Nobel et al., 2017). A key feature of ECRs is the ability to collect longitudinal medical information. A recent study analysed 10 years' worth of data, focusing on BP variability in T2D patients and the risk of peripheral arterial disease (Yeh et al., 2016); high risk patients had high systolic and diastolic inter visit variability compared to low risk individuals.

The use of ECRs is becoming increasingly prevalent in clinical research. The main limitation with ECR data sets is that they are reliant on health care staff to input all information deemed relevant (Carrington and Effken, 2011). A study in the USA assessed the accuracy of the International Statistical Classification of Diseases and Related Health Problems (ICD-9) coding system in ECR databases (Chiu et al., 2017); results indicated that major discrepancies exist between actual and recorded comorbidities and that common T2D conditions such as obesity were severely under reported.

1.7.1 Future direction of ECR research

As ECR technology develops the incorporation of more data sources will occur (Purvis, 2015). The most significant and beneficial development for clinical research will be the addition of data from connected devices, including wearable technology. There have been huge developments in real-time 24/7 glucose monitors, heart sensors and "smart" inhalers (Coda et al., 2017). These devices can provide live biomarker readings to ECRs that can relay important healthcare advice back to the patient via platforms such as mobile phone apps (Westerik et al., 2016).

The ultimate goal is to develop fully integrated ECRs that include all standard clinical information from primary and secondary care and from real time connected devices (Ventola, 2014). This vision is supported across Europe through the Connected care consortium (CONNECARE) project; this work is planning to release an adaptive integrated ECR for chronic care management (Ferrer et al., 2016). The group hopes to support and nurture adaptive management and personalised care, empowering the patient by providing them accurate recommendations on how to manage their disease. We propose using ECRs, held locally, to characterise T2D

patients recruited in clinical studies to gain a comprehensive overview of comorbidity, polypharmacy and disease management.

Aims of the Thesis

The aim of the work described in this thesis was to define peripheral proteomic and genetic biomarker panels, suggestive of chronic central processes relating to glycaemic control, multimorbidity, cognitive decline, and response to GLP-1Ra therapy in T2D. The hypothesis of this thesis states that current clinical diabetes measures insufficiently stratify patients, ultimately potentiating poor anti-diabetes pharmacological response, high levels of comorbidity and increased cognitive decline. The identification of novel peripheral biomarkers indicative of T2D and response to GLP-1 analogue therapy, and assessment of the impact of GLP-1 analogues on cognition in T2D may be used to stratify GLP-1 therapy towards responders, and provide rationale for the use of GLP-1 analogues earlier in the T2D disease course

Five results chapters; one preclinical, four clinical, describe results relating to biomarker identification and clinical analyses. Preclinical findings were attained utilising GLP-1 RKO, APP/PS1 and C57BL/6 mouse models. Clinical data was acquired after the recruitment of participants to two clinical studies, DiaStrat (n=500), and GLP-1 response study (n=69).

To investigate the main hypothesis the following aims were addressed:

I. To explore the molecular mechanisms downstream of GLP-1R activation in brain and whole blood in animal models to identify potential markers of GLP-1 response.
II. To assess the clinical profile of the DiaStrat cohort, including prescribing patterns, comorbidities and pharmacological adverse reactions and evaluate characteristics in those receiving GLP-1Ra treatment compared to other drug classes.
III. To identify proteins associated with glycaemic control, anti-diabetes drug response and comorbidity in T2D.
IV. To identify SNPs in genes related to insulin and leptin signalling associated with T2D and response to GLP-1Ra treatment.

V. To assess the effect of GLP-1Ra therapy on depression, anxiety, and cognitive function in T2D and to correlate observed performance with Body Mass Index (BMI), HbA_{1c} and Full blood Count (FBC).

Chapter II

Materials and Methods

Preclinical Methods

2.1 Animals

2.1.1 APP*swe*/PS1ΔE9 mouse model of Alzheimer's disease

The APP*swe*/PS1ΔE9 mouse model of AD (APP/PS1) were originally acquired from Jackson laboratories (USA). These transgenic mice were created by coinjection of mouse/human chimeric amyloid precursor protein (Mo/HuAPP695swe) and the exon-9-deleted variant of human presinilin one (PS1-dE9), controlled by the mouse prion protein promoters in order to direct expression primarily to the CNS. Each mutation has a different pathogenic affect, the APP mutation increases the affinity of APP for β-secretase resulting in increased Aβ production. The PS1 mutation causes a significantly greater Aβ42- Aβ40 ratio ensuring increased plaque deposition (Maia et al., 2013). APP/PS1 positive males were bred with C57BL/6 females from a colony maintained within the Biomedical and Behavioural Research Unit (BBRU) at Ulster University.

All male mice used were genotyped, using ear snips obtained at approximately 3 weeks of age, upon weaning. Animals were anaesthised briefly, using isofluorane (Abbott, Berkshire, UK), and surgical scissors were used to remove a small portion of tissue from the mouse ear, which was placed into a labelled 0.2 ml PCR tube (VWR International, Radnor, PA, USA, Cat. No. 732-0548) and kept on ice until needed. Scissors, forceps and worktops were cleaned with 70% ethanol between sampling in order to avoid cross-contamination. DNA was extracted using alkaline lysis buffer (75 µl; 25 mM NaOH, 0.2 mM Na_2EDTA; pH12) which was added to each tube and heated to 95^{o}C for 30 min using a TC-5000 gradient thermal cycler (Techne TC-5000, Davidson & Hardy Ltd UK). Samples were then cooled to 4°C and 75µl of neutralising buffer (40 mM Tris-HCL; pH5) was added to each tube and mixed thoroughly using pipette.

Polymerase chain reaction (PCR) was conducted using a Taq PCR master mix kit (Qiagen, West Sussex; Cat.No. 210443) using APP primers to detect the presence of the transgene (forward "GAATTCCGACATGACTCAGG", reverse "GTTCTGCTGCATCTTGGACA") and mPrP primers as a negative control (forward "CAGCAC CGCTGAAATCTAAT", reverse "TTGCTGCCAATACTGAGACA."

DNA (1 µl) from each animal was added to a prelabelled PCR tube with 12.5 µl taq PCR mastermix, 100 pmol of each of the primers above (0.5 µl of each) and 9.5 µl RNAse free H_2O Negative controls with RNAse free water (1 µl), and positive controls with DNA from a previously confirmed APP/PS1 positive mouse (1 µl) were also run. Samples were then placed in a PCR thermocycler for 5 min at 95°C, followed by 35 cycles of 45 sec at 95°C, 45 sec at 58°C and 45 sec at 72°C. Samples were then held at 72°C for 5 min, before the cycler was cooled to 4°C and samples removed.

Gel electrophoresis was then conducted using 1% agarose gels prepared in TAE buffer (40 mM Tris Base (pH 7.6), 20 mM acetic acid and 1 mM Na_2EDTA). Agarose was dissolved by microwave heating, and, on cooling, 5 µl per 100 ml ethidium bromide (Sigma-Aldrich, St Louis, Mo, USA; Cat. No. E1510) was added and the solution poured into casting trays (Embi Tec, San Diego; Cat No.EP1001) and left to cool.

The RunOne Electrophoresis System (Embi Tec, San Diego; Cat No.EP2000) was then filled with 1x TAE buffer and gels inserted. Samples and controls (12 µl) were mixed with 3 µl Orange G (20 mg Orange G in 10 ml of 30% glycerol) and carefully loaded. The gel was run at 100 V for 40 min. Gels were then visualised on a UV Transilluminator (Biorad, Richmond, CA, USA) and photographed using a digital camera (Kodak 1D^{TM}).

Positive males and wild-type controls were used for all experiments and kept individually in standard cages in a temperature (T: 21.5 ± 1°C) and light (12 h light/dark) controlled environment. Food and water access was *ad libitum.* For subsequent experiments mice were separated into two aged defined groups. The first group (n=5) was aged for 8 weeks before sacrifice and used to assess how the APP/PS1 genotype affected our genes of interest before any significant Aβ42 burden was apparent compared to age-matched wild-type (WT) controls (n=5). The second group was aged for 10 months (n=9) and separated into two subgroups treated (n=6) and untreated (n=3). These were used to assess the effect 7 days 25 nmol/kg body weight liraglutide treatment on our panel of genes after significant AD pathology develops, and were compared to age matched WT saline (n=6) treated controls.

2.1.2. GLP-1 RKO model

Brain tissue, and plasma, previously collected from GLP-1 RKO mice was used (n=4) in preclinical studies. Mice were bred in house at the Ulster University (UU), Coleraine, but the original generation is described elsewhere (Lamont et al., 2012). Phenotypically these mice are known to exhibit normal satiety, fasting hyperglycemia, normal peripheral glucose utilisation and increased insulin sensitivity (Ayala et al., 2010). Male mice were used for all experiments. Animals were housed individually and fed *ad libitum* in an air conditioned (T:21.5±1°C), light (12h light/dark) controlled room. Whole brains and plasma were obtained at 18 months and used to assess how our panel of appetite and insulin regulatory genes are affected in the absence of the GLP-1R.

2.1.3 C57BL/6 Wild type controls

C57BL/6 were used for further experiments due to them being used as the background strain for our transgenic models, and also because they are a well-established and commonly used WT model in the literature. They are easily bred, long lived, have a low tumor risk and high susceptibility to diet induced obesity and diabetes, making them a good model to assess anti-diabetic treatments (Mogil et al., 1999).

At 8 weeks of age mice were separated into two groups, one received 7-days saline treatment (n=12) and the other 7 days liraglutide treatment (n=12), Blood samples were obtained from the tail vein at baseline and on days 1-7. At the end of 7 days treatment whole brains and 2 aliquots of whole blood (1x 50 µl for gene analysis 1x 100 µl for plasma) were removed for genetic, proteomic and cytokine analysis.

Brain tissue from a previous experiment from 11 week old C57BL/6 mice who had received 21-day saline or liraglutide treatment from 8-11 weeks was also assessed (n=6 per group).

The purpose of these experiments was to determine mRNA alterations in brain in response to 7 and 21 days liraglutide treatment and to assess if brain changes may be detected from peripheral whole blood.

All animal experiments were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 and approved by the UU animal ethics committee. All precautions were taken to ameliorate any possible suffering. Animals were handled daily for 2 weeks prior to experiments, and sacrificed using inhaled anaesthetic isoflurane followed by cervical dislocation. All excised tissues were snap frozen in liquid nitrogen and then stored at -80°C until required.

2.2. Glucose Tolerance Test

A glucose tolerance was conducted to determine the acute effect of liraglutide. After an 8 hour fast, body weight was taken and liraglutide or saline was administered by intraperitoneal (i.p) injection in both C57BL/6 and APP/PS1 mice. Glucose was administered 30 min later (18 mmol/kg of body weight) in 0.9% NaCl. Blood was taken from the tail vein to measure basal glucose, then after glucose injection blood glucose reads were taken at 15, 30, 60 min, using the Ascencia Contour blood glucose meter (Bayer Health Care, UK).

2.3. Peptide

Liraglutide (MW 3751.202 g/mol) was obtained from EZ biolabs (Indiana, USA). Purity was analysed previously and determined to be > 99%. Aliquots were stored in polypropylene tubes at a concentration of 1 mg/ml diluted in ultrapure® water and kept at -80° C to permit fresh preparation, as required.

2.4. Quantitative polymerase chain reaction (qPCR)

This technique was used to measure the relative expression of 13 genes of interest. In relative qPCR all CT values must be normalised to a reference gene that is stable between genotypes and treatments. Common references are βACT, GAPDH, RNS16-18. These are then inputted into a mathematical model, known as the ΔΔCt method, which makes the assumption that the normalised CT value of the target gene is relative to the normalised reference gene.

2.4.1 RNA extraction

2.4.1.1 Brain tissue

RNA was obtained from whole brain tissue using the RNeasy lipid tissue mini kit (Qiagen Ltd, Manchester, UK; Cat. No. 78404). Each brain was thawed on ice before being homogenized using the Fisher Scientific™ disposable pestle system (Fisher Scientific, Loughborough, UK; Cat. 03-392-103) in 1 ml of QIAzol lysis reagent (Qiagen Ltd, Manchester, UK; Cat. No. 79306). Homogenisation continued until the sample was uniformly dispersed in lysis buffer and then left for 5 min at room temperature (20°C) to promote the dissociation of nucleoprotein. Chloroform ($CHCl_3$, 200 µl) was then added and the sample vortexed for 15 sec to ensure maximum phase separation. The homogenate was incubated for a further 3 min at room temperature before a 15 min centrifugation at 12,000g (4°C). Post centrifugation three distinct phases, organic phase (protein), interphase (DNA) and aqueous phase (RNA) were visible. The aqueous phase (approximately 500 µl) was removed without any DNA contamination and transferred to a new polypropylene tube. One volume of 70% ethanol was added and mixed by vortexing for 15 sec. The sample was transferred to an RNeasy mini spin column and placed in a 2 ml collection tube. It was then centrifuged for 15 sec at 8000g (20°C) and the flow through discarded. RNA captured on the RNeasy mini spin column membrane was then washed using 700 µl RW1 buffer (flow through discarded) and 500 µl RPE buffer (flow through discarded), each was spun through the column at 8,000 g (20°C) for 15 sec. The sample was then washed for a final time with 500 µl RPE buffer and centrifuged for 2 min at 8,000g (20°C). The RNeasy mini column was then transferred to a fresh polypropylene tube and total RNA eluted (8000 g, 20°C, 1 min) in 50 µl RNase free water. All samples were then stored at -80°C until required.

2.4.1.2 Whole Blood

RNA from 50 µl whole blood was obtained using the QIAamp RNA blood mini kit (Qiagen Ltd, Manchester, UK; Cat. No. 52304). Blood samples were thawed on ice then transferred to a fresh polypropylene tube and 250 µl of buffer EL (Qiagen Ltd, Manchester, UK; Cat. No. 79217) was added. All samples were then incubated for 30 min on ice, to allow for maximum red blood cells (RBC) lysis, before being centrifuged at 400 g (4^{o}C) for 10 min. This lysis step was repeated to ensure minimal RBC contamination. After discarding the supernatant the pellet was re-suspended in 1ml buffer RLT (Qiagen Ltd, Manchester, UK; Cat. No. 79216) plus 10 µl 98% 2-Mercaptoethanol (Sigma-Aldrich, Irvine, UK, Cat. No M3701) and vortexed at high speed to mix. The solution was then transferred to a QIAshredder spin column and placed in a 2 ml collection tube before being spun at 20,000 g for 2 min at 4^{o}C. The homogenized lysate was kept and 600 µl of 70% ethanol added. This was then transferred to a new QIAmp spin column and placed in another 2 ml collection tube and spun for 15 sec at 8000 g. RNA captured on the QIAmp spin column membrane was then washed using 700 µl RW1 buffer (flow through discarded) and 500 µl RPE buffer (flow through discarded), each was spun through the column at 8,000 g (20°C) for 15 sec. The sample was then washed for a final time with 500 µl RPE buffer and centrifuged for 3 min at 20,000g (20°C). The QIAmp spin column was then transferred to a fresh polypropylene tube and total RNA eluted (8000g, 20°C, 1 min) in 20 µl RNase free water. Samples were then stored at -80°C until required.

RNA quantity and quality was determined using a nanodrop (purity criteria was set at 260/280, >1.8, <2.2, and 260/230 >2.0, <2.2, Nanodrop 2000c, ThermoFisher Scientific, Wilmington, DE). Once quantified complementary DNA (cDNA) Synthesis was carried out immediately.

2.4.2 Complementary DNA (cDNA) Synthesis

For complementary DNA (cDNA) synthesis a Transcriptor First Strand cDNA synthesis kit was used (Roche Diagnostics Ltd., West Sussex, UK; Cat. No. 04379012001). A “master mix” was then prepared in a 1.5 ml polypropylene tube containing (‘individual volumes described’ x ‘number of samples’); 4 µl Transcriptor RT reaction

buffer 5x concentrated (final concentration 8 mM $MgCl^2$), 0.5 µl Transcriptor reverse transcriptase (RT) 20 U/µl (final concentration 10 U; Roche Diagnostics Ltd., West Sussex, UK; Cat. No. 03531317001), 0.5 µl Protector RNase inhibitor 20 U/µl (final concentration 20 U; Roche Diagnostics Ltd., West Sussex, UK; Cat. No. 03335399001), 2µl Deoxynucleotide Mix, 10 mM each (final concentration 2 mM each; Roche Diagnostics Ltd., West Sussex, UK; Cat. No. 11969064001), 2 µl random hexamer primer 600 pmol / µl (final concentration 60 µM; Roche Diagnostics Ltd., West Sussex, UK; Cat. No. 11034731001).

Total RNA (500 ng from brain, 100ng from whole blood) was added to a 200 µl PCR tube, with 9 µl of the "master mix" above and the final reaction volume brought to 20 µl in RNase-free water (Roche Diagnostics Ltd., West Sussex, UK; Cat. No. 03315932001). The reaction was conducted on a Techne prime thermo cycler (Applied Biosystems, USA) with cycling conditions of 10 min at 25°C, followed by 1 hour at 50°C and 5 min at 85°C.

2.4.3 qPCR

All qPCR reactions consisted of template cDNA (25 ng/µl for brain and 5 ng/µl for whole blood) and 9 µl of master mix. Master mix was composed of; 5 µl LightCycler® 480 Probes Master (Roche Diagnostics Ltd., West Sussex, UK; Cat. No. 04710436001), 1µl (10 pM/µl) gene-specific probes and 3µl of RNase free water (Roche Diagnostics Ltd., West Sussex, UK; Cat. No. 03315932001). Real-time ready Custom Single Assays were used for all experiments (Roche Diagnostics Ltd., West Sussex, UK) and consisted of; GLP-1 receptor (Glp1r *M. musculus,* Assay ID 314784, transcript length 1480bps, amplicon length 71 bps), GIP receptor (Gip *M. musculus*, Assay ID 316856, transcript length 1792 bps, amplicon length 92 bps), LEP-R (LEP-R *M. musculus*, Assay ID 310938, transcript length 4127, amplicon length 69 bps), STAT3 (Stat3 *M. musculus*, Assay ID 316898, transcript length 4516, amplicon length 93 bps), POMC (Pomc *M. musculus*, Assay ID 316887, transcript length 1031 bps, amplicon length 64bps), GHSR (Ghsr *M. musculus*, Assay ID 317858, transcript length 4433, amplicon length 89 bps), CREB1 (Creb1 *M. musculus*, Assay ID 300049, transcript length 8420 bps, amplicon length 76 bps), PRMT1 (Prmt1 *M. musculus*, Assay ID 317397,

transcript length 1360 bps, amplicon length 89 bps), IRS-1 (Irs1 *M. musculus*, Assay ID 301044, transcript length 9144 bps, amplicon length 93 bps), JAK2 (Jak2 *M. musculus*, Assay ID 300439, transcript length 5030, amplicon length 68 bps), SH2B1 (Sh2b1 *M. musculus*, Assay ID 318310, transcript length 3317 bps, amplicon length 72 bps), SOCS3 (Socs3 *M. musculus*, Assay ID 300387, transcript length 2552 bps, amplicon length 126 bps), AKT (Akt1 *M. musculus*, Assay ID 317539, transcript length 2690 bps, amplicon length 122 bps), RNS18 (Rn18s *M. musculus*, Assay ID 307906, transcript length 1870 bps , amplicon length 106 bps).

All qPCR reactions were pipetted onto a white, 96-well LightCycler® 480 Multi-well Plate (Roche Diagnostics Ltd., West Sussex, UK; Cat. No. 04729692001). The plate was then sealed with an adhesive plate cover and centrifuged at 1500 RPM for 2 min. The plate was then placed in Roche LightCycler 480® (Roche Diagnostics Ltd., Switzerland) and exposed to 95^{o}C for 10 min followed by 55 cycles of 95°C for 10 sec, 60°C for 30 sec and 1 second at 72°C. CT values were recorded on the accompanying software package (Roche, LightCycler 480 software, v1.5). Quantification was conducted by comparing mean CT values of target gene to housekeeping gene using the $2^{\Delta\Delta CT}$ method.

2.5. Western blot

2.5.1 Total protein extraction

Whole hemibrains were used for all protein extractions. Brains were defrosted on ice and homogenised using the Fisher Scientific™ disposable pestle system (Fisher Scientific, Loughborough, UK; Cat. 03-392-103) in a polypropylene tube containing 500 µl RIPA buffer (25 mM TrisHCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Thermo scientific, UK; Cat. No. 89900) and Pierce Protease Inhibitor (Thermo scientific, UK; Cat. No. A32965). Samples were kept on ice and vortexed at 5 min intervals for 20 min. Homogenates were centrifuged at 20,000 g for 20 min at 4^{o}C and supernatant transferred to a fresh polypropylene tube for quantification.

2.5.2 Determination of protein concentration

Protein concentration was determined using a Pierce™ BCA Protein Assay Kit (Thermo scientific, UK; Cat. No. 223227). Each protein sample was diluted 1:10 and 1:20 in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.4) and run in duplicate. Protein standards were prepared from Bovine Serum Albumin (BSA) over 9 concentrations; A- 2000 µg/ml, B- 1500 µg/ml, C- 1000 µg/ml, D- 750 µg/ml, E-500 µg/ml, F-250 µg/ml, G-125 µg/ml, H-25 µg/ml, I-blank. All samples and standards (10 µl) were added to individual wells on a 96 well plate and 15 µl of PBS added before the addition of 200 µl BCA working reagent (WR). The total WR volume was determined using *(# standards + # unknowns × # replicates × 200 µl) and* was prepared by mixing 50 parts BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and 1 part BCA reagent B (4% cupric sulphate; Thermo scientific, UK; Cat. No. 23227). Samples were then incubated at 30^{o}C for 30 min, before being allowed to cool for 2 min. Absorbance was then read on a BioTek Epoch Microplate Spectrophotometer (BioTek, UK, Cat. No. 15306176) at 562 nm. The absorbance values for the standards were used to plot a standard curve, from which unknown protein concentrations were determined.

2.5.3 Sample preparation

All samples (50 µg) were prepared in polypropylene tubes, to which 5 µl of NuPAGE™ LDS Sample Buffer (4X; Thermo scientific, UK; Cat. No. NP0008) and 2 µl NuPAGE™ Sample Reducing Agent (10X; Thermo scientific, UK; Cat. No. NP0004) was added. Samples were then made up to a final volume of 20 µl with deionised water, and heated at 70^{o}C for 10 min immediately prior to electrophoresis.

2.5.4 Western blot (SDS-page)

All gels were run in an XCell SureLock™ Mini-Cell Electrophoresis System (Thermo scientific, UK; Cat. No. EI0002), submerged in 1 litre of NuPAGE™ MOPS SDS Running Buffer (1X) (Thermo scientific, UK; Cat. No. NP0001) and 100 µl of NuPAGE™ Antioxidant (Thermo scientific, UK; Cat. No. NP0005). Samples were loaded into 10-

well NuPAGE™ 4-12% Bis-Tris, gradient protein gels (Thermo scientific, UK; Cat. No. NP0335BOX). Novex™ Sharp Pre-stained Protein Standard (7 µl; Thermo scientific, UK; Cat. No. LC5800) was loaded as a molecular weight control. Gels were then run for 20 min at 90 volts and a further 1 hour at 150 volts. They were then transferred on to a methanol (99.9%) soaked Polyvinylidene difluoride (PVDF) membrane (Thermo scientific, UK; Cat. No. LC2005) using the XCell II™ Blot Module (Thermo scientific, UK; Cat. No. EI0002). This was carried out in 1 litre of NuPAGE™ Transfer Buffer (1X + 10% methanol; Thermo scientific, UK; Cat. No. NP0006) at 30 volts for 1 hour. All membranes were blocked in 5% milk / 0.05% PBS-Tween (PBS-T) for 1 hour before overnight incubation at 4^{o}C with primary antibody. All overnight incubations were left on an analogue tube roller (Stuart, UK. Cat. No SRT6).

Antibodies were; β-actin (Cell Signalling, Cat. No 4970, Rabbit, MW45, dilution 1:1000); Total IRS-1 (Abcam, Cat. No ab52167, Rabbit, MW180, dilution 1:500); phospho IRS-1 S616 (Abcam, Cat. No ab4776, Rabbit, MW165, dilution 1:100); Total STAT3 (Cell Signalling, Cat. No 9132, Rabbit, MW79/82, dilution 1:1000); phospho STAT3 T705 (Cell Signalling, Cat. No 9145, Rabbit, MW79/86, dilution 1:1000); AKT (Cell Signalling, Cat. No 4685, Rabbit, MW60, dilution 1:1000); phospho FOXO1 (Cell Signalling, Cat. No 9461, Rabbit, MW82, dilution 1:1000); POMC (Cell Signalling, Cat. No 23499, Rabbit, MW10/28/32, dilution 1:1000); SOCS3 (Cell Signalling, Cat. No 2923, Rabbit, MW26, dilution 1:1000); GLP-1R (BIOSS, Cat. No bs1559R, Rabbit, MW53, dilution 1:100); GIP-R (BIOSS, Cat. No bs13292R, Rabbit, MW53, dilution 1:100).

After overnight incubation in primary antibody membranes were washed three times for 5 min with 0.05% PBS-T and then incubated at 20^{o}C for 1 hour in Anti-rabbit IgG, HRP-linked secondary antibody (Cell signalling, Cat. No 7074, Rabbit, dilution 1:1000). Blots were then washed another three times with 5% PBS-T before being incubated in SuperSignal Chemiluminescent Substrate (Thermo scientific, UK; Cat. No. 34080). Working Solution was generated by mixing equal parts of the Stable Peroxide Solution and the Luminol/Enhancer Solution. Blots were then imaged using the UVP Chemidock Imaging System (Analytik Jena, Germany, model It2 515, Cat. No 97-0685-04). Membranes were exposed for 10 min and densitometry carried out on ImageJ-1x (Schneider et al., 2012) open source software.

2.6. Meso Scale Discovery Proinflammatory panel 1 analysis

Plasma Cytokines IFNγ, IL1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, TNF-α and chemokine KC/GRO (CXCL1) were measured using the Meso Scale Discovery (MSD®) Multi-spot Assay Pro-inflammatory panel 1 (mouse) kit (Meso Scale Discovery, Rockville, MD, USA, Cat. No. K15048D), according to the manufacturer's instructions. Plasma (20 µl) was transferred onto a 10-spot Multi-spot® 96-well plate, which was pre-coated with capture antibodies on individual, well-defined assay spots. The plate was sealed with an adhesive plate cover and incubated at 4°C overnight to allow for maximum antibody binding. Excess sample was then decanted off and the plate washed three times using 150 µl 0.05% PBS-T per well. The 10 specific detection antibodies where then prepared with Diluent 45 in a master mix at 1:50 dilution. These were added (25 µl) to each well and the plate resealed with a fresh adhesive plate cover before shaking for 2 h at room temperature. The final step involved washing each well a further three times with PBS-T and adding the Read Buffer T (150 µl) at a working concentration of 2x. The plate was then read on the MSD instrument.

The MSD® is a desktop device that allows for highly sensitive (dynamic range; LLOD 2.7 pg/ml – UPOD 150-1500 pg/ml), rapid (90 sec read time), cost effective, multiplex cytokine analysis. MSDs electrochemiluminescence detection technology uses SULFO-TAG™ labels. These emit light after electrochemical stimulation, initiated at the electrode surface. Accurate, reproducible multiplex is achievable via MSD 10-spot® assay system.

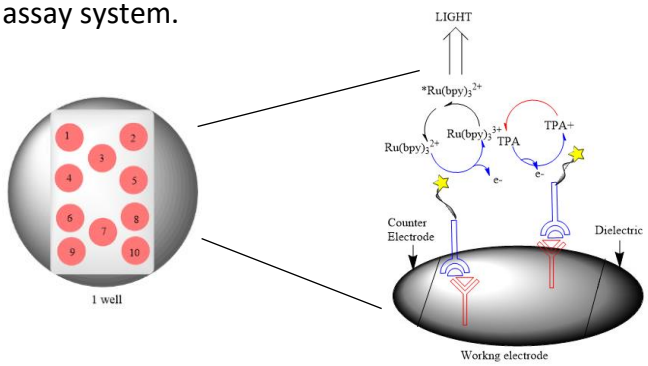


Diagram 2.1 - **MSD 10-spot® assay.** Each spot is coated with a specific antibody. After applying the sample and exciting with electricity a chemical reaction occurs involving Tris (bipyridine) ruthenium (Ru (bpy) and terephthalic acid (TPA), which ultimately generates light, emitted at 620 nm. There is very low background as stimulation mechanism is decoupled from the signal. Only labels bound to electrode surface are detected.

Clinical methods

2.7 Participant Recruitment

2.7.1 DiaStrat recruitment

Participants were identified by clinicians at the Altnagelvin Hospital, diabetes clinic, in Northern Ireland. Eligibility was determined by inclusion and exclusion criteria. Participants had to be clinically diagnosed with type 2 diabetes and be between the ages 18—80. If type 1, secondary or gestational diabetes was present individuals were excluded. Eligible participants were provided a Participant information sheet which included the aims and ethical considerations of the research. After a 48 hour cooldown period, participants were contacted regarding their willingness to participate. Those who agreed to participate were invited back to the clinic, to permit completion of the informed consent process and sample collection.

2.7.2 GLP-1 response study recruitment

Participants were identified by their clinician at the Altnagelvin Hospital, diabetes clinic, NI. Eligibility was determined by strict inclusion and exclusion criteria. Participants had to be clinically diagnosed with; 1. Non-Diabetes (n=19), 2. T2D + GLP-1Ra naïve (n=13), 3. T2D + GLP-1Ra responder (n=22), 4. T2D + GLP-1Ra non-responders (n=15), and be between the ages 18—100. GLP-1Ra response was determined by either a recent 1% reduction in HbA_{1c}, 3% reduction in body weight or both. If type I, secondary or gestational diabetes was present individuals were excluded. Eligible participants were provided a participant information sheet which included the aims and ethical considerations of the research. After a 48 hour cool down period, participants were then contacted regarding their willingness to participate. Those who agreed were invited back to the clinic, to permit completion of the informed consent process, questionnaires and screening instruments and blood sample collection. Non-diabetes controls were identified and sampled at the University of the Third Age (U3a), Derry, NI. This centre aims to stimulate and educate retired members of the community, and is an international organisation.

2.8 Sample Collection

2.8.1 DiaStrat

2.8.1.1 Buccal Swab sampling

Samples were taken in a clinical room after informed consent had been obtained. A single buccal swab was acquired using a MasterAmp™ buccal brush (Epicentre, Madison, WI USA; Cat no. 4459). Each cheek of the participant was lightly brushed, and the swab was then placed in a 15 ml conical polypropylene tube for DNA extraction.

2.8.1.2 Blood sampling

Blood samples were obtained using 21G Vacuette® safety needles (Greiner Bio-One, Stonehouse, UK; Cat no. 450091), coupled with a Vacuette® Leur adapter (Greiner Bio-One, Stonehouse, UK; Cat no. 450070) and BD vacutainer® (BD, Oxford, UK; Cat no. 364815). Approximately 26 ml of blood was collected into 2 x 9ml EDTA (K3E K3EDTA) coated Vacuette® tubes (Greiner Bio-One, Stonehouse, UK; Cat no. 455036) and 1 x 8ml Serum (Z Serum Sep Clot Activator) tube (Greiner Bio-One, Stonehouse, UK; Cat no. 455071). Samples were stored and transported in a cooled (approx. 4^{o}C) medical blood transportation bag (Versapak, Kent, UK; Cat no. BLD1_RDS). Blood samples were processed immediately after collection.

2.8.1.3 Blood processing

Prior to sample processing, 10 polypropylene tubes (per sample) were prelabelled using Item Tracker© software (ItemTracker Software Ltd, Suffolk, UK), ensuring compliance with the Human Tissue Act 2004 regulations. One 9 ml EDTA blood sample was also labelled as whole blood and immediately frozen at -80^{o}C for future DNA extractions. The remaining 9 ml EDTA and 8 ml Serum blood samples were centrifuged at 4000 rpm (4^{o}C) for 15 min. The resulting EDTA sample separated into three distinct layers, the upper layer was designated plasma, middle layer, white blood cells (WBC) and the bottom layer, RBC. The plasma was aliquoted into four 1.5ml prelabelled Eppendorfs (Plasma A-D). WBCs were then carefully removed and

placed in a polypropylene tube, washed once with PBST (1 ml) and split between two prelabelled tubes (Protein, RNA) before being washed again with PBST (1 ml). WBCs in the protein tube were stored in 1 ml Mammalian Protein Extraction Reagent M-PER™ (Thermo Scientific, UK; Cat no. 78501) and WBCs in the RNA tube in 1ml RNAlater (Thermo Scientific, UK; Cat no. AM7020). The supernatant from the 8ml serum tube was designated serum, and aliquoted into four 1.5 ml Eppendorf tubes (Serum A-D). All samples were then frozen at -80^{o}C and were separated between primary and secondary freezers. Sample maps were updated after each day of recruitment to ensure sample traceability and compliance with HTA.

2.8.2 GLP-1 response Study

2.8.2.1 Blood sampling

All blood samples were obtained using 21G Vacuette® safety needles (Greiner Bio-One, Stonehouse, UK; Cat no. 450091), coupled with a Vacuette® Leur adapter (Greiner Bio-One, Stonehouse, UK; Cat no. 450070) and BD vacutainer® (BD, Oxford, UK; Cat no. 364815). Approximately 50ml of blood were extracted into 2x 9 ml EDTA (K3E K3EDTA) coated Vacuette® tubes (Greiner Bio-One, Stonehouse, UK; Cat no. 455036), 2x 4 ml EDTA (K3E K3EDTA) coated Vacuette® tubes (Greiner Bio-One, Stonehouse, UK; Cat no. 455021) and 1x 8 ml Serum (Z Serum Sep Clot Activator) tube (Greiner Bio-One, Stonehouse, UK; Cat no. 455071). Any human tissue was stored and transported in a cooled (approx. 4^{o}C) medical blood transportation bag (Versapak, Kent, UK; Cat no. BLD1_RDS). All blood samples were processed immediately after collection.

2.8.2.2 Blood processing

Prior to sample processing, 10 polypropylene tubes (per sample) were prelabelled using Item Tracker© software (ItemTracker Software Ltd, Suffolk, UK), ensuring compliance with the Human Tissue Act 2004 regulations. One 9 ml EDTA blood sample was also labelled as whole blood and immediately frozen at -80^{o}C for future DNA extractions. The remaining 9 ml EDTA and 8 ml Serum blood samples were

centrifuged at 4000 rpm (4^{o}C) for 15 min. The resulting EDTA sample separated into three distinct layers, the upper layer was designated plasma, middle layer, white blood cells (WBC) and the bottom layer, red blood cells (RBC). The plasma was aliquoted into corresponding prelabelled tubes (Plasma A-D). WBCs were then carefully removed and placed in a polypropylene tube, washed once with PBST and split between two prelabelled tubes (Protein, RNA) before being washed again with PBST. WBCs in the protein tube were stored in 1 ml Mammalian Protein Extraction Reagent M-PER™ (Thermo Scientific, UK; Cat no. 78501) and WBCs in the RNA tube in 1 ml RNAlater (Thermo Scientific, UK; Cat no. AM7020). The supernatant from the 8ml serum tube was designated serum, and aliquoted between corresponding tubes (Serum A-D). All samples were then frozen at -80^{o}C and were separated between primary and secondary freezers. Sample maps were updated after each day of recruitment to ensure sample traceability and compliance with HTA.

The two 4 ml EDTA (K3E K3EDTA) coated Vacuette® tubes (Greiner Bio-One, Stonehouse, UK; Cat no. 455021) were transported to Altnagelvin hospital labs for HbA_{1c} and full blood count analysis.

2.9 DNA extraction

2.9.1 Buccal Swab DNA extraction

DNA was extracted from MasterAmp^{TM} buccal brushes (epicentre, Madison, WI USA; Cat no. 4459) using the QIAamp DNA blood mini kit (Qiagen, Manchester, UK; Cat no. 51106). Each brush was incubated at 56^{o}C for 10 min in; 600 µl PBS, 400 µl buffer AL (Qiagen, Manchester, UK; Cat no. 19075) and 20 µl protease stock solution (Qiagen, Manchester, UK; Cat no. 1021055). Throughout the incubation samples were intermittently vortexed on high power for 5 sec. Ethanol (400 µl, >96-100%) was added to each sample and tubes vortexed to precipitate the DNA. The mixture was then transferred to a QIAamp mini spin column in a 2 ml collection tube and centrifuged at 6000 g for 1 min at room temperature (20^{o}C). DNA was captured on the QIAamp mini spin column membrane and any flow through was discarded. The DNA was then washed using 500 µl of buffer AW1 (Qiagen, Manchester, UK; Cat no.

19081), spun through the column at 6000 g for 1 min (20^{o}C). Each sample was then washed a final time using buffer AW2 (Qiagen, Manchester, UK; Cat no. 19072), centrifuged at 20,000 g for 3 min (20^{o}C). The QIAamp mini spin column was then inserted into a clean polypropylene tube and total DNA was eluted (6000 g, 1 min, 20^{o}C) in 50 µl buffer AE (Qiagen, Manchester, UK; Cat no. 19077). Samples were stored at -80^{o}C until required.

2.9.2 Whole blood DNA extraction

DNA was extracted from EDTA whole blood samples using the Gentra Puregene blood kit (Qiagen, Manchester, UK; Cat no. 158445). Samples were thawed at room temperature (20^{o}C) immediately before the procedure. Once defrosted, 900 µl of RBC Lysis solution (Qiagen, Manchester, UK; Cat no. 158904) was dispensed into a 1.5 ml polypropylene tube, to which 300 µl of whole blood was added and mixed by inversion 10 times. Samples were then incubated in solution for 5 min (20^{o}C) to maximize lysis. After incubation samples were centrifuged for 20 sec at 13,000 g to pellet remaining cells. The supernatant was carefully discarded, leaving approximately 10 µl of residual liquid. The cell pellet was then resuspended by vortexing before the addition of 300 µl Cell Lysis Solution (Qiagen, Manchester, UK; Cat no. 158906). To ensure homogeneity and removal of any cell clumps, samples were incubated at 37^{o}C for 15 min. RNase A solution (1.5 µl) (Qiagen, Manchester, UK; Cat no. 19101) was then added and mixed by inversion (25x). Samples were then incubated for a further 15 min at 37^{o}C and then quickly cooled for 1 minute on ice. Protein precipitate solution (Qiagen, Manchester, UK; Cat no. 158910) was then added (100 µl) to each sample and mixed by vortexing for 10 sec, prior to a 1 min centrifugation at 13,000g (20^{o}C). In a clean 1.5 ml polypropylene tube, 300 µl >99.9% isopropanol (Sigma-Aldrich, UK; Cat no. 437522) was added. The supernatant from the centrifugation step was then carefully poured into the isopropanol and mixed by inverting 50 times, before being centrifuged at 13,000 g for 1 min (20^{o}C). The DNA was then visible as a white pellet. The supernatant was removed and 300 µl 70% ethanol added to wash the DNA pellet. A final centrifugation was conducted at room temperature for 1 min at 13,000 g and ethanol removed. The DNA pellet was allowed to air dry for 5 min before 100 µl DNA hydration solution (Qiagen,

Manchester, UK; Cat no. 158914) was added. Samples were then incubated at 65^{o}C for 5 min to dissolve the DNA and left at room temperature overnight, before being stored at -80^{o}C for future use.

2.9.3 DNA quantification

DNA was quantified using a Qubit®Fluorometer (ThermoFisher Scientific, UK; Cat no. Q33216) and Qubit™ dsDNA HS Assay kit (ThermoFisher Scientific, UK; Cat no. Q32854). DNA extracted from whole blood samples was thawed at room temperature before use. Qubit® assay tubes (ThermoFisher Scientific, UK; Cat no. Q32856) were labelled prior to working reagent preparation. The working reagent consisted of a 1:200 dilution of Qubit® dsHS reagent (200 x concentrate in Dimethyl sulfoxide, DMSO, $(CH_3)_2SO$) in Qubit® dsDNA HS buffer. Qubit® DNA standards and samples were then prepared, in individual Qubit® assay tubes, 10 µl of each standard (standard 1, 0 ng/µL in TE; standard 2, 10 ng/µL in TE buffer), followed by 2 µl of each sample. Once all DNA was aliquoted into individual tubes, Qubit® working reagent was added to each; 190 µl to standards and 198 µl to unknown samples. The final volume for all reactions was 200 µl. Tubes were then incubated at room temperature (20^{o}C) for 2 min before being read on the Qubit®Fluorometer.

2.10 Enzyme-Linked Immunosorbent Assays (ELISA)

2.10.1 Insulin sandwich ELISA

Plasma insulin was quantified using human Mercodia insulin ELISA kit (Mercodia, Uppsala, SE; Cat no. 10-1113-10). All plates (96 well) were precoated with mouse monoclonal anti-insulin antibody. Calibrators 0-5 and unknown samples (human plasma) were then thawed at room temperature (20^{o}C), briefly vortexed and 25 µl pipetted in duplicate onto the plate. A 1X enzyme conjugate solution was prepared by diluting 11X Enzyme conjugate in Enzyme Conjugate buffer, 100 µl was then added to each well and left to incubate on a microtitre plate shaker (Stuart, UK., Cat. No SSM1) for 1 h at 700 rpm (20^{o}C). Each plate was then manually washed 6 x with 200 µl, 1X wash buffer, prepared from a 21X stock in ultra-distilled water (ELGA,

Wycombe, UK; Cat no. PF2XXXXM1). Once complete 200 µl 3,3', 5,5;-tetramethylbenzidine (TMB) solution was added to each well and left to incubate at room temperature (20^{o}C) for 15 min. This reaction was light and time sensitive so precise timings were ensured with each plate. Stop solution (0.5 M, H_2SO_4, 50 µl) was then added to each well. All plates were briefly incubated on microtitre plate shaker (Stuart, UK., Cat. No SSM1) to ensure mixing, then absorbance was read on a BioTek Epoch Microplate Spectrophotometer (BioTek, UK, Cat. No. 15306176) at 450 nm. Insulin concentration for all samples was determined by extrapolating corresponding absorbance readings from the calibrator standard curve (0-5) on a log/log scale.

2.10.2 C-peptide sandwich ELISA

Plasma C-peptide was measured using human Alpco C-peptide ELISA kit (Alpco, Salum, US; Cat no. 80-CPTHU-E01.1, E10). Plates were first equilibrated to room temperature (20^{o}C) before 25 µl of each standard (Blank-0, A-20, B-100, C-300, D-1000, E-3000 pM) and diabetes controls supplied control 1 & 2 were pipetted into designated wells. Unknown samples were then thawed at room temperature (20^{o}C) and briefly vortexed, before 25 µl was pipetted, in duplicate, into designated wells. Assay buffer (50 µl) was then added to each well before plates were sealed (Roche, UK; Cat. No 04729757001) and left to incubate for one hour on a microtitre plate shaker at 700 rpm (Stuart, UK., Cat. No SSM1). Wells were washed 6 times with 1X wash buffer (200 µl per well), prepared from a 21 x concentrate in ultra-distilled water (ELGA, Wycombe, UK; Cat no. PF2XXXXM1). A 1 x enzyme conjugate solution was prepared by diluting 11 x Enzyme conjugate in Enzyme Conjugate buffer, 100 µl of which was then added to each well and left to incubate on a microtitre plate shaker (Stuart, UK., Cat. No SSM1) for 1 h at 700 rpm (20^{o}C). Plates were washed another 6 times with 1X wash buffer (200 µl), before the addition of 100 µl TMB. The plate was then sealed and incubated for 15 min at 20^{o}C. Stop solution (100 µl) was added to each well and absorbance read on a BioTek Epoch Microplate Spectrophotometer (BioTek, UK, Cat. No. 15306176) at 450 nm. C-peptide plasma concentration for all samples was determined by extrapolating corresponding

absorbance readings from the assay standard curve using a 5 parameter logistic (pl) model.

2.11. Clinical database construction

2.11.1 DiaStrat

Relevant clinical information for all participants, who provided consent, was obtained from Western Health and Social Care Trusts, Hicom Diamond.NET diabetes management system (Hitcom, Surrey, UK) and Orion Health technologies, Northern Ireland Electronic Care Record (NIECR) (Orion health, Hammersmith, UK). Both computer systems allowed collation of longitudinal data from primary and secondary care. Data collected included; Sex, DOB, age at diagnosis, first diabetes appointment, current high and low HbA_{1c}, weight, BP systolic/diastolic, HDL, LDL, total cholesterol and BMI, alcohol units per day and comorbidities and the related ICD-10 codes. In addition we collected all current diabetes drugs including dose, date started, time on (days) and all other prescribed medication including dose, date started, time on (days). Unscheduled care records including, reason for attendance and date, drug reaction and allergies were also noted. All data was inputted into Microsoft excel for future analysis.

2.11.2 GLP-1 response Study

Relevant clinical information for all participants was obtained from Western Health and Social Care Trusts, Hicom Diamond.NET diabetes management system (Hitcom, Surrey, UK) and Orion Health technologies, Northern Ireland Electronic Care Record (NIECR) (Orion health, Hammersmith, UK). Data collected was: Sex, DOB, unscheduled care records including clinic attended, date and reason, current diabetes and non-diabetes prescriptions, cognitive and mood screening questionnaire data (*Chapter 2, section 2.14)*, education, personal and family health information, $HbA_{1c,}$ full blood count (*Chapter 2, Section 2.8.2.1),* weight (Kg), height (cm) and BMI (Kg/m^2). Data was collated into a Microsoft excel spreadsheet for future analysis.

2.12. Multiplex proteomics

Protein analysis on 374 type 2 diabetes samples and 20 controls was out-sourced to OLink proteomics (OLink, Uppsala, SW). Four panels were assessed including Cardiovascular II, Cardiovascular III, Inflammation and Immune response, each with 92 proteins. OLink utilises unique Proximity Extension Assay (PEA) technology that enables 92plex biomarker research. All wells are treated with 96 pairs of unique oligonucleotide labelled antibodies. Specific probes bind to target proteins in the sample via a homogeneous assay and when pairs are in close proximity create a unique DNA reporter sequence (OLink, 2017). These DNA polymerization events are detected and quantified using qPCR and remove any risk of cross reactivity seen on other 10plex technologies.

EDTA plasma samples were thawed at room temperature (20^{o}C), before 50 µl of each pipetted into 96-well, clear PCR plates. Row 12 of each plate (n=5) was left blank for OLink control plasma. All plates were sealed using adhesive cover slips and shipped on dry ice (CO^2, -78^{o}C).

OLink conducted a two-step internal quality control (QC) that evaluated each sample against the standard deviation of the internal controls and the degree of deviance from the median of the internal controls. Only samples above 0.2 Normalised Protein Expression (NPX) and samples that deviate less than 0.3 NPX passed QC. All data is presented as NPX values, OLink Proteomics' arbitrary unit on a log2 scale. Analyses on raw data returned from OLink was conducted in excel, SPSS, and Prism to assess proteins that were significantly altered in T2D, T2D patients prescribed specific anti-diabetes drugs, related comorbidities and associated treatment plans.

2.13. SNP genotyping using the Affymetrix UK Biobank array

2.13.1 Sample Preparation

Whole blood samples were obtained (Section 2.8.2.1) and DNA was extracted (Section 2.9.2). All samples were quantified using the Qubit®Fluorometer (ThermoFisher Scientific, UK; Cat no. Q33216) and QubitTM dsDNA HS Assay kit (ThermoFisher Scientific, UK; Cat no. Q32854) (Section 9.3). A total of 266 T2D, and 19 non-diabetes control DNA samples were added to ABgene 96 Square Well Storage plates (ThermoFisher Scientific, UK), and cap mats were used to seal the plates. Final concentration was 5 ng/µl and total mass per well was 100 ng. All samples were diluted in EDTA TE buffer (10 mM Tris-HCL pH8.0, 0.1 mM EDTA).

2.13.2 Data generation

Sample plates were shipped to Cambridge Genomic Services (CGS, UK) on dry ice (CO^2). The Affymetrix UK Biobank SNP array was run onsite at CGS, and the final data output was returned for analysis.

2.13.3 Variant Calling

Raw data was returned in .CEL files and SNPs were identified using Axiom's Genotype Console software, version 4.2 (Affymetrix, UK). Samples with a SNP call rate of less than 97.2% or a QC value of <0.82 were considered to have failed QC and were excluded from subsequent analyses. SNP lists were generated for 20 genes of interest (GLP-1R, GIPR, INSR, IRS-1, IGFBP2, AKT, MAPK1, MTOR, LEP-R, STAT3, SH2B1, POMC, IL6, IL10, IL18, IL1RN, HGF, HAVCR1, SORT1, CASP3). Comparisons were made between non-diabetes controls and T2D patients and also between GLP-1Ra responders (patients with good glycaemic control (<53 mmol/mol HbA_{1c})) and non-responders (poor glycaemic control (>65 mmol/mol HbA_{1c})).

2.14. Mood and cognition screening

2.14.1 Beck Anxiety Inventory

Participants self-scored 21 symptoms, according to how much each one had bothered them the previous week. There were 4 possible answers; Not at All; Mildly (It did not bother me much); Moderately (It was very unpleasant, but I could stand it), and; Severely (I could barely stand it). Values are assigned to each response: Not at All = 0; Mildly = 1; Moderately = 2, and; Severely = 3. The total sum of the answers indicates the level of anxiety experienced. A total score of 0 - 7 is indicative of "Minimal" anxiety; 8 - 15 as "Mild"; 16 - 25 as "Moderate", and 26 - 63 as "Severe". Full inventory can be found in *Appendices; Supplementary Screen 1*.

2.14.2 Beck Depression inventory

Participants self-scored 21 questions ranking severity from 0-3. The minimum score is 0 and maximum score is 63. Higher scores are indicative of greater symptom severity. Standardised cut offs are; 1-10 normal ups and downs, 11-16 mild mood disturbance, 17-20 borderline clinical depression, 21-30 moderate depression, 31-40 severe depression, over 40 extreme depression. Full inventory can be found in *Appendices; Supplementary Screen 2*.

If participants indicated symptoms of either anxiety or depression, a notification was sent to their General Practitioner (GP).

2.14.3 Standardized Mini-Mental State Examination (SMMSE)

Before conducting the SMMSE, all props were prepared (pen, paper, visual cues) and hearing and vision checks were made with the participant. The examination was conducted with Dr Molloy's proposed script (Molloy and Standish, 1997). The test consisted of 12 questions, was scored out of 30 and took approximately 10 min. It measures 2 domains of cognitive function; orientation and cognition. Strict cut off points have been developed to allow for the maximum sensitivity and specificity. Generally any score above 26 is seen as normal, 20-25 is indicative of MCI, 10-19

moderate cognitive impairment and 0-9 severe impairment or developed AD. Full test can be found in *Appendices; Supplementary Screen 3*.

2.14.4 Quick mild cognitive impairment screen (qMCI)

The questionnaire consists of 6 questions and is designed to address 6 domains of cognition; orientation, registration, clock drawing, verbal memory (VM), verbal fluency (VF) and logical memory (LM). The greatest weight of marks is associated with VM, VF and LM. The examination is scored out of 100; scores <62 are indicative of MCI, and scores <36 indicative of dementia. The screen takes approximately 5 min to administer and score, and is easy to learn and use. The qMCI can be used in an informal setting, reducing patient stresses and testing time in the clinic. It also compares favourably to other established cognitive screens (O'Caoimh et al., 2012). Full test can be found in *Appendices; Supplementary Screen 4.*

2.15. Statistics

All statistical analysis was conducted using Graphpad Prism software (Graphpad Software Inc., La Jolla, CA, USA; v6.0h), and graphs represent mean values ± standard deviation of the mean.

Initial gene expression data (Chapter 3) was analysed using Student's *t* tests, enabling comparisons to be made between two experimental groups. Ordinary one-way ANOVA was later used to detect differences in gene expression between 3-7 groups. Tukey's post-hoc test was utilised to determine differences between groups. A P value less than 0.05 was deemed significant. Cytokine profiling and western blot experiments in Chapter 3, also had 3 and 4 groups respectively. Ordinary one-way ANOVA with Tukey's post-hoc analysis was used here.

For comparing patient group demographics in Chapters 4 (males vs. females) and 5 (Diabetes vs. non-diabetes controls), Student's *t* tests were used to determine differences in age, HbA_{1c} and BMI. Student's t tests were also used to determine changes in a number of variables in specific comorbidities in patients positive for that comorbidity compared to those without the condition (Chapter 4).

Correlation analysis was conducted in Chapter 4 to determine any relationships between currently measured biomarkers. Two tailed, nonparametric Spearman's correlation analysis (95% CI), was conducted as normal distribution could not be guaranteed. Linear regression was used in Chapter 5, to assess the relationship between protein expression and disease duration, LDL, total cholesterol, C-peptide, number of comorbidities and diabetes medications. A line of best fit was generated, origin was automated and confidence interval set at 95%. Non-linear regression with normal (Gaussian) distribution was used to correlate protein levels to HDL. Least squares ordinary fit was used as recommended by the software.

Receiver Operating Characteristics (ROC) graphs (logistic regression) were used in Chapter 5 to assess the specificity and sensitivity of proteins associated with diabetes vs non-diabetes controls; an area under the curve (AUC) value of 0.7 was deemed a fair distinction in line with recommendations. All protein comparisons made between 3+ groups in Chapter 5 were made using Ordinary one-way ANOVA with Tukey's post-hoc test, and dual comparisons were made using Student's *t* tests.

In chapter 6 genotyping experiments significant SNPs were identified using the Z score test for two proportions. This analysis was used to compare SNP genotype proportions for T2D vs. non-diabetes controls, and T2D GLP-1Ra responders vs. non-responders for all SNPs across 20 genes of interest. The Z-test was also used in Chapter 4 to assess proportion differences in comorbidities, adverse reactions and unscheduled care in those receiving GLP-1Ra therapy vs. those receiving other diabetes drugs,

Analysis of cognitive screening examination (SMMSE, qMCI) scores in chapter 7 was conducted using repeated measures two-way ANOVA; this enabled variation between 4 groups and multiple questions to be identified. Holm-Šídák's multiple comparisons post-hoc test was applied. All correlations between Beck depression and anxiety inventories, SMMSE and qMCI with BMI a HbA_{1C} and Neutrophils were conducted using Two tailed, nonparametric Spearman correlation analysis (95% CI). In all statistical tests a p value of less than 0.05 was considered significant.

Chapter III

Targeted discovery of plasma and brain biomarkers indicative of GLP-1 analogue response

Abstract

Type II diabetes (T2D) and AD share several pathophysiologies and brain insulin resistance is characteristic of both diseases. GLP-1Ras are known to increase insulin sensitivity, are used in the treatment of T2D and show promise in AD. This study was designed to explore the molecular mechanisms downstream of GLP-1R activation in brain. We identified potential insulin and leptin signalling-associated genetic and proteomic markers of GLP-1 response. GLP-1 RKO and APP/PS1 mouse models were used to assess the importance of GLP-1 signalling and the impact of brain insulin resistance on gene expression. In brain, GLP-1R, LEP-R, GHSR, CREB1, STAT3, POMC, PRMT1 and GIP were affected in both genotypes ($P<0.05$ - $P<0.0001$). AD progression had a profound effect on all genes; 8 week old APP/PS1 mice displayed increases ($P<0.05$-$P<0.0001$), and 10 month APP/PS1 mice had global reductions ($P<0.05$-$P<0.0001$).

The effect of liraglutide treatment on genes of interest was analysed in C57BL/6 mice. In brain, genes of interest were upregulated after 7 days ($P<0.05$ - $P<0.001$), and downregulated after 21 days ($P<0.0001$). A daily peripheral expression pattern for all genes of interest was detected over 7 day's liraglutide treatment in C57BL/6 mice. Peripheral and central expression patterns differed, but early changes in periphery were predictive of gene expression patterns observed in brain after 3 weeks liraglutide administration.

In APP/PS1 and WT mice differences in baseline peripheral expression of GLP-1R and LEP-R were detected and liraglutide treatment normalised this. IL-10 and IL-4 were increased in APP/PS1 liraglutide treated mice, suggestive of anti-inflammatory effects. Protein levels of GLP-1R, IRS-1, AKT, STAT3α, POMC, SOCS3 and GIPR were consistent in C57BL/6, APP/PS1, GLP-1RKO, and APP/PS1 after 7 days liraglutide treatment. Post-translational modifications differed significantly between genotypes in IRS1 s616 and FOXO1 s256 ($P<0.05$ - $P<0.001$). These results indicate that leptin signalling is important in response to liraglutide and that gene expression changes in the periphery may be predictive of chronic effects of drug therapy in brain.

3.1 Introduction

Type 2 diabetes (T2D) and Alzheimer's disease (AD) are linked by mechanisms that are not fully understood (Ramirez et al., 2015). It is estimated that 4.5 million people suffer from T2D in the United Kingdom (Diabetes UK, 2016) and that these patients are at a 3 fold increased risk of developing AD (Arvanitakis et al., 2004). The incidence and prevalence of both diseases increases with age and both share several pathophysiological abnormalities including insulin resistance, oxidative stress, dyslipidaemia, advanced glycation end products and changes to hormonal signalling (Malkki, 2015).

Insulin is essential for the maintenance of physiological and biochemical processes in the brain and periphery (Robertson et al., 2004). It can alter feeding behaviour and numerous aspects of metabolism (Khodabandehloo et al., 2016). Insulin binds to its receptor, the IR, causing the tyrosine phosphorylation of IRS-1 (Waters and Pessin, 1996). In the central nervous system (CNS) this interaction affects body weight, cell growth, and glucose homeostasis by activating a number of signalling cascades including Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), MAPK (Sugano et al., 2006) and a number of other transcription factors involved in apoptosis and cell differentiation (Burgos-Ramos et al., 2012). Much of insulin's activity is mediated by AKT (Spolcova et al., 2014). It is involved in glucose transport to the plasma membrane via GLUT4 (Chang et al., 2004), but it is also involved in a number of anti-inflammatory pathways (Orellana et al., 2015). AKT inhibits the phosphorylation of GSK3 and phosphorylates FOXO1 at serine 256; activity which alters a number of transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) and B-cell lymphoma 2 (BcL2) which are involved in gene expression, cell viability and apoptosis (Niyomchan et al., 2015).

In T2D insulin transport across the BBB, irrespective of blood concentration, is negatively affected (Banks et al., 1997). This lowers the level of brain insulin and has been shown to impact insulin signalling affecting central and peripheral metabolism, exacerbating metabolic disorders and insulin resistance (Kaiyala et al., 2000). Much of this T2D pathogenesis has also been shown in the brains of Alzheimer's mouse models (Malkki, 2015). In human clinical trials reduced insulin

sensitivity has been shown to be a characteristic of AD and prolonged insulin resistance is significantly linked to late stage AD (Chen and Zhong, 2013). Molecularly both disease have a destabilized IR/IRS-1/PI3K pathway and a link is no longer in doubt (Talbot et al., 2012a).

In neuron-specific IR knockout mice (NIRKO), increased levels of insulin, leptin, lipids and moderate insulin resistance is common and has been shown to result in peripheral insulin resistance and impaired suppression of gluconeogenesis, which leads to hyperglycaemia (Obici et al., 2002c). In the brain of NIRKO mice impaired IR/IRS-1/P1-3K signalling leads to the phosphorylation of tau protein which is a central mechanism linking T2D to AD (Koch et al., 2010).

Insulin signalling may be augmented by increasing insulin concentrations, reducing insulin resistance or activating parallel signalling mechanisms (Kahn et al., 2006). Injectable insulin is not effective in this case due to the limitations in transport across the BBB, and significant risk of hypoglycaemia (Banks et al., 1997). There are now other pharmaceutical options that have been shown to increase brain insulin signalling (Salcedo et al., 2012). One that has gained particular interest, due to its mode of action and particularly mild adverse event profile, is Glucagon-like peptide-1 receptor agonist (GLP-1Ra), liraglutide. This drug is a synthetic incretin hormone, and in clinical trials has been shown to increase insulin secretion, insulin sensitivity and affect appetite and weight loss (Ard et al., 2016). GLP-1Ra have central and peripheral effects, acting as neurotransmitters and growth factors (Hayes, 2012). Liraglutide has been shown to induce significant insulin secretion in response to nutrient ingestion via c-AMP (Iepsen et al., 2015b) and it can penetrate the BBB to interact with the insulin and leptin signalling cascades (Blonde and Russell-Jones, 2009). The main mechanism by which it achieves this is by inhibiting PTP1B (Tiganis, 2013). PTP1B is insulin's main negative regulator, but is also linked to leptin pathways (Zabolotny et al., 2002a). Leptin has recently been shown to be capable of activating PI3K and affecting insulin resistance (Ren et al., 2007) but is also critical in the regulation of energy intake and metabolism. Classically leptin activates its receptor (LEP-R) inducing activation of Janus kinase 2 (JAK2) and STAT3 but recently it has been shown that the binding of SH2B1 to JAK2 allows for leptin mediated IRS-1 phosphorylation and downstream PI3K activation (Duan et al., 2004).

The LEP-R/JAK/SH2B1 complex can stimulate genes that code for IR and IRS proteins, and inhibition of SH2B1 increases insulin resistance (Chua, 2010). This has been shown to be an important process; alterations in SH2B1 result in abnormal metabolism, insulin resistance and obesity (Doche et al., 2012). Leptin signaling has also been implicated in neuronal damage. Alterations lead to impaired synaptic plasticity and insulin sensitivity in the hypothalamus (Perez-Gonzalez et al., 2014). Therefore GLP-1Ra stimulation of leptin signalling may be one mechanism by which liraglutide mediates its reported neuroprotective effects, reducing synapse loss and restoring neuronal function (McClean and Holscher, 2014).

Anti-inflammatory effects, of GLP-1 analogues may also mediate some of the reported neuroprotective properties. It has been shown that liraglutide reduces microglia and pro-inflammatory cytokines in an x-ray inflammation model (Parthsarathy and Hölscher, 2013). Liraglutide-induced reductions in inflammation have been shown in APP/PS1 mice to be directly linked to learning and memory (McClean et al., 2015). Anti-inflammatory effects have also been observed in a mouse model of intracerebral haemorrhage (ICH) (Hou et al., 2012) and a rat model of traumatic brain injury (TBI) (Hakon et al., 2015). Interestingly in the rat it was shown to improve integrity of the BBB (Hakon et al., 2015).

GLP-1R are widely expressed in the brain and on the BBB and stimulation by GLP-1 has been shown to have significant effects on insulin sensitivity, neurogenesis and energy metabolism (Kanoski et al., 2011). Due to the close link between insulin and leptin neuronal signalling and GLP-1's ability to interact with both systems (Akieda-Asai et al., 2014), it is now considered a good pharmaceutical candidate to stimulate both pathways (Adamska et al., 2014). The present study was designed to explore the relationship between incretin receptor expression and leptin signalling in the absence of the GLP-1 receptor, using GLP-1 RKO mice and in a model of brain insulin resistance, the APP*swe*/PS1ΔE9 mouse model of AD. Subsequently we assessed response to acute (7 days) or chronic (21 days) liraglutide treatment on the same panel of genes in brain in a C57BL/6 mouse model. Finally we investigated whether gene expression profiles observed in brain may be detected, or predicted, in peripheral whole blood.

3.2 Methods

3.2.1 Animals

APP/PS1 mice were aged and treated with liraglutide as described in *Chapter 2; Section 2.1.1.* GLP-1 RKO mice are described in *Chapter 2; Section 2.1.2. C56BL/6* mice are described in *Chapter 2; Section 2.1.3.* All experiments were conducted in accordance with the UK home office Animals (Scientific Procedures) Act.

3.2.2 Total RNA extraction for gene expression analysis

3.2.2.1 Brain tissue

The RNeasy lipid tissue mini kit (Qiagen Ltd, Manchester, UK; Cat. No. 78404) was used to extract total RNA from whole hemibrain, as described in *Chapter 2; Section 2.4.1.1*

3.2.2.2 Whole blood

Whole blood was obtained from the tail vein and RNA was extracted from a 50 µl aliquot using the QIAamp RNA blood mini kit (Qiagen Ltd, Manchester, UK; Cat. No. 52304), as described in *Chapter 2; Section 2.4.1.2*

RNA quality and quantity was determined immediately before cDNA synthesis using a nanodrop (Nanodrop 2000c, ThermoFisher Scientific, Wilmington, DE).

3.2.3 Complementary DNA (cDNA) Synthesis

Reverse transcription was conducted using a Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics Ltd., West Sussex, UK; Cat. No. 04379012001). Total RNA was dependant on sample type (Brain (25 ng) and blood (5 ng) per reaction), as described in *Chapter 2; Section 2.4.2*.

3.2.4 Quantitative Polymerase Chain Reaction (qPCR)

All qPCR experiments were normalised to a stable housekeeping gene (RNS18 *M. musculus*, Assay ID 307906, transcript length 1870 bps, amplicon length 106 bps) in whole blood and brain and under various treatment conditions, using the $2^{\Delta\Delta CT}$ method. Full real-time ready probe list, reagents and method are available in *Chapter 2; Section 2.4.3.*

3.2.5 Total protein extraction and sample preparation for proteomic analysis

Protein was extracted from one hemisphere of mouse brain using RIPA lysis buffer (*Chapter 2; Section 2.5.1*). Concentration was determined using a Pierce™ BCA Protein Assay Kit (Thermo scientific, UK; Cat. No. 223227) and BioTek Epoch Microplate Spectrophotometer (BioTek, UK, Cat. No. 15306176), according to manufacturer's instructions. Full protocol outlined in *Chapter 2; Section 2.5.2*.

3.2.6 Western blot SDS-page

Protein samples were run on a 10-well NuPAGE™ 4-12% Bis-Tris, gradient protein Gel (Thermo scientific, UK; Cat. No. NP0335BOX) and transferred on to Polyvinylidene difluoride (PVDF) membranes (Thermo scientific, UK; Cat. No. LC2005) before being blocked (5% milk/ 0.05 %TBS-T) and incubated in primary antibody overnight. Proteins were developed using Anti-rabbit IgG, HRP-linked secondary antibody (Cell signalling, Cat. No 7074, Rabbit, dilution 1:1000) and SuperSignal Chemiluminescent Substrates (Thermo scientific, UK; Cat. No. 34080). Full protocol and reagent inventory outlined in *Chapter 2; Section 2.5.4.*

3.2.7 Meso Scale Discovery proinflammatory array

Cytokine and chemokine analysis was conducted using the Meso Scale Discovery (MSD®) Multi-spot Assay Pro-inflammatory panel 1 (mouse) kit (Meso Scale Discovery, Rockville, MD, USA, Cat. No. K15048D) according to the manufacturer's instructions, as described in in *Chapter 2; Section 2.6.*

3.2.7 Statistical analysis

All statistical analysis was conducted using Graphpad Prism software (Graphpad Software Inc., La Jolla, CA, USA; v6.0h). Student *t* tests and one way ANOVA with tukey's post-hoc test were used as described in *Chapter 2; Section 2.15.*

3.3 Results

3.3.1 Leptin and Insulin regulatory genes are upregulated in the brain of GLP-1R KO and young APP/PS1 mice.

3.3.1.1 Incretin receptor compensation in GLP-1RKO and APP/PS1 mice

Both GLP-1R and GIP-R expression levels were significantly altered in GLP-1R knockout (KO) mice. GLP-1R mRNA levels were reduced by 70 ± 3% compared to wild type ($P<0.05$; Fig. 3.1A). Although not completely 'knocked out', GLP-1R knockdown had an effect on GIP-R levels which were increased by 64 ± 13% ($P<0.01$; Fig. 3.1H). In 8 week old APP/PS1 mice GLP-1R expression was significantly increased by 152 ± 30% compared to WT ($P<0.001$; (Fig. 3.1A), however GIP-R expression was not significantly different compared to WT (88 ± 19%; Fig. 3.1H). GIPR expression was significantly increased in GLP-1 RKO mice compared to APP/PS1 mice ($P<0.01$).

3.3.1.2 GLP-1R –LEP-R crosstalk

In GLP-1 RKO mice GHSR was increased by 261 ± 16%, ($P<0.0001$; Fig. 3.1C) and LEP-R increased 153% ± 41 %, ($P<0.005$; Fig. 3.1B), compared to WT mice. In the APP/PS1 model we recorded a similar significant increase in GHSR (224 $\pm$ 34%; $P<0.0001$), however LEP-R expression was comparable to controls.

As expected, increased LEP-R expression in the GLP-1 RKO model had a significant impact on downstream components STAT3 (+158 ± 46%; $P<0.005$; Fig. 3.1E), and POMC (+84 ± 3%; $P<0.05$; Fig. 3.1F). In the APP/PS1 model STAT3 mRNA levels were comparable to WT and significantly reduced compared to GLP-1 RKO mice (-102 ± 16%, $P<0.05$; Fig. 3.1D). POMC expression was increased in the APP/PS1 model compared to control (+101 ± 34%, $P<0.01$; Fig 3.1E).

3.3.1.3 Neuroprotective effect

GLP-1 RKO mice exhibited a 144 ± 24% increase in CREB1 ($P<0.0001$; Fig. 3.1D). CREB1 was also significantly increased in the APP/PS1 model (173 ± 13%; $P<0.001$). Interestingly the major regulator of CREB1 transcription in pancreas, PRMT1 (Fig.

3.1G), was also significantly upregulated in both GLP-1R KD and APP/PS1 mice by 178 ± 41% ($P<0.001$) and 67 ± 9% ($P<0.05$), respectively.

3.3.2 Downstream cell regulators maintained at normal levels in GLP-1 RKD and APP/PS1 mice

In the brain of GLP-1 RKD mice, IRS-1 (103 ± 5%; Fig. 3.2A) and AKT (115 ± 14.35%; Fig. 3.2E) expression was not significantly different from WT mice. Similarly, key genes involved in Leptin signalling; JAK2 (95 ± 7%, Fig. 3.2B), SH2B1 (98 ± 12%, Fig. 3.2C) and SOCS3 (106 ± 12%, Fig. 3.2D) were unaffected in this genotype.

The 8 week old APP/PS1 mice exhibited a similar expression profile to GLP-1R KD mice, showing normal leptin gene expression levels and therefore IRS-1 (93 ± 11%, Fig. 3.2A), AKT (133 ± 28%, Fig. 3.2E), JAK2 (84 ± 20.11%, Fig. 3.2B), SH2B1 (97 ± 16.6%, Fig. 3.2C) and SOCS3 (123 ± 22%, Fig. 3.2D) were comparable to WT and GLP-1RKO mice.

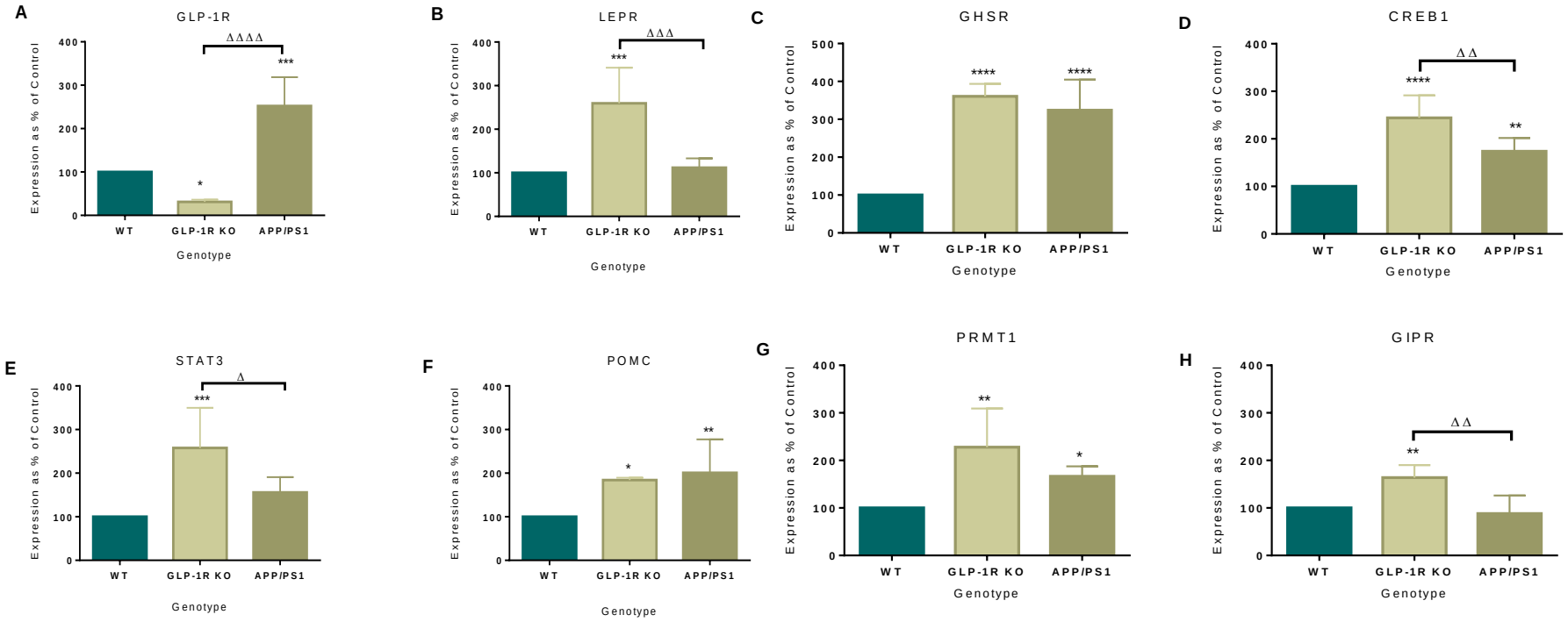


Figure 3.1 – **Insulin and appetite regulatory genes are upregulated in the brain of GLP-1RKO and APP/PS1 mouse models.** GLP-1R (A), LEP-R (B), GHSR (C), CREB1 (D), STAT3 (E), POMC (F), PRMT1 (G) and GIPR (H) gene expression was determined by quantitative real-time PCR and normalised to internal housekeeping control RNS18. GLP-1R 'knockout' resulted in increased LEP-R and GIPR which corresponded with increased related and downstream genes GHSR, CREB1 ,STAT3, POMC PRMT1 ($p<0.05$-$p<0.001$). APP/PS1 mice had increased GLP-1R and unchanged LEP-R and GIPR but experienced the same significant downstream increases in GHSR, CREB1, POMC, PRMT1 ($p<0.05$-$p<0.001$), highlighting a regulatory relationship between GLP-1R and LEP-R. Wild Type (WT, n=6), GLP-1R KO (n=4) and APP/PS1 (n=5). Statistical significance determined using one-way ANOVA and Tukey's multiple comparisons test. **P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs WT control.* Δ P<0.05, ΔΔ P<0.01, ΔΔΔ P<0.001 and ΔΔΔΔ P<0.0001 vs. APP/PS1.

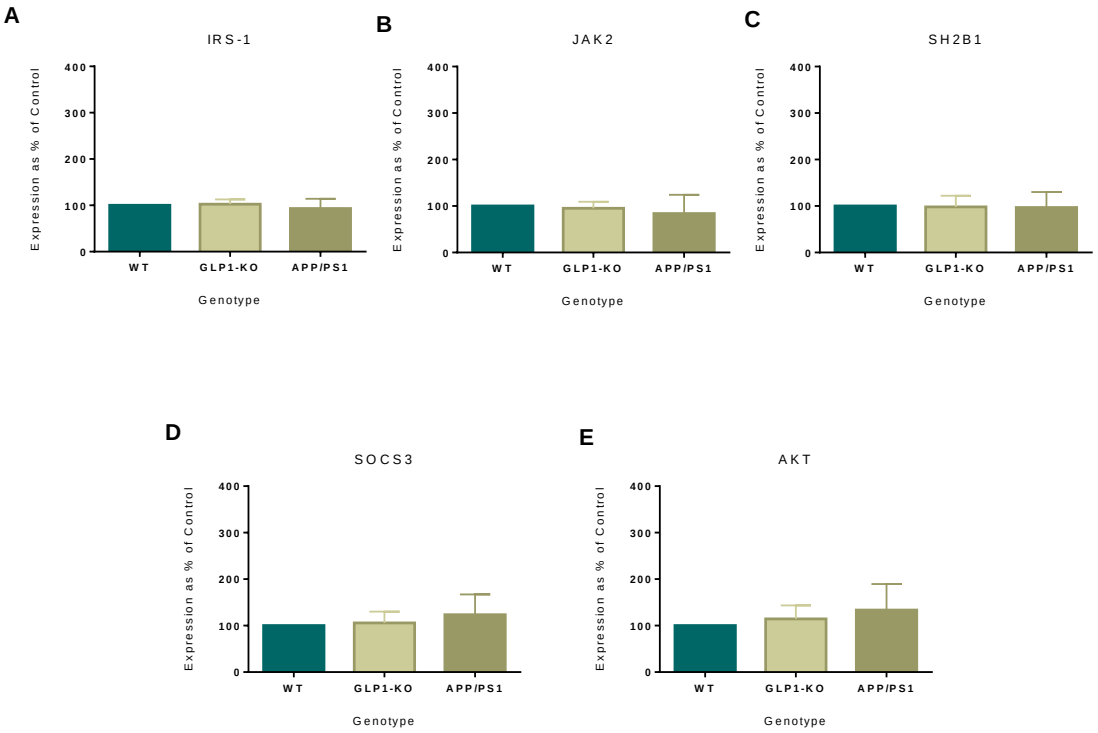


Figure 3.2 – **Expression levels of cell regulators that link leptin and insulin signalling are unaltered in GLP-1RKO and APP/PS1 mouse models**. IRS1 (A), JAK2 (B), SH2B1 (C), SOCS3 (D) and AKT (E) gene expression was determined by quantitative real-time PCR and normalised to internal housekeeping control RNS18. No significant change was observed in any of the cell regulators in either genotype compared to WT controls. WTS, GLP1-RKO and APP/PS1, n=3 per group. Expression changes compared to WT control.

3.3.3 Alzheimer's disease progression has a negative effect on key regulatory genes involved in Leptin and insulin signalling

In young APP/PS1 mice (8 week) the mRNA levels of major regulators of Leptin and insulin signalling were maintained, likely a result of the significant upregulation of the related upstream genes observed. To further characterise the effect of the APP/PS1 genotype on genes of interest we measured mRNA levels of the key regulators in 10 month APP/PS1 mice that phenotypically have a higher levels of Aβ and plaque formation (Maia et al., 2013).

3.3.3.1 Incretin signalling disruption

In early AD (8 week, APP/PS1) GLP-1R expression (Fig. 3.1A) was increased however in aged APP/PS1 mice at 10 months both GLP-1R (Fig. 3.3F) and GIP-R (Fig. 3.3G) were significantly downregulated, 66 ± 18% ($P<0.01$) and 54 ± 15% ($P<0.05$), respectively, characteristic of impaired incretin action in brain.

3.3.3.2 Major disruption of downstream cell regulators in 10 month APP/PS1 mice

Expression of the major leptin regulating genes in 10 month APP/PS1 mice contrasts the expression pattern observed in younger mice. The LEP-R binding protein JAK2 (Fig. 3.3B) was reduced 66 ± 17% ($P<0.05$) and SH2B1 61% ± 15% ($P<0.05$, Fig. 3.3C). IRS-1 was reduced 74 ± 5%, ($P<0.0001$, Fig. 3.3A) and it downstream target AKT reduced by 62% ± 5%, ($P<0.005$, Fig. 3.3E), indicative of substantial alterations in insulin signalling (Cong et al., 1997). SOCS3 was not significantly affected (45% ± 21.5%; $P< 0.10$) in aged APP/PS1 mice.

3.3.4 Liraglutide differentially affects leptin and insulin signalling genes dependent on treatment duration

3.3.4.1 Liraglutide affects GLP-1R but not GIP-R transcription.

To further explore potential insulin sensitising mechanisms in brain we treated C57BL/6 mice with liraglutide for 7 and 21 days. GLP-1R mRNA (Fig. 3.4A) was unaffected by liraglutide treatment after 7 days (23 ± 15%, $P< 0.15$) however a significant decrease was apparent after 21 days (82 ± 16%, $P<0.0001$). GIP-R expression was unchanged in response to 7 day and 21 day liraglutide treatment (Fig. 3.4H).

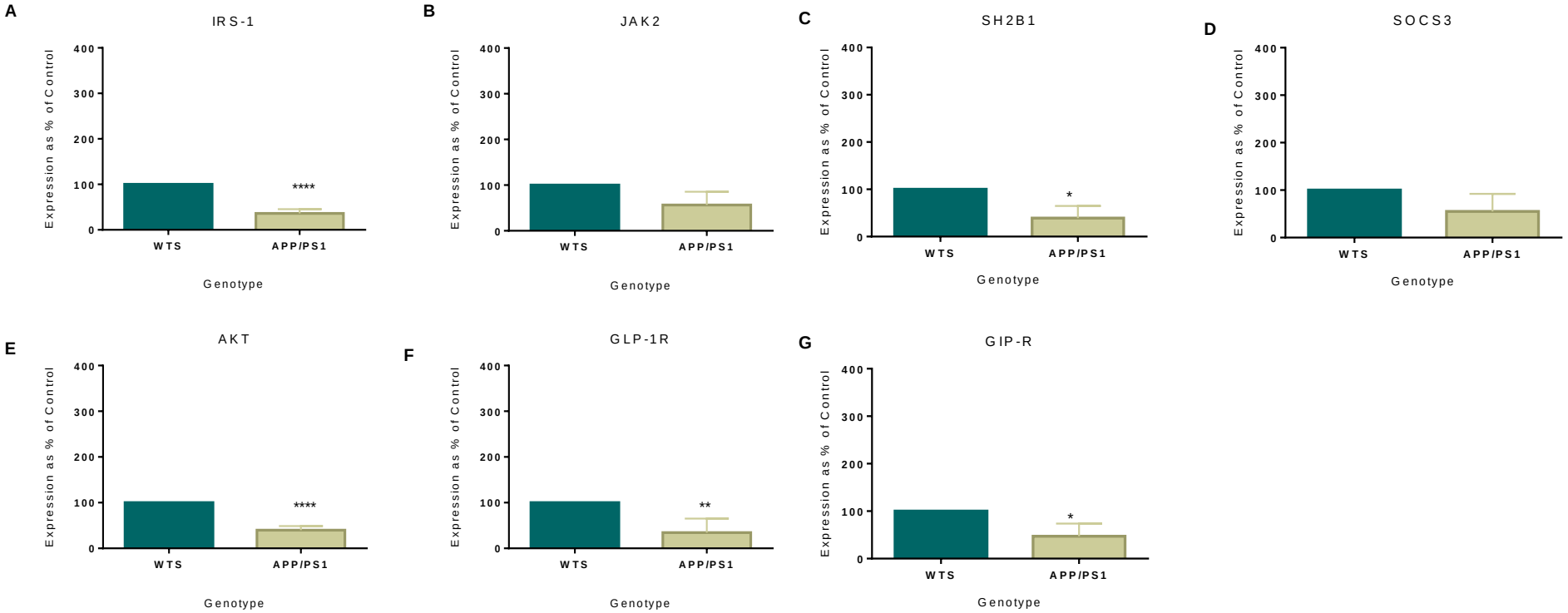


Figure 3.3 – **Expression levels of cell regulators that link leptin and insulin signalling in brain from APP/PS1 mice with significant Aβ deposition (10month).** IRS-1(A), JAK2 (B), SH2B1 (C), SOCS3 (D), AKT (E), GLP-1R (F), GIP-R (G) gene expression was determined by quantitative real-time PCR and normalised to internal housekeeping control RNS18. Contrasting what was observed in young APP/PS1 mice, before beta amyloid deposits develop, IRS-1, SH2B1, AKT, GLP-1R and GIPR were all significantly downregulated ($p<0.05$-$p<0.001$), suggestive of severely dysregulated leptin and insulin signalling. WTS and APP/PS1 n=3 per group. Statistical significance determined using student's T-test. **P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. WT control*

3.3.4.2 Liraglutide increases brain mRNA of Leptin and insulin signalling genes after 7 days treatment

After 7 days liraglutide treatment, LEP-R expression (Fig. 3.4B) was upregulated by 36 ± 6% ($P<0.05$) which resulted in a significant increase in its downstream target STAT3 ($P<0.05$; Fig. 3.4E) by 15 ± 7%. The neuronal peptide POMC (Fig. 3.4F) was also significantly affected ($P<0.05$) with mRNA levels reduced by 48 ± 11% compared to untreated controls. GHSR (Fig. 3.4C) mRNA levels were comparable between 7-day and untreated controls. CREB1 (Fig. 3.4D) was upregulated by 22 ± 6% ($P<0.05$), as was the neuroprotective enzyme PRMT1 (+48 ± 10%, $P<0.0001$).

3.3.4.3 Global down regulation of Leptin and insulin signalling genes in brain after 21 days Liraglutide treatment

All genes of interest were down regulated after 21 days liraglutide treatment. LEP-R expression was significantly reduced by 78 ± 18% ($P<0.0005$, Fig. 3.4B), followed by an 81 ± 4% decrease in STAT3 expression (Fig. 3.4C, $P<0.0001$) and an 84 ± 4% decrease in POMC ($P<0.0001$; Fig. 3.4F) mRNA. Unexpectedly the GHSR was also significantly reduced by 86 ± 5% compared to UT controls, indicative of maintained appetite suppression ($P<0.001$; Fig. 3.4C). CREB1 (Fig. 3.4D) and PRMT1 (Fig. 3.4G) were both significantly reduced ($P<0.0001$) by 77 ± 5% and 75 ± 4%, respectively, after 21 days liraglutide treatment.

3.3.5 Downstream cell regulators exhibit contrasting expression profiles in brain after 7 and 21 days treatment

Neuronal cell regulator mRNA was maintained at a normal levels after 7 days liraglutide treatment. We found that JAK2 (93 ± 5%; Fig. 3.5B), SH2B1 (100 ± 9%; Fig. 3.5C), SOCS3 (121 ± 9%; Fig. 3.5D) were not affected by treatment. As a result neither IRS-1 (92 ± 6%; Fig. 3.5A) nor AKT expression levels (102 ± 14%; Fig. 3.5E), were altered after 7 days treatment.

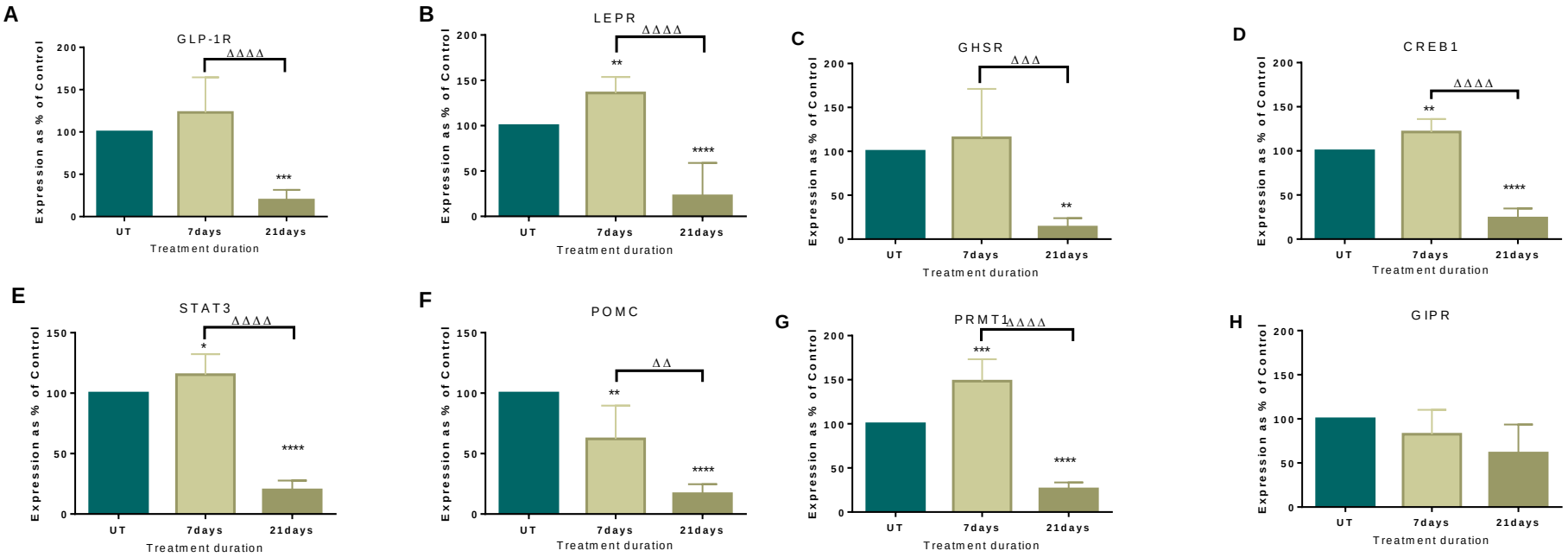


Figure 3.4 – **Leptin and insulin genes are differentially expressed in brain of WT mice treated with liraglutide (25 nmol/Kg) after 7 and 21 days.** GLP-1R (A), LEP-R (B), GHSR (C), CREB1 (D), STAT3 (E), POMC (F), PRMT1 (G) and GIP-R (H) gene expression was determined by quantitative real-time PCR and normalised to internal control gene RNS18. A significant upregulation in LEP-R, CREB1, STAT3 and PRMT1 (P<0.05-P<0.001), and a significant down regulation in POMC (P<0.01) was observed after 7 days treatment. In contrast after 21 days a significant down regulation in GLP-1R (P<0.001), LEP-R (P<0.0001), GHSR (P<0.01) CREB1 (P<0.0001), STAT3 (P<0.0001), POMC (P<0.0001) and PRMT1 was observed (P<0.0001). Untreated (UT), 7 day treated, and 21 day treated, n=6 per group. Statistical significance was determined using one-way ANOVA and Tukey's multiple comparisons test *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. untreated control. ΔΔ P<0.01, ΔΔΔ P<0.001 and ΔΔΔΔ P<0.0001 vs. 7-day treated group

Similar to what we observed with upstream components, treating the mice for 21 days caused a completely different expression profile. IRS-1 (14 ± 5%; Fig. 3.5A) and AKT (25 ± 9%; Fig. 3.5E) were both significantly downregulated, P<0.0001 and P<0.001 respectively. Leptin signalling components JAK2 (74 ± 11%; P<0.001; Fig. 3.5B), SH2B1 (86 ± 5%; P<0.0001; Fig. 3.5C) and SOCS3 (72 ± 8%; P<0.0001; Fig. 3.5D) were also significantly downregulated by 21 days liraglutide treatment.

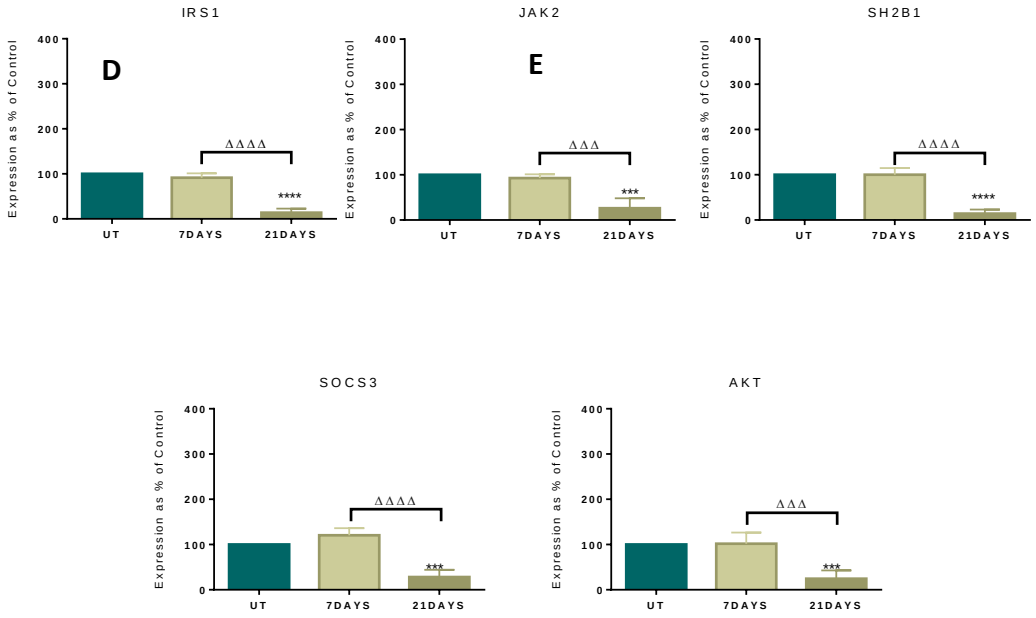


Figure 3.5 – **Expression levels of key cell regulators linking leptin and insulin signalling are down regulated after 21 days liraglutide treatment.** IRS1 (A), JAK2 (B), SH2B1 (C), SOCS3 (D) and AKT (E) gene expression was determined by quantitative real-time PCR and normalised to internal housekeeping control RNS18. No significant change was observed after 7 days but expression of all genes was significantly reduced (P<0.001-P<0.0001) after 21 days treatment when compared to UT WT controls. UT, 7 and 21 day n=3 per group. Statistical significance was determined one-way ANOVA with tukey's post-hoc test. ***P<0.001, ****P<0.0001 vs. untreated control. ΔΔΔ P<0.001 and ΔΔΔΔ P<0.0001 vs. 7-day treated group.

3.3.6 Peripheral detection and assessment of Leptin and insulin signalling genes after 7 days liraglutide treatment

3.3.6.1 Detection of Leptin and insulin signalling genes is possible in whole blood

Peripheral gene expression is rapidly affected by liraglutide treatment. Expression of genes of interest was significantly altered after 24 h and a peak effect occurred after 48 h. After 30 min GLP-1R expression was reduced and LEP-R expression increased however significant variability in response (GLP-1R, Fig. 3.6A) had a SEM of ± 69% and the LEP-R (Fig. 3.6B) of ± 83% negated significance. After 24 h mRNA levels of GLP-1R, 74 ± 26% ($P<0.01$), and the LEP-R, 14.9% ± 1.9%, were downregulated. GLP-1R mRNA levels declined further over time with the lowest level being recorded at 72 h (5 ± 5%, $P<0.0001$) before being undetectable on the 7th day. Expression of LEP-R remained constant after 24 h, until the 7th day, when mRNA could no longer be detected. GHSR (Fig. 3.6C) could not be detected in whole blood.

3.3.6.2 Peripheral Leptin and insulin gene expression exhibits a specific expression profile over 7 days Liraglutide treatment

Many studies have shown the fast pharmacodynamic profile of Liraglutide 2 h initiation, 18 h duration) (Astrup et al., 2012). Only GLP-1R and LEP-R were not rapidly affected 30 min after a first liraglutide injection. Leptin receptor and GLP-1 receptor expression was affected after 24 h and peripheral mRNA for both could not be detected after 168 h (*section 3.3.6.1*). STAT3 (Fig. 3.6E) mRNA was significantly reduced ($P<0.01$) by 45 ± 11% after 30 min this declined a further
49 ± 2% ($P<0.01$) after 24 h before a maximum reduction of 98 ± 0.3% ($P<0.0001$) was recorded after 48 h treatment.

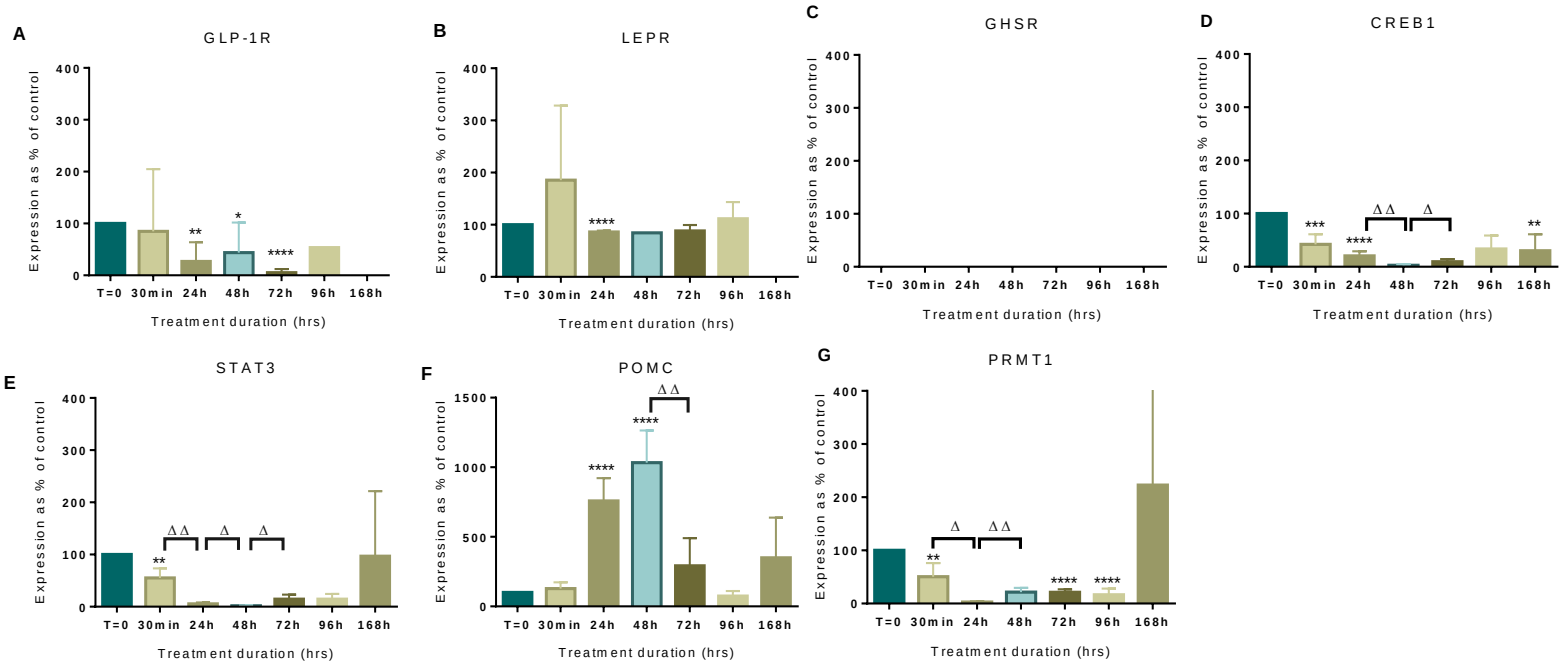


Figure 3.6 – **mRNA expression profile of leptin and insulin regulatory genes in whole blood from WT liraglutide treated mice over 7 days.** GLP-1R (A), LEP-R (B), GHSR (C), CREB1 (D), STAT3 (E), POMC (F) and PRMT1 (G) gene expression in whole blood 30 min, 24 h, 48 h, 72 h, 96 h and 168 h after first administration of liraglutide was determined by quantitative real-time PCR and normalised internal control RNS18. Animals received a once daily injection of liraglutide at 15.00. Liraglutide exerted a maximum effect on gene expression 24-48hrs after initiation of treatment. At 24 h significant down regulation was observed in GLP-1R (P<0.01), LEP-R (P<0.0001), CREB1 (P<0.0001), STAT3 (P<0.0001) and PRMT1 (P<0.0001), while POMC was significantly upregulated (P<0.0001). This, excluding the acute effect on POMC, shows a contradictory peripheral expression profile compared to what was observed in brain after 7 day treatment with liraglutide, but mirrors gene expression in brain after 21 day. GHSR was undetectable in peripheral blood. N=5, Statistical significance was determined using one-way ANOVA and Tukey's multiple comparisons test *P<0.05, **P<0.01,***P<0.001 and ****P<0.0001 vs. UT control. ΔP<0.05 and ΔΔP<0.01 vs. indicative time point.

This expression pattern directly contrasted neuronal peptide POMC (Fig. 3.6F), which was unchanged at 30 min but significantly increased ($P<0.0001$) by 658 ± 82% after 24 h and 1055 ± 115% ($P<0.0001$) after 48 h. This relationship is seen throughout the duration of treatment; at 72 h when STAT3 mRNA begins to increase, POMC expression significantly decreases ($P<0.01$) 291 ± 100%.

The same 24 / 48 h transcription profile was also evident with CREB1 (Fig. 3.6D). CREB1 was significantly decreased 58% ± 11% after 30 min ($P<0.001$) and continued to reduce after 24 h reaching a maximum reduction after 2 days (97 ± 0.9%, $P<0.0001$) when compared to time point 0. The same trend was recorded for PRMT1, which was decreased (50 ± 15%, $P<0.005$) after 30 min and most significantly reduced after 24 h by 97 ± 1% ($P<0.0001$).

3.3.7 Liraglutide differentially affects peripheral gene expression in WT and APP/PS1 mice

3.3.7.1 Baseline GLP-1R and LEP-R expression is different in WT and APP/PS1 mice

Initial peripheral gene expression profile (day 0) of WT and APP/PS1 mice was not significantly different for most genes of interest (GHSR Fig. 3.7C, CREB1 Fig. 3.7D, STAT3 Fig. 3.7E, POMC Fig. 3.7F, PRMT1 Fig. 3.7G). GLP-1R expression was significantly reduced in APP/PS1 (Fig. 3.7A; -6 ± 2;, $P<0.0001$), compared to WT mice. LEP-R expression was significantly increased (4 ± 1; $P<0.05$; Fig. 3.7B). GHSR was undetectable in blood (Fig. 3.7C. All data presented on Log10 scale, % of control.

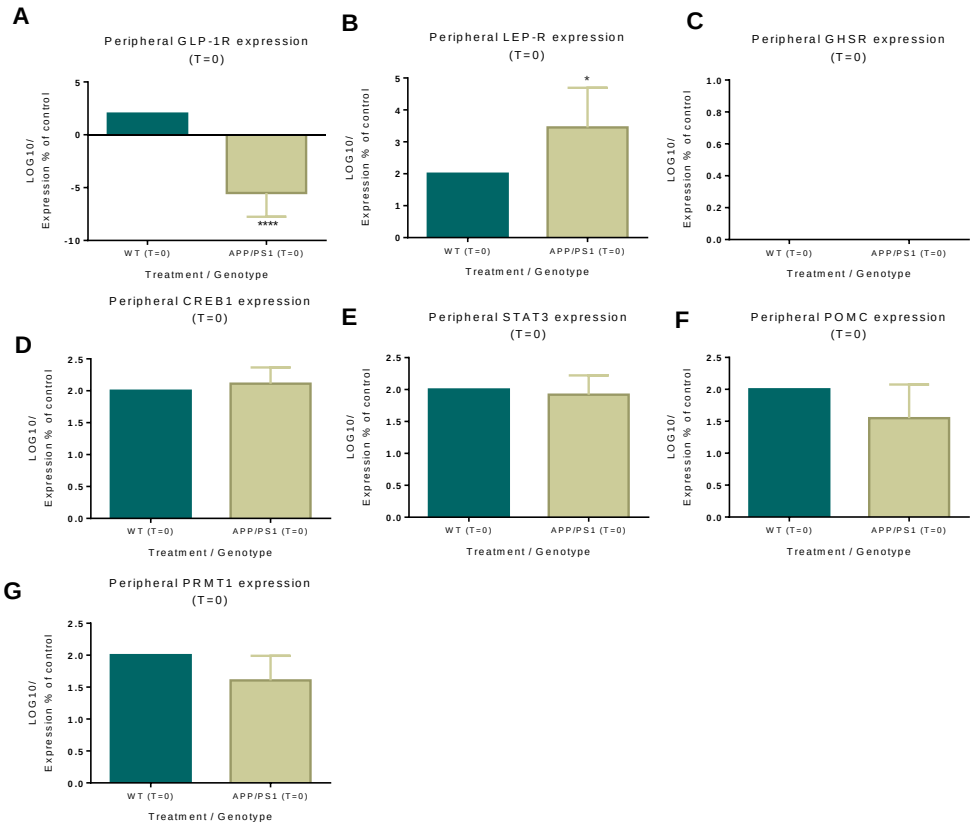


Figure 3.7 - **Peripheral GLP-1R and LEP-R expression is different in WT and APP/PS1 mice at 8 weeks of age.** Whole blood (WB) (A) GLP-1R, day 0, (B) WB LEP-R, day 0, (C) WB GHSR, day 0. (D) WB CREB1, day 0, (E) WB STAT3, day 0, (F) WB POMC, day 0. (G) WB PRMT1, day 0. Gene expression in WB was determined by quantitative real-time PCR and normalised internal control RNS18. Expression at day 0 (T=0) for APP/PS1 is shown as a percentage of WT control. WT and APP/Ps1 baseline expression level was opposite for GLP-1R, LEP-R. There was no difference in any of the other genes. GHSR was undetectable in peripheral blood. Statistical significance was determined using students T-test *P<0.05, ****P<0.0001 vs. WT saline control.

3.3.7.2 Peripheral gene expression after 7 days liraglutide treatment in WT and APP/PS1 mice

After 7 days liraglutide treatment GLP-1R (-1 ± 0, P<0.0001, Fig. 3.8A) and CREB1 (0.7 ± 0.4, P<0.0001, Fig. 3.8C) were downregulated, POMC was upregulated (Fig. 3.8F, P<0.01) and STAT3 (Fig. 3.8E) and PRMT1 (Fig. 3.8J) were unchanged in WT mice. The expression of LEP-R opposed that of the GLP-1R, and was significantly upregulated in WT treated mice (3 ± 0.02; P<0.0001; Fig. 3.7B). It is worth noting that this data corresponds with the expression trends seen in our previous peripheral gene experiments using WT mice (Fig. 3.6).

Interestingly APP/PS1 mice had an opposing expression profile of GLP-1R and LEP-R after 7 days liraglutide treatment. GLP-1R (Fig. 3.8A) was significantly upregulated in the blood of APP/PS1 mice (1 ± 0.01; P<0.0001) and LEP-R (Fig. 3.8B) was significantly downregulated in blood of APP/PS1 mice (0.86 ± 0.02, P<0.0001). All other genes in APP/PS1 mice exhibited the same expression profile as WT treated mice after 7 days liraglutide treatment.

3.3.7.3 Comparison of blood/brain gene expression profiles after 7 days liraglutide treatment in WT and APP/PS1 mice

Figure 3.4 demonstrated that genes of interest increased in brain after 7 days liraglutide treatment in WT mice. The present data (Fig. 3.8) supports this and the central (brain) gene expression profile that was identified in APP/PS1 mice at 8 weeks, illustrated in Figure 3.1. We expand on this data by showing the relationship between brain and peripheral (blood) gene expression; generally and in response to liraglutide treatment. Expression of all genes of interest significantly increased in brain after 7 days treatment in all groups when compared to 7-day treated blood from the same group. GLP-1R expression (P<0.0001, Fig. 3.8A) and LEP-R (P<0.0001, Fig. 3.8B) were increased most. No significant difference was detected between APP/PS1 treated mice and WT saline treated mice for any genes of interest in brain. GHSR was detected in brain, but not blood (Fig. 3.7F).

3.3.8 Liraglutide treatment in the APP/PS1 model induces a greater anti-inflammatory response than in WT mice

Expression of 10 cytokines in plasma from WT saline, WT liraglutide and APP/PS1 liraglutide-treated mice is illustrated in Fig. 3.9. No significant differences were observed between genotype or in response to liraglutide in IL-12P70 (F) IL-2 (G), IL-6 (H), IL-5 (I) OR KC/GRO (J). As illustrated in Fig. 3.9A, IL-10 was significantly increased from day 0 to day 7 in all groups, WT saline group (P<0.0001), WT liraglutide group (P<0.0001) and APP/PS1 liraglutide group (P<0.00001). IL-10 was also significantly increased in the APP/PS1 liraglutide-treated group on day 7 compared to the wild-type saline-treated group on day 7 (P<0.01).

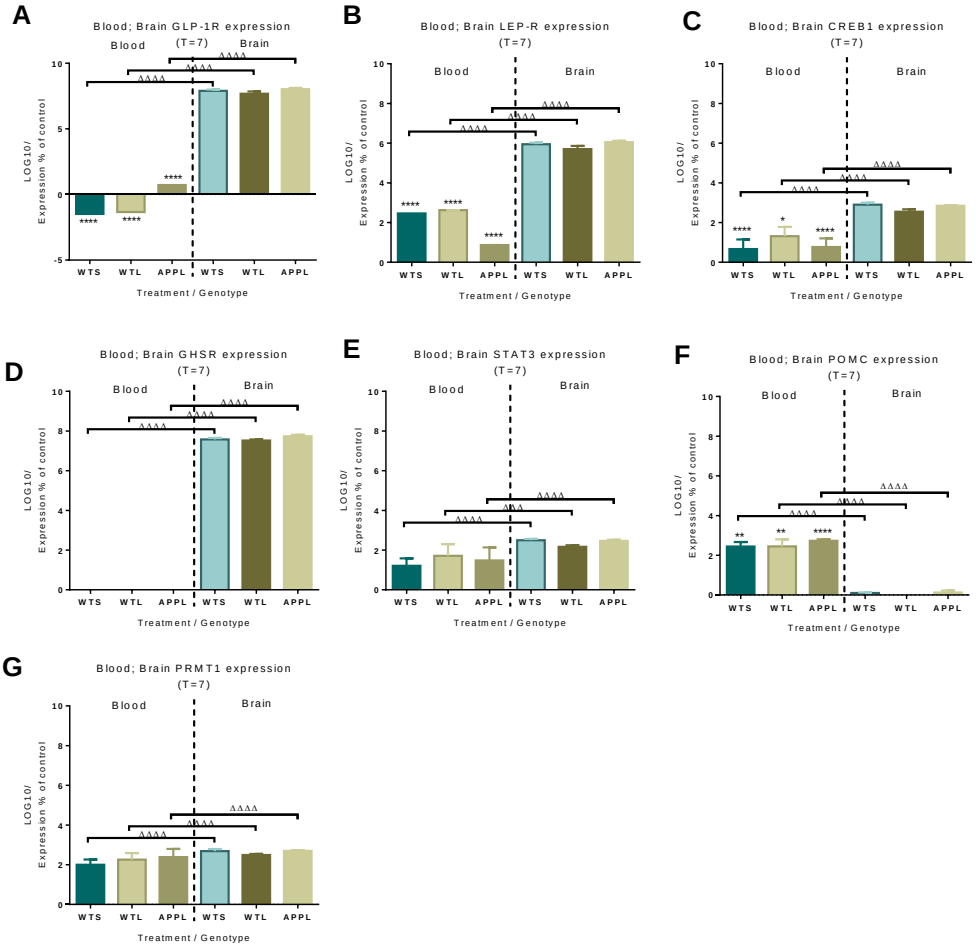


Figure 3.8 - **Peripheral receptor expression is different in APP/PS1 mice and is modulated by liraglutide treatment.** (A) GLP-1R, day 7, (B) LEP-R, day 7, (C) GHSR, day 7. (D) CREB1, day 7, (E) STAT3, day 7, (F) POMC, day 7. (G) PRMT1, day 7. Gene expression in whole blood and whole hemibrain was determined by quantitative real-time PCR and normalised internal control RNS18. After 7 days treatment (T=7) WT expression was determined by comparing WT T=7 to WT T=0 and APP/PS1 T=7 to APP/PS1 T=0. In T=7 brain expression is presented as a percentage change of the treatment groups peripheral T=7 expression. Animals were aged for 8 weeks then received a once daily injection of liraglutide at 15.00 for 7days. GHSR was undetectable in peripheral blood. Statistical significance was determined using one-way ANOVA and Tukey's multiple comparisons test *P<0.05, **P<0.01, ****P<0.0001 vs. T=0 peripheral genotype specific control. ΔP<0.05 and ΔΔΔP<0.001, ΔΔΔΔP<0.0001 vs. 7th day peripheral genotype specific expression.

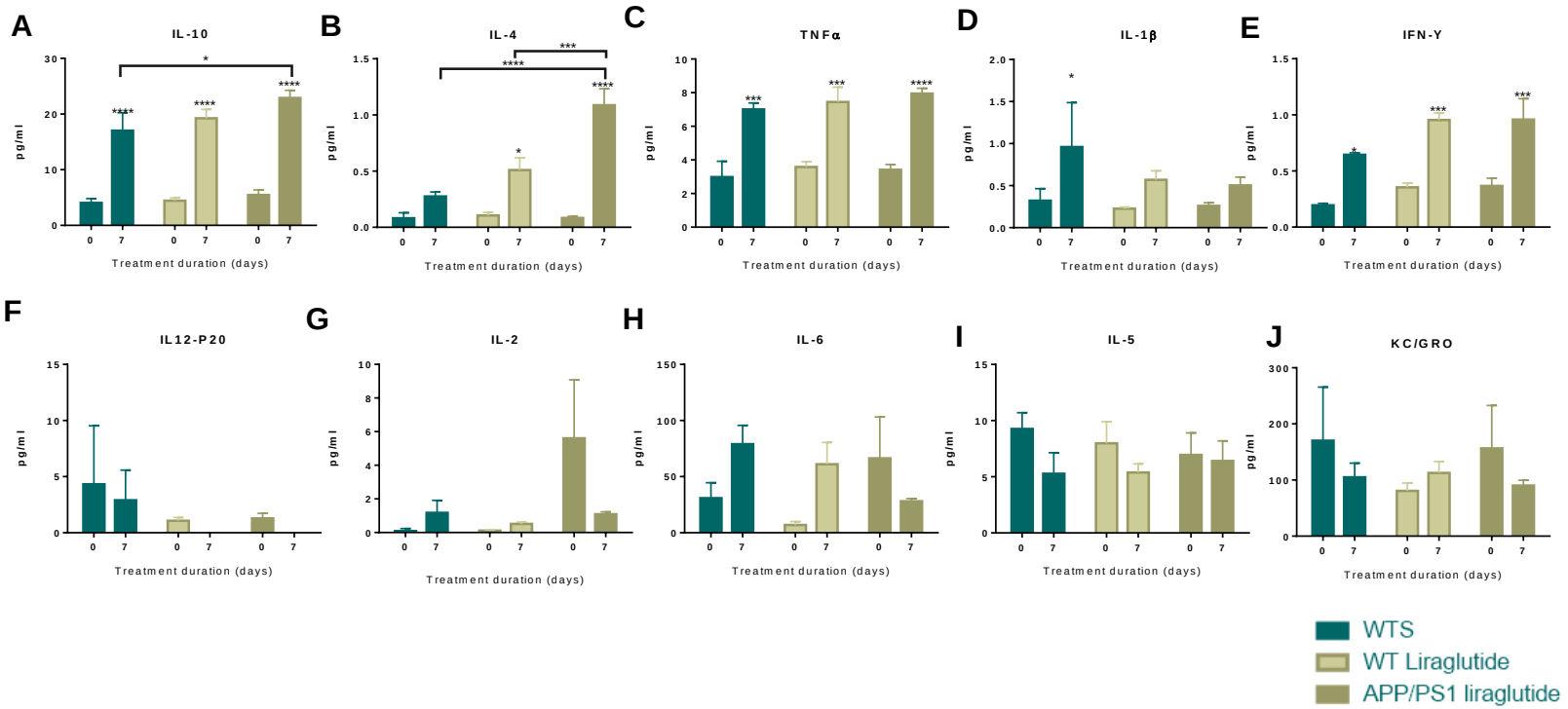


Figure 3.9 – **Plasma cytokine profile of WT saline, WT liraglutide and APP/PS1 liraglutide treated mice.** IL-10 (A), IL-4 (B), TNFα (C), IL-1β (D), IFN-Y (E), IL-12-P70 (F), IL-1(G), IL-4 (H), IL-5 (I) and KC/GRO (H) plasma concentrations were measured, using the Meso Scale Discovery (MSD®) Multi-spot Assay Pro-inflammatory panel 1, in WT mice treated with saline (n=3) or liraglutide (n=5) and APP mice treated with liraglutide (n=6). Animals received a once daily injection of liraglutide, or saline at 15.00 for 7 days. Statistical significance was determined using one-way ANOVA and Tukey's multiple comparisons test. *P<0.05 ***P<0.001, ****P<0.0001 vs. day 0 control.

IL-4 was significantly increased from day 0 to day 7 upon liraglutide administration in WT ($P<0.05$) and APP/PS1 mice ($P<0.0001$; Fig. 3.9B), while levels were unaltered by 7 days saline administration. No significant difference was observed between WT saline and WT Liraglutide groups after 7 days, however the APP/PS1 saline-treated group had increased IL-4 compared to both WT saline ($P<0.0001$) and WT liraglutide-treated mice ($P<0.001$).

3.3.9 Liraglutide affects leptin and insulin associated proteins in the brain of APP/PS1 mice

3.3.9.1 Liraglutide reduces phosphorylation of IRS-1 s616 in APP/Ps1 mouse brain

There was no difference in the total protein level of GLP-1R (Fig. 3.10.1. A), IRS-1 (Fig. 3.10.1. C) or GIPR (Fig. 3.10.1. B) in APP/PS1 mice treated with liraglutide (7 days), GLP-1 RKO mice, untreated APP mice or WT saline treated (7 days) mice. Levels of phosphorylated IRS-1 s616, a putative marker for insulin resistance, varied between genotypes. The levels in the WT saline mice were very low, and had a densitometric value of 0.029 ± 0.006. Levels were significantly increased in the APP/PS1 genotype (0.231 ± 0.054, $P<0.05$), highly significantly increased in the GLP-1RKO model (0.568 ± 0.064, $P<0.001$) compared to WT saline-treated mice. Seven days treatment with liraglutide abolished serine phosphorylation of IRS-1 as s616 (Fig. 3.10.1. D), while levels of total IRS-1 were unchanged in APP mice (Fig. 3.10.1. C).

3.3.9.2 Liraglutide increases total STAT3β in APP/PS1 mouse brain

We measured both STAT3 isoforms (α, β) and STAT3 activation (t705). STAT3α was unchanged across groups (Fig. 3.10.2. A), however STAT3β (Fig. 3.10.2. B) was comparable between WT and GLP-1 RKO mice but undetectable in untreated APP/PS1 mice ($P<0.001$). Seven-day liraglutide treatment in APP/PS1 mice had a profound effect; STAT3β levels were significantly increased ($P<0.001$) compared to WT saline-treated mice. Many studies have found STAT3β to be directly linked to reduced inflammation (Maritano et al., 2004).

3.3.9.3 Liraglutide increases pFOXO1 in APP/PS1 mouse brain

POMC (Fig. 3.10.3. C) and SOCS3 (Fig. 3.10.3. D) protein levels were not significantly different in APP/PS1 mice or the GLP-1R KD model compared to controls. Liraglutide treatment also had no effect on these proteins in the present experiment (Fig. 3.10.3. A, B).

AKT was not significantly affected by genotype or treatment (Fig. 3.10.3. A) but downstream pFOXO1 was (Fig. 3.10.3. B). In the APP/PS1 mice treated with liraglutide pFOXO1 protein levels were significantly increased compared to WT saline-treated mice, indicative of cytoplasmic retention and increased CREB activity (Mohammad et al., 2011).

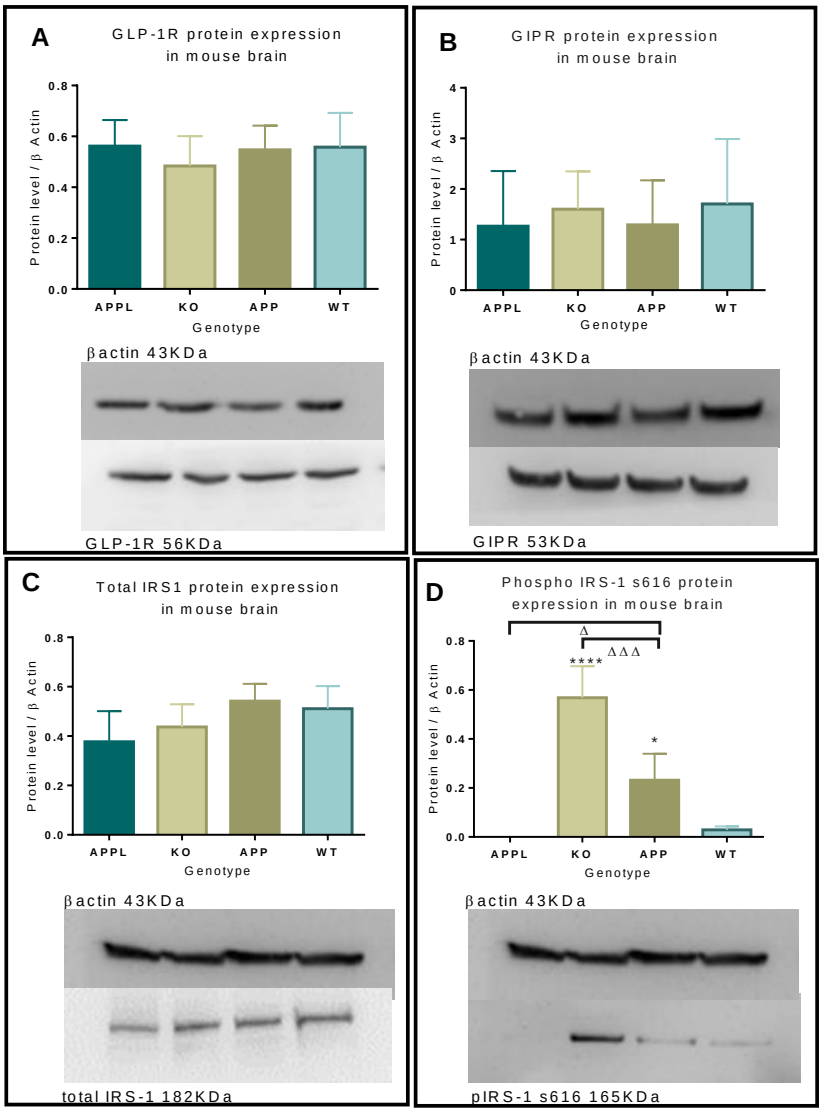


Figure 3.10.1 – **Incretin receptor and IRS1 protein levels in C57BL/6, APP/PS1 and GLP-1RKO mice and after liraglutide-treatment in the APP/PS1 model.** Total protein levels of GLP-1R (A), GIPR (B), total IRS1 (C), phospho IRS1 s616 (D) were measured via western blot. C57 (WT), APP/PS1 (APP) and APP/PS1 treated with liraglutide (APPL) mice were 12 weeks of age, GLP-1RKO (KO) mice were 18 months old. Treated animals received a once daily injection of liraglutide at 15.00 for 7 days. Densitometry was conducted using imagej software. N=4 per group and replicated n=2. Statistical significance was determined using one-way ANOVA and Tukey's multiple comparisons test. *P<0.05, ****P<0.0001 vs. WT saline control. ΔP<0.05, ΔΔΔP<0.001, vs. genotype.

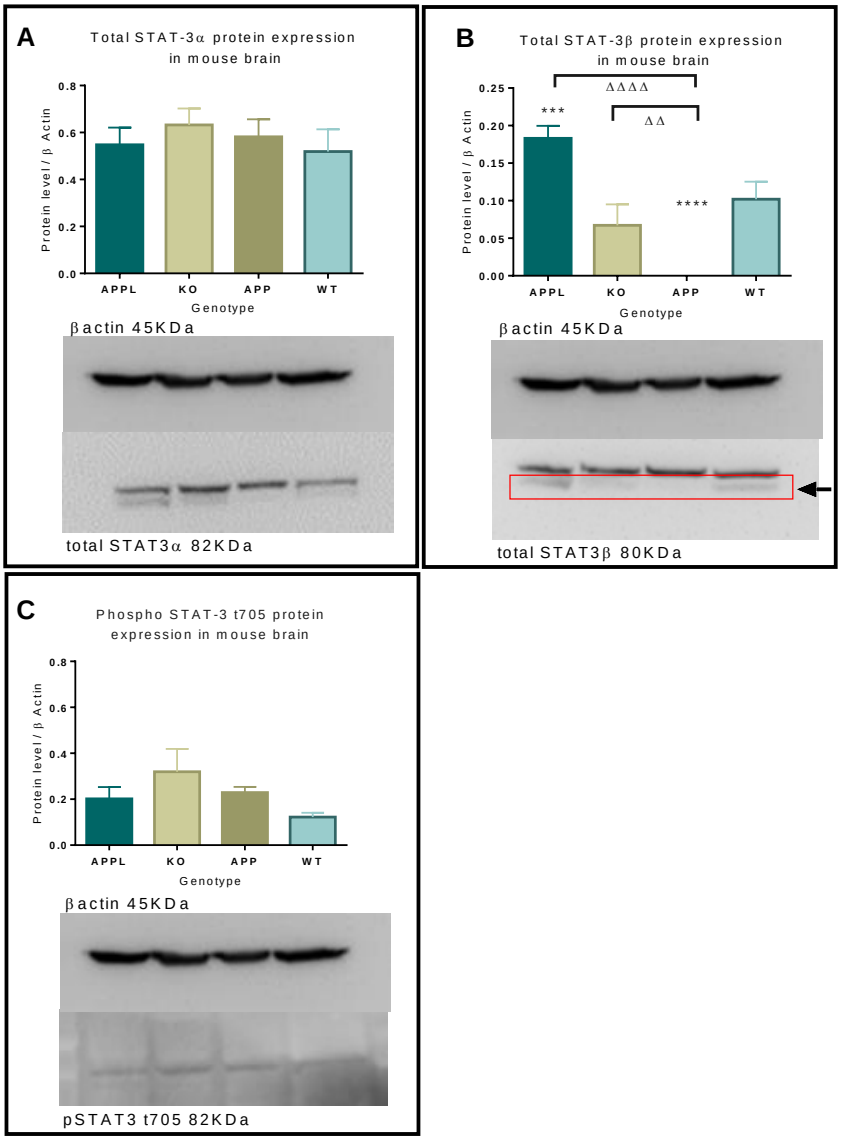


Figure 3.10.2 – **Total and phosphorylated STAT3 protein levels in C57BL/6, APP/PS1 and GLP-1RKO mice and after liraglutide-treatment in the APP/PS1 model** Total protein levels of STAT3α (A), STAT3β (B), phospho STAT3 t705 (C) were measured via western blot. C57 and APP/PS1 mice were 12 weeks of age. GLP-1RKO mice were 18 months old. C57 (WT), APP/PS1 (APP) and APP/PS1 treated with liraglutide (APPL) mice were 12 weeks of age, GLP-1RKO (KO) mice were 18 months old. Treated animals received a once daily injection of liraglutide at 15.00 for 7 days. Densitometry was conducted using imagej software. N=4 per group and replicated n=2. Statistical significance was determined using one-way ANOVA and Tukey's multiple comparisons test. ***P<0.001, ****P<0.0001 vs. WT saline control. ΔΔP<0.01, ΔΔΔΔP<0.0001, vs. genotype.

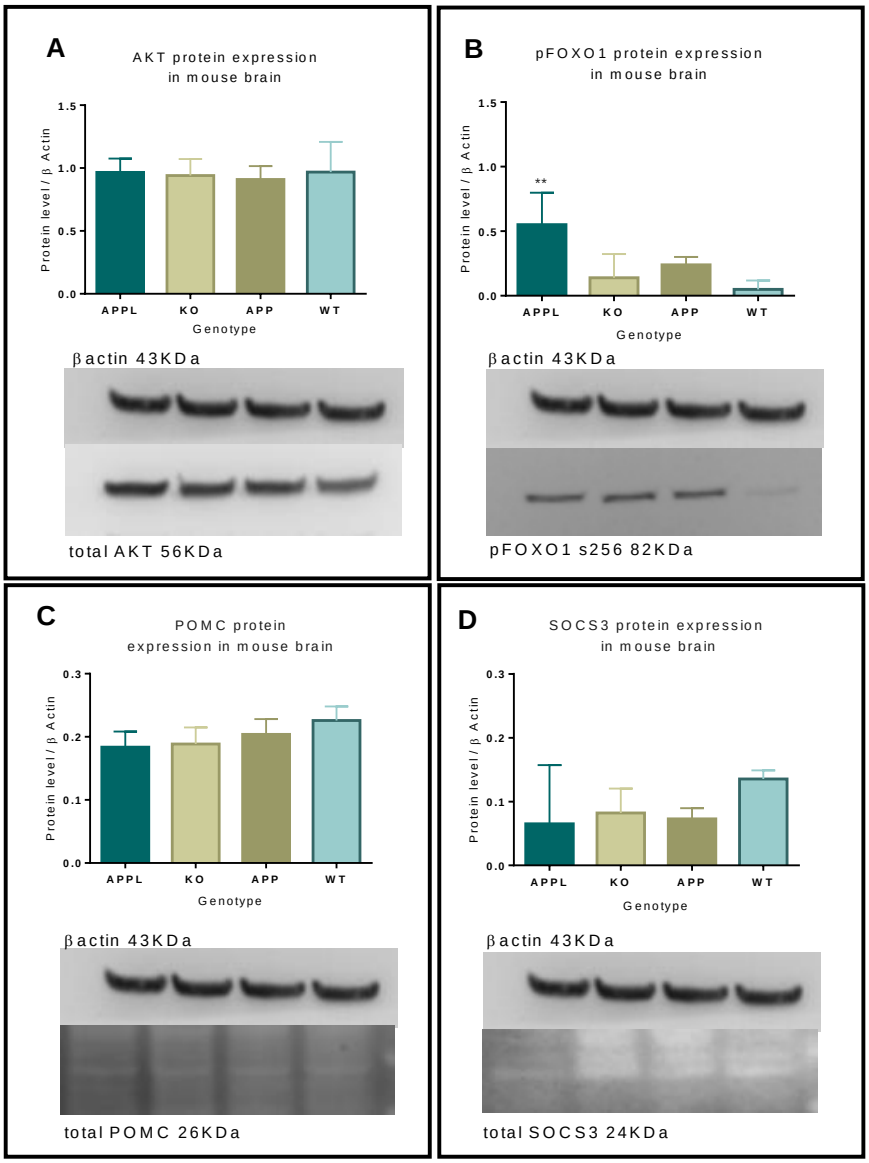


Figure 3.10.3 – **Phosphorylation levels of FOXO1 are elevated in APP/PS1 mice treated with liraglutide.** Total protein levels of AKT (A) phosphoFOXO1 (B), POMC (C) and SOCS3 (D) were measured via western blot. C57 and APP/PS1 mice were 12 weeks of age. GLP-1RKO mice were 18 months old. Treated animals received a once daily injection of liraglutide at 15.00 for 7 days. Densitometry was conducted using imagej software. N=4 per group and replicated n=2. Statistical significance was determined using one-way ANOVA and Tukey's multiple comparisons test. *P<0.05 vs. WT saline control.

3.4 Discussion

This data demonstrates that leptin and insulin gene expression profiles are affected in the brain in the absence of the GLP-1 receptor and during the progression of AD. It also illustrates that the same panel of genes are altered in response GLP-1Ra (liraglutide) treatment, with different effects dependent on treatment duration. The relationship between brain and peripheral gene expression was also identified, and indicates tighter control of transcriptional alterations in brain than in blood, presumably as a protective mechanism.

This, to our knowledge, is the first study to explore the effect of liraglutide treatment on a wide array of genetic and protein biomarkers in WT and APP/PS1 mice in the brain and periphery. Previous work within our laboratory has highlighted liraglutide's ability to improve aspects of learning and memory and to reduce classic AD pathology (McClean et al., 2015), however mechanistic insights behind these effects are poorly described.

Others have indicated a close relationship between leptin and GLP-1 (Akieda-Asai et al., 2014) demonstrating that each peptide can modulate the other to elucidate tissue specific responses in C57BL/6 intestine (Anini and Brubaker, 2003) and obese (*ob/ob)* mouse brain (Ronveaux et al., 2015b). The present study assessed this concept in the brain of GLP-1 RKO mice and found that reducing GLP-1R resulted in significantly increased LEP-R expression and that this translated to its downstream targets. We also observed GIP-R expression was increased in the GLP-1R KO model, and published data supports this finding (Preitner et al., 2004, Pederson et al., 1998).

The GLP-1R was not completely 'knocked out' in the GLP-1RKO model but the significant reduction had a major impact on LEP-R expression and appetite related signalling, increasing downstream genes STAT3 and POMC (Ernst et al., 2009). This is interesting as a number of *in vivo* studies have shown co-stimulation of LEP-R and GLP-1R control food intake in an addictive manor and blocking GLP-1R reduces the LEP-R inhibitory effect (Reidelberger et al., 2012). Other *in vitro* studies have shown that knockout of the GLP-1R and subsequent stimulation with a GLP-1Ra increases LEP-R signalling by directly affecting leptin's negative regulator PTP1B (Kanoski et al., 2015). The present study supports initial characterisations of GLP-1

RKO mice that showed them to have elevated GIP (Preitner et al., 2004) and normal satiety (Lamont et al., 2012), but further adds to this knowledge by defining the close relationship between incretin and leptin signalling.

This transcriptional relationship between GLP-1R and LEP-R signalling pathways, and a desire to understand changes in incretin signalling in a model of Alzheimer's disease, with known brain insulin resistance, provided the rationale to characterise the same panel of genes in an APP/PS1 model and assess the effect of disease progression on expression. Interestingly, in 8 week old APP/PS1 mice, GLP-1R expression was significantly increased and LEP-R expression comparable to control. The elevated expression of GLP-1R in APP/PS1 mice may represent a response to inflammation. Studies have shown that GLP-1 is secreted in response to inflammatory stimuli (Kahles et al., 2014), and at 8 weeks these mice have activated microglia, low level Aβ and high levels of pro-inflammatory cytokines such as TNFα (Malkki, 2015). It is possible that these conditions induce secretion of GLP-1 from both intestinal L cells (Ronveaux et al., 2015a) and microglia in the brain to reduce inflammation (Kappe et al., 2012). As a secondary effect high levels of GLP-1 have been shown to cause hyperactivation of the LEP-R (Zhao et al., 2012), increasing leptins downstream components (Akieda-Asai et al., 2014). Hyperactivation has been linked to internalisation, which can occur as a regulatory mechanism (Kuna et al., 2013). Such internalisation has also been demonstrated with the GLP-1R under chronic GLP-1R agonist treatment (Thompson and Kanamarlapudi, 2015). Internalisation of the Leptin receptor could explain why downstream components of leptin signaling are upregulated, yet the receptor is not. Receptor internalisation is likely an essential process for maintaining the steady state of expression of key genes relating to insulin sensitivity, in both the GLP-1 RKO and APP/PS1 models.

The same panel of genes were measured in 10-month-old APP/PS1 mice to assess the effect of disease progression. At this age these mice phenotypically have significant plaque formation, synaptic loss and cognitive impairment (Malkki, 2015). All genes were down regulated indicative of significant insulin desensitisation. Other research has detailed IRS1/PI3K and AKT signalling disruption in the APP/PS1 model (Talbot et al., 2012a), however, to our knowledge there are no reports of dysregulation of leptin signalling in this model.

To further characterise the GLP-1/leptin relationship focus was shifted to the effect of GLP-1 analogue treatment (liraglutide) on the same panel of genes in C57BL/6 mice after 7 and 21 days. Differential expression was observed dependent on treatment duration; genes involved in leptin and insulin signalling were significantly upregulated after 7 days and significantly downregulated after 21 days treatment. The expression profile after 7 days is consistent with what has been described in other work, with raised LEP-R/STAT3 expression indicative of appetite suppression (Kanoski et al., 2015) as well as increased mRNA levels of genes involved in neurogenesis and insulin gene transcription (Bao et al., 2015). It has been reported that GLP-1R agonists reduce the cleavage of membrane bound LEP-R (Iepsen et al., 2015a). This effect results in decreased soluble receptors and increased free leptin, which would maintain weight loss and prevent the normal appetite increase and energy expenditure decrease associated with decreased weight or fat mass (Rosenbaum et al., 2008).

After 21 days liraglutide treatment, GLP-1R mRNA was significantly reduced in brain. Other *in vitro* work has shown liraglutide increases GLP-1R expression in a dose and time dependent manner although in much shorter time frames (h) (Zhao et al., 2015). *In vivo* studies have shown GLP-1 analogues increase GLP-1R expression in tissues including the heart, pancreas and kidneys, although most experiments were short (1 week) (Noyan-Ashraf et al., 2009). There is *in vivo* evidence that prolonged treatments (6 weeks), reduces GLP-1R activity, possibly indicative of reduced expression (Bock et al., 2003). It is possible that chronic GLP-1Ra therapy, such as the 3 weeks treatment reported here may cause receptor internalisation. A number of pre-clinical studies have shown G protein-coupled receptors (GPCRs) internalise upon activation (Baggio et al., 2004). This reduces cell surface expression, and is a way of reducing biological response, to re-sensitise the receptor (Marchese et al., 2003). This recycling could have a significant downstream effect for all related pathways, including LEP-R/JAK2. Circulating peptide levels (GLP-1, Leptin, insulin) were not measured, however bodyweight was not significantly different between groups. The present data suggest that liraglutide has a profound effect on Leptin and incretin receptor gene expression in brain and lends support to other studies that

have indicated GLP-1 mediates weight loss and affects insulin sensitivity via the LEP-R (Iepsen et al., 2015a).

Mechanistic insights are important, however detecting central changes in the periphery increases clinical relevance of such findings considerably (Thambisetty and Lovestone, 2010). As such we assessed the relationship between gene expression in the brain and in peripheral whole blood to determine differences between genotype (APP/PS1 vs. WT) and in response to liraglutide administration. This analysis indicated that peripheral expression is not perfectly correlated with gene expression in the brain for the genes of interest. Findings supported work that proposed some genes are appropriate and some are not for peripheral analysis (Glatt et al., 2005) as GHSR could not be detected peripherally. Results indicated an inverse relationship between central and peripheral gene expression after 7 days treatment for GLP-1R, CREB1, STAT3 and POMC in both WT and APP/PS1 treated mice, while blood was more predictive of chronic changes observed in brain at 3 weeks. Identification of genes capable of being detected with any surrogate relationship with the brain is thought to be difficult (Tsuang et al., 2005). The complexity of the brain and BBB is well established (Kadakkuzha and Puthanveettil, 2013) but whole blood is also a complex multi cell tissue, with cells that vary in function, morphology and lineage (Watkins et al., 2009). There is growing evidence that brain gene expression affects peripheral tissues via the CNS and circulatory system (Luykx et al., 2016). This work suggests that blood mRNA changes in genes of interest may be predictive of those observed in brain in response to chronic liraglutide administration, however more work is required to support this hypothesis.

The effect of liraglutide on brain and peripheral whole blood gene expression in WT and APP/PS1 was assessed. The APP/PS1 transgenes had a profound effect on peripheral gene expression at baseline. Expression of GLP-1R was reduced and LEP-R increased compared to WT controls. This further supports a relationship between GLP-1R and LEP-R (Akieda-Asai et al., 2014) and also highlights that pathology associated with the APP/PS1 transgenes in brain (Maia et al., 2013) affects whole blood gene expression. Liraglutide treatment differentially affected the peripheral expression of GLP-1R and LEP-R after 7 days in the APP/PS1 model. GLP-1R was increased and LEP-R decreased. This peripheral expression change resulted in

significantly more brain GLP-1R and LEP-R which ultimately resulted in increased expression of all downstream genes of interest, with the exception of POMC. There was no difference in brain gene expression between APP/PS1 and WT saline treated mice. This could indicate that liraglutide impacts peripheral leptin and insulin receptor expression to normalise central gene expression. This would not be unusual; cells maintain homeostasis by regulating surface expression of genes and synthesis of their products (Rue and Martinez Arias, 2015).

Cytokines have been shown to be key mediators of T2D and AD. Quantification of 10 plasma cytokines in response to liraglutide treatment in wild-type and APP/PS1 mice identified differences between the genotypes with respect to response to liraglutide treatment. We observed increased plasma TNFα over the course of treatment in all groups however no differences were observed between genotypes. APP/PS1 mice have been reported to have high levels of TNFα (Montgomery and Bowers, 2012), however such differences were not observed here. Interleukin 1 beta (IL-1β) was significantly increased in 7-day saline-treated WT mice, but not WT or APP/PS1 liraglutide-treated animals, indicative of an anti-inflammatory effect (Schmidt et al., 2008). APP/PS1 mice treated with liraglutide had significantly higher plasma Interleukin 10 (IL-10) and Interleukin 4 (IL-4) than WT mice treated with saline or liraglutide, indicating that the APP/PS1 model is more susceptible to liraglutide mediated increases of IL-10 and IL-4, both of which have been linked to insulin signalling and inflammation (Ropelle et al., 2010). IL-10 is an anti-inflammatory cytokine; in T2D it is reported to function as a pro-inflammatory inhibitor (Dagdeviren et al., 2017), and can act as an activator of the LEP-R, mediating downstream insulin related affects (Niemand et al., 2003). IL-10 is also involved in the adaptive immune response and can induce the differentiation of CD4+ T cells to T helper cells (Mitchell et al., 2017). Both of these characteristics are beneficial in T2D and AD, and could be a mechanism by which GLP-1Ras mediated anti-inflammatory and neuroprotective effects previously observed (McClean and Holscher, 2014). The increase in IL-4 is supported by other research that has shown liraglutide to increase IL-4 and Signal transducer and activator of transcription 6 (STAT6) activity (Kaplan et al., 1996). These proteins can reduce ROS and NF-Kb signalling in diabetes (Clarke et al., 1998). This protective effect is also seen in

APP/PS1 mice with increased IL-4 being directly linked to reduced Aβ peptide and deposition and improved neurogenesis (Kiyota et al., 2010). IL-4 has also been linked to improved learning and memory (Derecki et al., 2010).

The present study indicates liraglutide has anti-inflammatory effects in wild-type and APP/PS1 mice, with more significant increases in IL-10 and 1L-4 in the transgenic model, which is worthy of additional study. An obvious limitation of the present study is the absence of an APP/PS1 saline-treated group. Future studies will assess the cytokine profile in a saline-treated APP/PS1 control group. The increased levels in the majority of cytokines after 7 days saline-treatment in wild-type mice was surprising, and may represent a stress response at the time of sample (Mormède et al., 2002). Despite this differences between groups are considered valid as all mice experienced the same procedure.

A number of proteins related to our pathways of interest were quantified in the present study. Surprisingly total protein levels for all genes of interest were not affected by genotype (C57BL/6, APP/PS1, GLP-1RKO) or by treatment in the case of the APP/PS1 model. This highlights a possible disconnect between transcription and translation with respect to the leptin and insulin genes of interest. There is a body of work that has shown a large number of mRNAs are not equal in terms of protein translation (Schwanhausser et al., 2011). These genes tend to be related to key cellular processes involved in metabolism and homeostasis; genes that have a high gene-protein correlation tend to be stable housekeeping such as β-actin. Many studies have shown mRNA to be variable but protein to be constant. A steady state is thought to be mechanistically homeostatic and for many proteins represents ‘healthy’ (Vogel and Marcotte, 2012). Interestingly, it is thought to be post-transcriptional and translational modifications that compensate for changing mRNA and bring mRNA levels back to ‘normal’ (Shebl et al., 2010). The results from this study support post-translational modification of proteins of interest. Protein levels of phosphorylated FOXO1 s256 and IRS-1 s6161 are different between genotypes and in liraglutide-treated mice. Phosphorylation of FOXO1 at s256 was only significantly increased in the APP/PS1 mice treated with liraglutide. Pre-clinical work has shown this protein to be a key mediator of insulin gene transcription via CREB activity (Yamagata et al., 2008). The opposite effect was observed for IRS-1 activation;

treating APP/PS1 mice with liraglutide abrogated s616 phosphorylation. IRS1 pS616 has been associated with brain insulin resistance (Talbot et al., 2012a), reduced AKT signalling (O' Neill, 2013) and progression of AD pathology (Talbot et al., 2012a). Others have reported liraglutide-induced reductions in IRS1 pS616 in older APP/PS1 mice treated with liraglutide for 2 months (Long-Smith et al., 2013). The present data suggests IRS1pS616 may be detected early in the AD disease process and is altered after short treatment duration.

Interestingly, IRS-1 pS616 was expressed at its highest level in the GLP-1R KO mice; studies have shown that these mice usually have increased peripheral insulin sensitivity (Moffett et al., 2014) but phosphorylation of IRS-1 at this residue is a widely accepted marker of insulin resistance (Talbot et al., 2012b). It may be that brain insulin resistance is a feature of the GLP-1RKO model. Indeed, GLP-1R KO mice are more susceptible to neuronal injury (Belsham et al., 2009) and studies have shown these mice to have impaired synaptic plasticity and memory (Abbas et al., 2009), features which may be attributable to high pIRS-1 s616 in brain. Future work will assess IRS-1 phosphorylation in each of the models in more detail.

The leptin signalling pathway is important for liraglutide's insulin sensitising and anti-inflammatory effects in both T2D and AD. A direct relationship exists between the GLP-1R and LEP-R in brain, which is dysregulated by knockout of the GLP-1 receptor and in APP/PS1 mice with brain insulin resistance. Modulation of associated genes is observed in response to liraglutide treatment which affected central and peripheral gene expression. Gene expression changes observed in blood in response to liraglutide treatment after 24 h were not predictive of brain changes within 1 week, but reflective of changes observed after 3 weeks treatment for most genes of interest. Further studies will identify their efficacy as potential markers of response to therapy.

Chapter IV

Glycaemic control, comorbidity and the effect of GLP-1 analogue therapy in a Northern Irish type II diabetes cohort (DiaStrat)

Abstract

In type II diabetes (T2D), chronic hyperglycaemia causes short and long-term complications, and mortality is often a result of multimorbidity. This study aimed to assess the clinical profile of T2D patients in Northern Ireland (NI) and evaluate the effect of GLP-1Ra treatment on common diabetes measures (HbA_{1c}, BMI and lipids). Prescribing patterns, comorbidity and pharmacological adverse reactions were also analysed. Five hundred T2D patients were enrolled; clinical data was obtained from ECR systems, and primary data analysis of insulin and C-peptide levels.

Glycaemia was poorly managed in the DiaStrat cohort who had an average HbA_{1c} of 65.08 mmol/mol (8.4%). Ninety three percent of patients were overweight and 40% were on insulin therapy. Majority (~70%) were on 2 + anti-diabetes medications, and there was major deviation from NICE stepwise guidance. Over 60% of the cohort had more than 4 comorbidities and 69% had a rare combination affecting <3 patients. Cardiovascular comorbidity was common accounting for 25% of recorded comorbidity. Surprisingly, recent blood lipid tests showed that LDL and total cholesterol were higher ($P<0.0001$) in patients without a CVD diagnosis. Anti-hypertensive and lipid regulating medications made up 47% of all non-diabetes prescriptions, indicative of aggressive cardiovascular risk management, both were overprescribed when compared to recorded diagnosis ($P<0.0001$). There was a high incidence of noted adverse events for these medications.

Patients receiving GLP-1Ra were younger and heavier ($P<0.01$) while HbA_{1c} was no different from the rest of the cohort. There were fewer ($P<0.05$) GLP-1Ra patients with no comorbidity and significantly more with CVD ($P<0.05$); GLP-1Ra patients were prescribed more antihypertensive and lipid regulating drugs ($P<0.01$). This data shows that T2D patients in NI are treated with complex polypharmacy for a wide range of comorbidities. Treatment does not follow NICE guidance or correlate strongly with biochemical measures. C-Peptide correlated with diabetes regime intensification and the assessed comorbidities, and would be a beneficial measure in disease management.

4.1 Introduction

Type II diabetes (T2D) affects 3.2 million 'diagnosed' individuals in the UK (Diabetes UK, 2016), at a cost to the economy £14 billion per year (Hex et al., 2012). Obesity is the greatest risk factor for T2D, with 80% of patients being overweight or obese (BMI>30 kg/m^2) (NICE, 2017). T2D and obesity alone are associated with the development of comorbidities and complications (Choby, 2017), but patients suffering from both have a greatly increased risk of developing heart disease, hyperlipidaemia, hypertension and other microvascular complications (Alexopoulos et al., 2016).

T2D is characterised by chronic hyperglycaemia (Gajos and Mostowik, 2016) and has been shown to worsen with weight gain and improve with weight loss (Sattar et al., 2016). Significant weight loss, such as that linked to gastric bypass surgery, has been shown to lead to partial or total remission of T2D and associated conditions (Schauer et al., 2016). Comorbidities linked to diabetes and obesity affect systems all around the body, and have been traditionally categorised as macrovascular and microvascular (Lu et al., 2009). Such characterisation can overshadow other complications such as endocrine, digestive, musculoskeletal and those associated with cognitive function and mental health (Pentakota et al., 2012). It is thought that in the UK most diabetes patients have at least one comorbidity and 40% are thought to have at least three (Khan, 2017). It has been suggested that treatment of comorbidities may shift priority away from diabetes (Piette and Kerr, 2006). This is supported by large retrospective studies (n=42,826), focusing on patients with new onset diabetes which indicate that those with comorbidities sharing a similar pathogenesis or management plan to diabetes received increased diabetes care testing (LDL, $HbA_{1C,}$) than those with discordant conditions such as musculoskeletal disorders (Pentakota et al., 2012).

The treatment options available to T2D patients have increased in recent years evident by the expanding guidance by the NICE (NICE, 2015), American Diabetes Association (ADA) (ADA, 2016) and European Association for the Study of Diabetes (EASD) (European Association for the Study of Diabetes, 2015).

NICE recommend a uniform stepwise intensification of therapy to achieve adequate HbA_{1c} control (NICE, 2017), while the EASD are now recommending a personalised approach (European Association for the Study of Diabetes, 2015). This is thought to be due to failings in diabetes control despite comprehensive guidance and numerous therapeutic options (Iglay et al., 2016). Studies in the US have indicated ~55% of T2D patients have an HbA_{1C} greater than 53 mmol/mol (7%) (Menon and Ahluwalia, 2015), while in the UK ~51.3% of patients are thought to exceed the 53 mmol/mol (7%) threshold (Mauricio et al., 2017).

A personalised approach to treatment of multiple chronic diseases can be difficult. Clinicians are often required to treat a constellation of diseases with different management approaches and pharmacological options (Pentakota et al., 2012). NICE have guidelines for the prescription of glycaemic medications and non-glycaemic medications which takes into account drug safety and tolerability profiles (NICE, 2015).

Electronic Care Records (ECR) are a dependable source of clinical information that encompass a wealth of patient data related to treatment strategies, comorbidity and a range of biochemical readings (Franzen et al., 2016). Individuals with three comorbidities are prescribed on average 9 medications (Patel et al., 2017), see multiple clinicians and receive 15 home visits a year (Hing et al., 2008). ECR systems promote coordination between primary and secondary health care providers enabling clinicians to exchange and update clinical information. This reduces duplicate hospital tests and data storage, and improves patient support and clinical decision making (Sulmasy et al., 2017).

As a secondary use, ECR systems facilitate clinical research (Cowie et al., 2017). Many systems are being used to conduct epidemiologic and observational research (Bower et al., 2017). ECR systems can be used as standalone data sets or used to link up with primary research data, proving integrated datasets with information spanning the patient's life (Häyrinen et al., 2008). Such studies enable longitudinal analysis of disease progression and drug efficacy. Some countries have linked ECRs at the patient level, to secondary population data sets including; disease registries, education, social care and criminal justice systems (Casey et al., 2016).

Such comprehensive data linkage allows for greater understanding of factors such as socioeconomic status on disease.

Safety and surveillance research is an emerging application for ECR systems (Cederholm et al., 2015). ECR can provide real world information (outside of clinical trial reports) on adverse events or reactions (Trifiro et al., 2009). The European Exploring and Reporting Adverse Reactions by Integrative Mining of Clinical Records and Biomedical Knowledge (EU-ADR) project encompasses 8 databases for the analysis of choice target adverse reaction numbers (Trifiro et al., 2009). This project works closely with the European Medicines Agency (EMA) to inform the European Network of Centres for Pharmacoepidemiology and Pharmacovigilance (ENCePP) on post marketing drug risk (Eichler et al., 2008). In the United States the Food and Drug Administration (FDA) also uses ECR to similar ends (Ball et al., 2016).

Clinical research utilises ECR in a number of ways, the most common is the facilitation of patient recruitment, enabling efficient generation of patient lists that meet specific inclusion and exclusion criteria (Köpcke et al., 2013). These systems can also be used to collect and reuse information used in routine clinical care such as demographic, prescription and comorbidity data. As such data is inputted at point of care by a health professional it is thought to have a reduced risk of transcriptional error (Thadani et al., 2009). In a clinical trial setting, ECR systems have been shown to reduce timelines by removing manual data entry and enable remote data observation (Fordyce et al., 2015). Initiatives and online tools such as the Innovative Medicine Initiative (IMI) Electronic Health Records for Clinical Research project are examples of systems that simplify the reuse of clinical data for research (De Moor et al., 2015).

Challenges and limitations exist when utilising ECR data. Data quality and validation issues have been reported by clinical studies, predominantly related to inaccurate information and coding errors (Hersh et al., 2013). As data entry is reliant on various medical personnel, across multiple centres, input parameters and methodologies vary. This can cause errors and lead to incomplete data capture.

Current screening measures for multimorbidity require numerous patient-clinic visits for effective management. This requirement exceeds currently available clinic hours in the UK (Baxter et al., 2016), and results in many conditions that would

usually be treated in an inpatient setting being treated on an outpatient basis (O'Connor et al., 2016). Optimisation of care strategies to deal with and support to multimorbid patients is critical (Khan, 2017). Defining the effect Multiple Comorbidity Clusters (MCC) have on mortality, therapy and choice of therapy is of significant importance (Li et al., 2013).

Diabetes glycaemic control, prescription trends, comorbidity and associated prescribing patterns have not been assessed in Northern Ireland, nor has the effect of glucagon-like peptide-1 receptor analogue (GLP-1Ra) therapy on such measures. The present study was designed to assess currently available ECR recorded data on glycaemic control, lipid profile, BMI and recorded comorbidity, prescription data, unscheduled care and noted adverse reactions in 500 type 2 diabetes participants. Additionally insulin and C-peptide, which are not routinely measured in clinical practice, were measured to identify their utility as a measure of disease progression or management. GLP-1R analogue prescribed patients were also characterised, to better inform clinical practice on best stratification policies of this phenotypically severe group.

4.2 Methods

4.2.1 Participant recruitment

All participants were identified by diabetes clinicians at the Altnagelvin Hospital, in Northern Ireland, after ORECNI ethical approval and Western Trust governance approvals were in place, as described in *Chapter 2, Section 2.7*. All participants were given a 48 hour cool down period prior to sample collection.

4.2.2 Sample collection

4.2.2.1 Buccal Swab

A single buccal swab was obtained using a MasterAmp™ buccal brush (epicentre, Madison, WI USA; Cat no. 4459), as described in *Chapter 2, Section 2.8.1.1.*

4.2.2.2 Blood sampling

Blood samples were obtained using 21 G Vacuette® safety needle (Greiner Bio-One, Stonehouse, UK; Cat no. 450091), as described in *Chapter 2, Section 2.8.1.2* Approximately 26 ml of blood was extracted in total into 2 x 9 ml EDTA (K3E K3EDTA) coated Vacuette® tubes (Greiner Bio-One, Stonehouse, UK; Cat no. 455036) and 1 x 8ml Serum (Z Serum Sep Clot Activator) tube (Greiner Bio-One, Stonehouse, UK; Cat no. 455071). All blood samples were processed immediately.

4.2.3 Blood processing

Polypropylene tubes were labelled using Item Tracker© software (ItemTracker Software Ltd, Suffolk, UK), prior to processing for serum, plasma, protein, RNA and whole blood, as described in *Chapter 2, Section 2.8.1.3*. Samples were stored at -80^{o}C in HTA compliant freezers.

4.2.4 DNA extraction

4.2.4.1 Buccal Swabs

DNA was extracted from MasterAmp™ buccal brushes (epicentre, Madison, WI USA; Cat no. 4459) using the QIAamp DNA blood mini kit (Qiagen, Manchester, UK; Cat no. 51106), as described in *Chapter 2, Section 2.9.1*.

4.2.4.2 Whole blood

EDTA whole blood samples were thawed at room temperature (20°C) and DNA extracted using the Gentra Puregene blood kit (Qiagen, Manchester, UK; Cat no. 158445), as described in *Chapter 2, Section 2.9.2*.

4.2.4.3 DNA quantification

DNA was quantified using a Qubit®Fluorometer (ThermoFisher Scientific, UK; Cat no. Q33216) and Qubit™ dsDNA HS Assay kit (ThermoFisher Scientific, UK; Cat no. Q32854), as described in *Chapter 2, Section 2.9.3*.

4.2.5 Enzyme-Linked Immunosorbent Assays (ELISA)

4.2.5.1 Insulin sandwich ELISA

Plasma insulin (25 µl) was quantified using human Mercodia insulin ELISA kit (Mercodia, Uppsala, SE; Cat no. 10-1113-10), as described in *Chapter 2, Section 2.10.1*.

4.2.5.2 C-peptide sandwich ELISA

Plasma C-peptide (25 µl) was measured using human Alpco C-peptide ELISA kit (Alpco, Salum, US; Cat no. 80-CPTHU-E01.1, E10), as described in *Chapter 2, Section 2.10.2*.

4.2.6 Clinical database construction

Relevant clinical information for all participants (n=500) was obtained from Western Health and Social Care Trusts, Hicom Diamond.NET diabetes management system

(Hitcom, Surrey, UK) and Orion Health technologies, Northern Ireland Electronic Care Record (NIECR) (Orion health, Hammersmith, UK). All data was used in compliance with research ethics and Data Protection Act 1998. Full details on database construction are described in *Chapter 2, Section 2.11.1.*

4.2.7 Statistical analysis

All statistical analysis was conducted using Graphpad Prism software (Graphpad Software Inc., La Jolla, CA, USA; v6.0h). Student's *t* tests and one way ANOVA with Tukey's post-hoc test, and Z-test proportions were used as described in *Chapter 2; Section 2.15.*

4.3 Results

The descriptive characteristics of the Diastrat Cohort are presented in Table 4.1. Data are presented for the total cohort, and also separated by gender. The cohort was predominantly male; of the 494 eligible participants 310 were male and 184 female. Obese females had a significantly higher BMI than obese males (39.3 ± 10 vs. 35.8 ± 5 kg/m^2; P<0.001). Females also started insulin therapy at a significantly younger age than males (53.97 ± 3, vs 57.45 ± 11; p><0.05). There were no sex differences in measures related to glycaemic control, blood pressure (BP) and lipids. Subsequent analyses were therefore conducted on the cohort as a whole. The mean age was 62 ± 11 years, 86% of participants were classified as being above HbA_{1c} target (>48.9 mmol/mol, 6.6%) and 93% were overweight (BMI >25 Kg/m^2). Blood pressure (BP) was generally well managed. Mean systolic BP was 132 ± 14 mmHg and diastolic of 76 ± 9 mmHg. Two hundred and nine participants had a clinically diagnosed lipid abnormality. Mean HDL was 1.13 mmol/l; LDL 1.93 mmol/l and total cholesterol 3.91 mmol/l indicative of good management of hyperlipidaemia within the cohort. Overall 190 participants were prescribed exogenous insulin injections.

DiaStrat	Complete Cohort			Male			Female		
	Total	Mean (SD)	%	Total	Mean (SD)	%	Total	Mean (SD)	%
Number of Participants	494			310			184		
Age (Years)	491	62.75 (11)	99.4	308	63.1 (11)	99.4	183	62.12 (12)	99.5
Duration of Diabetes	395	12.43 (8)	80.0	251	12.48 (8)	81.0	144	12.36 (9)	78.3
HbA_{1c}	454	65.08 (17)	91.9	282	63.91 (17)	91.0	172	65.21(19)	93.5
Below target <47.9 mmol/mol	56	42.38 (4)	12.3	31	42.91 (3)	11.0	25	41.72 (5)	14.5
Target 48 mmol/mol	9	48 (0)	2.0	4	48 (0)	1.4	5	48 (0)	2.9
above target >48.9 mmol/mol	389	68.75 (15)	85.7	247	68.02 (14)	87.6	142	70.01 (17)	82.6
BMI	360	33.93 (8)	72.9	224	33.71(6)	72.3	136	34.69(10)	73.9
Underweight <18.4	0	0 (0)	0.0	0	0 (0)	0.0	0	0 (0)	0.0
Healthy 18.5-24.9	26	23.30 (1)	7.2	15	23.82 (1)	6.7	11	23.82 (1)	8.1
Overweight 25-29.9	84	27.87 (1)	23.3	50	27.94 (1)	22.3	34	27.77 (1)	25.0
Obese >30	250	37.07 (7)	69.4	159	35.8 (5)	71.0	91	39.39 (9)***	66.9
BP	323		65.4	204		65.8	119		64.7
Systolic	323	132 (14)		204	131.6 (14)		119	132.8 (11)	
Diastolic	323	76 (9)		204	76.3 (9)		119	76.4 (9)	
Target <130/80 mmHg	68	120/71	21.1	41	121/71	20.1	27	119/71	22.7
Diagnosed Lipid abnormality	209		41.8	124		40.0	85		46.2
HDL	447	1.13(1)	100.0	281	1.05(1)	90.6	166	1.26(1)	87.4
LDL	444	1.93(1)	100.0	278	1.83(1)	89.7	166	2.08(1)	87.4
Total Cholesterol	447	3.91(2)	100.0	281	3.77(2)	90.6	166	4.11(2)	87.4
Number on Insulin	190		38.5	118		38.1	72		39.1
Time on Insulin (Years)	190	7.06 (5)		118	6.76 (5)		72	7.54 (5)	
Age at Insulin introduction	188	56.13 (12)	98.9	117	57.45 (11)	99.2	71	53.97 (13)*	98.6
Delay from diagnosis to insulin introduction	184	9 (7)	96.8	115	9.44 (7)	97.5	69	8.27 (8)	95.8

Table 4.1 – **Demographics and clinical features of Type 2 Diabetes patients in Northern Ireland.** Data is presented as Complete Cohort, Males and Females. Each column describes total number of participants, mean ± SD and % of total for each measure. All participants were aged 18-80 years. Targets used for HbA_{1C}, BMI and BP classification were obtained from NICE. Significance was determined by Student's t-test, *P<0.05, ***P<0.001 vs. males.

4.3.1 Diabetes treatment regimes

Illustrated in Figure 4.1 are the number of anti-diabetes medications per participant (A) and number of participants on each class of anti-diabetes therapy (B). Most participants were prescribed 3 (140) or 2 (129) anti-diabetes medications (Fig. 4.1A). Eight individuals were on 5 anti-diabetes drugs and 57 were not taking any anti-diabetes medication (Fig. 4.1A). Biguanides were the most prescribed drug class (365), followed by SU (166), short acting insulin (133), and DDP IV inhibitors (95). NICE advised 4th line therapy, GLP-1Ra, were prescribed to 81 participants (Fig. 4.1B).

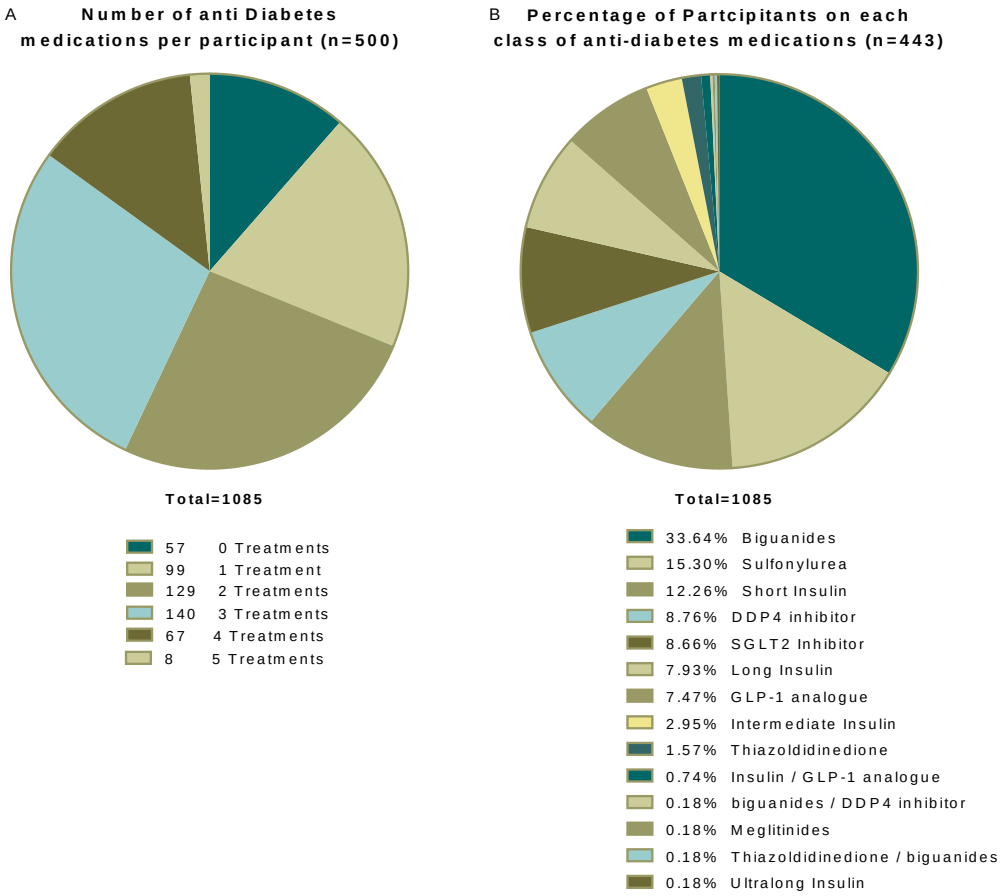


Figure 4.1 – **Number of anti-diabetes drug treatments and frequency of prescription of anti-diabetes drug classes in the DiaStrat cohort.** (A) Total number of participants on 0-5 anti-diabetes drugs (n=500). (B) Total number of prescribed drugs per diabetes drug class (n=443 participants, n=1085 recorded medications). All drugs were classified according to the British National Formulary (BNF).

Across the cohort 443 participants were prescribed at least 1 anti-diabetes medication; 362 were on a regime that included metformin (+) and 81 were on a metformin negative (-) regime (Table 4.2). Participants were sub-grouped into 4 classifications dependant on treatment intensification stage specified by NICE (monotherapy, dual therapy, triple therapy or quad plus therapy). Metformin monotherapy was prescribed to 71 individuals (19.6% of the total cohort), while insulin therapy (short, intermediate, long) was the most frequently prescribed treatment (18, 64.3%) if metformin was not tolerated. The most common additions for dual therapy in metformin positive regimes were; short acting insulin (28, 29.8%), sulphonylurea (SU) (22, 23.4%) and DDP IV inhibitors (17, 18.1%). In the metformin negative (-) group the most common dual therapy combinations were; long + short acting insulin (16, 45.7%) and DDP IV inhibitor + sulphonylurea (6, 17.1%).

Majority of the cohort were on a triple therapy metformin positive (+) regime (124). Within this classification most were prescribed metformin plus; DDP IV inhibitor + SU (23, 18.5%), long + short acting insulin (18, 14.5%) or GLP-1Ra + SU (17, 13.7%). Within the metformin negative (-) group the most frequently prescribed regime was a long + short acting insulin + a SGLT2 inhibitor (4, 25%). There were 75 individuals prescribed four or more anti-diabetes medications in metformin positive (+) regimes. The most commonly prescribed were metformin plus: GLP-1Ra + SGLT2 inhibitor + SU (12, 16.4%), DDP IV inhibitor + SGLT2 inhibitor + SU (8, 11%), long + short acting insulin + SGLT2 inhibitor (8, 11%). There were no quad therapy regimes in the metformin negative (-) group.

DiaStrat Cohort			n=500	Total on Metformin (+)		362	82%
Participants on Diabetes Medication			n=443	Total on Metformin (-)		81	18%
	Metformin *(+)*				**Metformin *(-)***		
		Total	**%**			**Total**	**%**
Mono therapy	Metformin	71	100.0		Short acting insulin	11	39.3
					Intermediate Insulin	4	14.3
					DDP IV inhibitor	4	14.3
					Long acting insulin	3	10.7
					Sulphonylurea	4	14.3
					SGLT2 inhibitor	1	3.6
					GLP-1R agonist	1	3.6
	Total	71	19.6	**Total**		28	34.6
Dual therapy	Short acting insulin	28	29.8		Long + Short acting insulin	16	45.7
	Sulphonylurea	22	23.4		DDP IV inhibitor + Sulphonylurea	6	17.1
	DDP IV inhibitor	17	18.1				
	GLP-1R agonist	11	11.7				
	Intermediate insulin	8	8.5				
	SGLT2 inhibitor	3	3.2				
	Thiazolidinediones	2	2.1				
	Insulin + GLP-1 mix	2	2.1				
	Long acting insulin	1	1.1		*Other combinations (≤2)*	*13*	37.1
	Total	94	26.0	**Total**		35	43.2
Triple therapy	DDP IV inhibitor + Sulphonylurea	23	18.5		Long +Short acting insulin +SGLT2 inhibitor	4	25.0
	Long + Short acting insulin	18	14.5		DDP IV inhibitor + SGLT2 inhibitor + Sulphonylurea	2	12.5
	GLP-1R agonist + Sulphonylurea	17	13.7				
	SGLT2 inhibitor + Sulphonylurea	10	8.1				
	GLP-1R agonist + SGLT2 inhibitor	8	6.5				
	GLP-1R agonist + Short acting insulin	5	4.0				
	DDP IV inhibitor + Short acting insulin	5	4.0				
	DDP IV inhibitor + SGLT2 inhibitor	5	4.0				
	SGLT2 inhibitor +Short acting insulin	5	4.0				
	Short acting insulin + Sulphonylurea	4	3.2				
	Long acting insulin + Sulphonylurea	4	3.2				
	Other combinations (≤3)	*21*	16.9		*Other combinations (1)*	*10*	62.5
	Total	124	34.3	**Total**		16	19.8
Quad plus therapy	GLP-1R agonist + SGLT2 inhibitor + Sulphonylurea	12	16.4				
	DDP IV inhibitor + SGLT2 inhibitor + Sulphonylurea	8	11.0				
	Long + Short acting insulin + SGLT2 inhibitor	8	11.0				
	Long acting insulin + SGLT2 inhibitor + Sulphonylurea	6	8.2				
	GLP-1Ra + Long + Short acting insulin	4	5.5				
	SGLT2 inhibitor + Short acting insulin + Sulphonylurea	3	4.1				
	Other combinations (1)	*32*	43.8		*Other combinations (1)*	*2*	100.0
	Total	73	20.2	**Total**		2	2.5

Table 4.2 – **Prescription trends for anti-diabetes medications in the DiaStrat cohort.** Data is presented as a Metformin positive or Metformin negative and split into four groups as stipulated (Mono, Dual, Triple, Quad therapy) by NICE. Total is equal to the total count of participants in that drug/ combination, percentage is equal to total count / total number in treatment group.

4.3.1.1 Relationship between number of anti-diabetes medications and clinical measures of diabetes severity

The number of anti-diabetes medications prescribed was positively correlated with HbA_{1c} (R=0.40, P<0.0001; Fig. 4.2A). The mean HbA_{1c} of individuals on 0 anti-diabetes drugs was 50.5 ± 8.4 mmol/mol; if prescribed 5 medications HbA_{1c} was significantly higher at 78.0 ± 13.3 mmol/mol (P<0.0001). HbA_{1c} was significantly higher in patients prescribed two or more anti-diabetes medications when compared to those on none (P<0.0001).

C-peptide was negatively correlated with the number of diabetes medications (R= -0.23, P<0.0001; Fig. 4.2B). The average plasma C-peptide value for participants not receiving anti-diabetes drugs was 7.6 ± 7.4 ng/ml. A decline in C-peptide concentration was recorded as the number of prescribed anti-diabetes medications increased; 3 medications (4.9 ± 5.1 ng/ml), 5 medications (3.6 ± 1.7 ng/ml). C-peptide levels were significantly reduced in individuals prescribed 4 anti-diabetes medications compared to those on none (P<0.01).

BMI was positively correlated with the number of anti-diabetes medications (R=0.22, P<0.0001; Fig. 4.2C). Those on no medication had a BMI 31 ± 5 and those on 4 medications had a BMI of 36 ± 7 (P<0.05). Interestingly, irrespective of the number of anti-diabetes medications all groups had a mean BMI >30.

LDL was negatively correlated with the number of anti-diabetes medications (R=-0.2, P<0.0001), while HDL and total cholesterol were not (Fig. 4.2D). Participants on 3 and 4 anti-diabetes medications had significantly lower LDL cholesterol and total cholesterol compared to those on no anti-diabetes medications. (LDL values 0 medications 2.1 ± 0.7 pg/ml, 3 medications 1.7 ± 0.7 pg/ml and 4 medications 1.5 ± 0.6 pg/ml). Total cholesterol (0 = 4.1 ± 0.7 pg/ml, 3 = 3.6 ± 0.9 pg/ml, 4 = 3.5 ± 0.7 pg/ml)

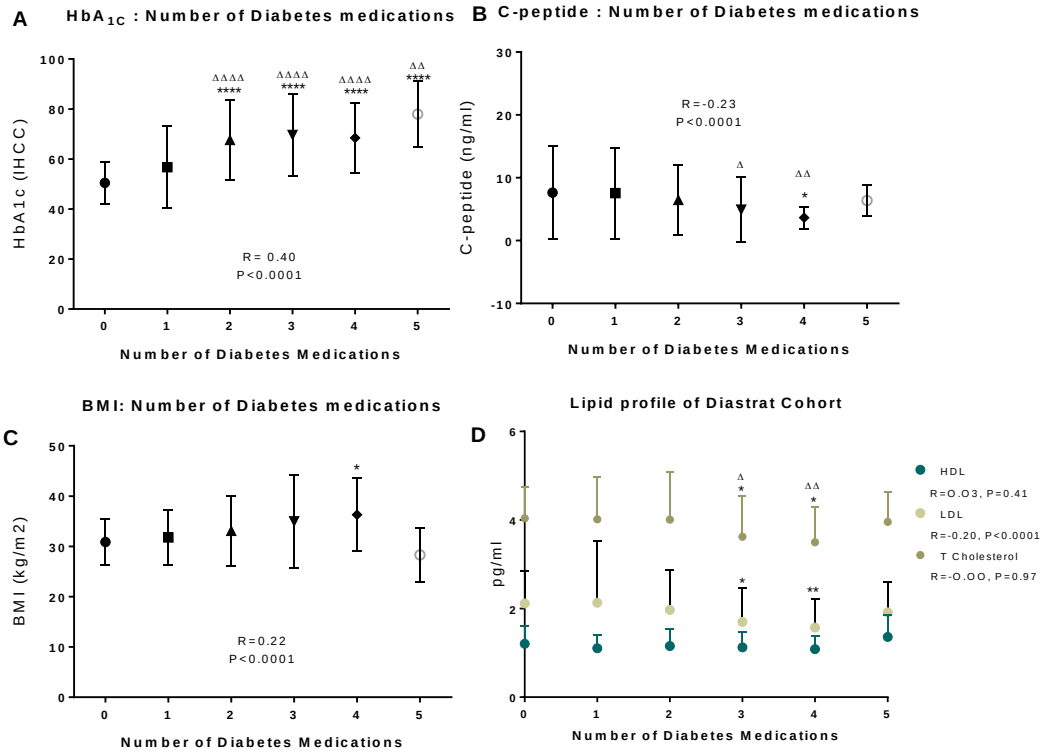


Figure 4.2 – **Relationship between number of anti-diabetes medications and clinical measures of diabetes severity.** (A) HbA_{1c} n= 454; (B) C-peptide n=368; (C) BMI n=360; (D) Lipid profile (HDL, LDL, Cholesterol) n=447. Participants were grouped dependant on the number of anti-diabetes medications prescribed and the mean value and standard deviation of HbA_{1c}, C-peptide, BMI, LDL, HDL and total cholesterol plotted. Two tailed Spearman's correlation analysis between number of diabetes mediations and each measure were calculated. Analysis of grouped data was conducted using one-way ANOVA and Tukey's multiple comparisons test. *P<0.05, ****P<0.0001 vs. 0 medications. ΔP<0.05 and ΔΔP<0.01, ΔΔΔΔP<0.0001 vs 1 medication.

4.3.1.2 The inter-relationship of C-peptide with HbA_{1c}, BMI and insulin levels

Plasma C-peptide levels negatively correlated with HbA_{1c} (R= -0.12, P<0.05, Fig. 4.3A) and were not correlated with BMI (R=0.02, P<0.66, Fig. 4.3B). Plasma C-peptide and insulin were positively correlated in participants receiving exogenous insulin injections (R= 0.68, P<0.0001, Fig. 4.3C). C-peptide levels were 2.4 ng/ml when insulin was between 0.1 ng/ml and significantly increased to 6.3 ng/ml when insulin was 4+ ng/ml (P<0.0001). C-peptide levels were higher in participants not receiving exogenous insulin therapy, and also positively correlated with plasma insulin levels (R= 0.48, P<0.0001, Fig. 4.3D). C-peptide levels were 4.7 ng/ml when insulin was between 0-1 ng/ml and significantly increased (P<0.05) to 10.73 ng/ml when insulin was 4+ ng/ml

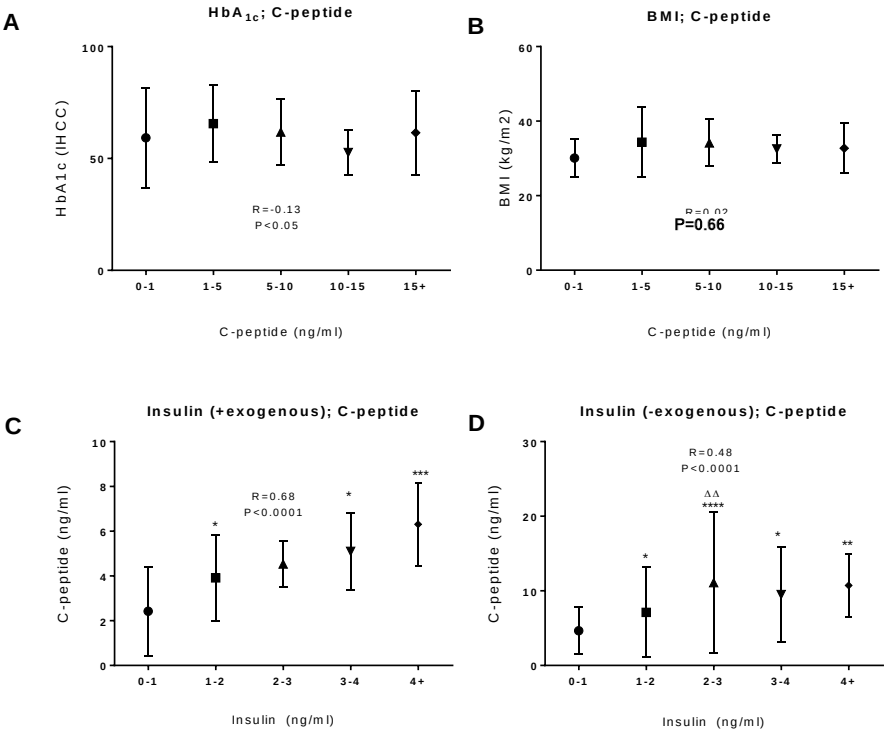


Figure 4.3 – **The inter relationship of C-peptide with HbA_{1c}, BMI and insulin levels** (A) HbA_{1c}; C-peptide, n= 333 (B) BMI; C-peptide, n=241 (C) C-peptide; Insulin (+exogenous); n= 112 (D) C-peptide; Insulin (-exogenous), n=260. Participants were grouped dependant on plasma levels of either C-peptide or insulin, the mean value for each group and standard deviation was plotted. Two tailed Spearman's correlation analysis between; plasma C-peptide, HbA_{1c}, BMI and insulin was conducted. Statistical significance was determined using one-way ANOVA and Tukey's multiple comparisons test. ***P<0.001 vs. 0-1 ng/ml insulin. ΔP<0.05 and ΔΔP<0.01 vs 1-2 ng/ml insulin.

4.3.1.3 Effect of GLP-1R agonist therapy on markers of diabetes control

Participants treated with a GLP-1Ra (4^{th} line treatment) were compared to those who were not. There was no significant difference in duration of diabetes or HbA_{1c} levels (Fig. 4.4A, C), however GLP-1Ra positive patients were significantly younger (59.4 ± 11.2 years, P<0.01, Fig. 4.4B) and BMI was significantly higher (P<0.05) than participants treated with other anti-diabetes medications. GLP-1Ra (+) participants had a mean BMI of 35.72 ± 7.1 compared to GLP-1Ra (-) participants who had a BMI of 33.45 ± 7.8. Plasma insulin and C-peptide levels were not affected regardless of the presence of exogenous insulin or GLP-1Ra treatment (Fig. 4.5).

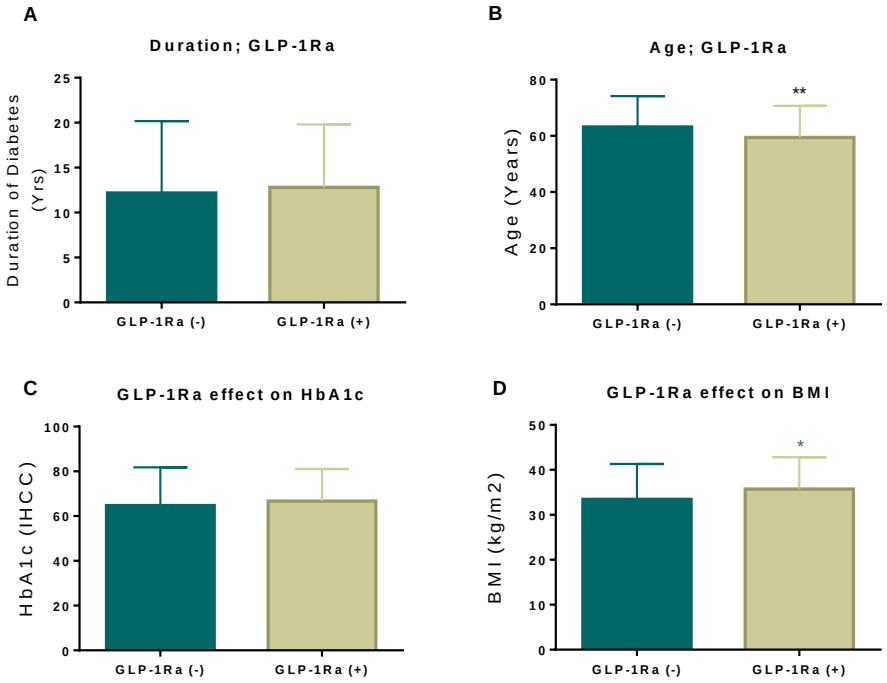


Figure 4.4 – **GLP-1Ra patient characteristics; Duration, age, HbA_{1c} and BMI.** (A) Duration: GLP-1Ra (+) n=80, GLP-1Ra (-) n=315. (B) Age: GLP-1Ra (+) n=88, GLP-1Ra (-) n=412 (C) HbA_{1c}: GLP-1Ra (+) n=85, GLP-1Ra (-) n=371. (D) BMI: GLP-1Ra (+) n=77, GLP-1Ra (-) n=283. Participants were clustered dependant on the presence, or absence, of a GLP-1Ra in their anti-diabetes treatment regime. The mean duration, age, HbA_{1c} and BMI values were plotted including standard deviation. Participants most recent HbA_{1c} and BMI was used for analysis. Statistical significance was determined Student's t-test. *P<0.05 vs. GLP-1R (-).

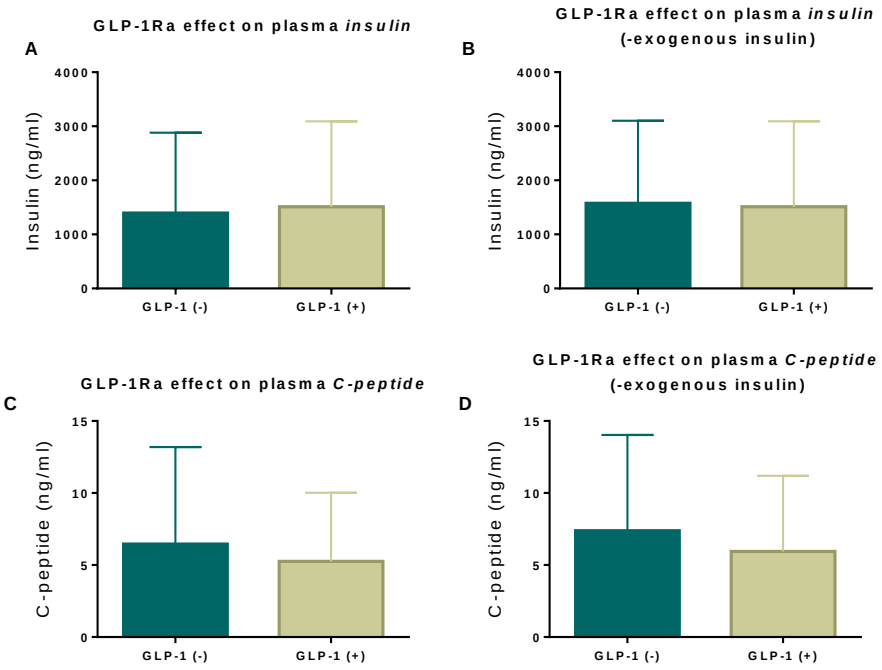


Figure 4.5 – **Effect GLP-1R agonist therapy on plasma insulin and C-peptide plus or minus exogenous insulin administration.** (A) Plasma Insulin: GLP-1Ra (+) n=57, GLP-1Ra (-) n=311. (B) Plasma Insulin – exogenous insulin: GLP-1Ra (+) n=57, GLP-1Ra (-) n=311. (C) Plasma C-peptide GLP-1Ra (+) n=57, GLP-1Ra (-) n=311. (D) Plasma C-peptide minus exogenous insulin: GLP-1Ra (+) n=57, GLP-1Ra (-) n=311. Participants were clustered dependant on the presence of a GLP-1Ra in their anti-diabetes treatment regime. The mean plasma insulin or C-peptide was plotted including standard deviation. Insulin and C-peptide values were obtained from a blood sample taken from non-fasted participants on the day of recruitment, Statistical significance was determined Student's t-test. **P<0.01 vs. GLP-1Ra (-).

4.3.2 Comorbidity in the DiaStrat cohort

ICD-10 recorded comorbidities in the DiaStrat cohort were identified from the Northern Ireland Electronic Care Record. The most frequent number of comorbidities per participant was between 4-6 (133). Eighty four participants had no recorded comorbidity and thirteen participants had between 16-18 recorded comorbidities (Fig. 4.6A). The most common type of comorbidity was disease of the circulatory system, accounting for 24% of all comorbidity. This was followed by endocrine, nutritional and metabolic diseases (16%), diseases of the digestive system (11%) and diseases of the musculoskeletal system (7%) (Fig. 4.6B).

Microvascular complications retinopathy, nephropathy and neuropathy are associated with diseases of the eye and adnexa (4.1%), diseases of the genitourinary system (5.3%), and diseases of the nervous system (2.2%), respectively. Each had a relatively low incidence compared to macrovascular complications and other comorbidity, however diseases of the genitourinary system were most common in this cohort indicting nephropathy may represent the most common microvascular comorbidity.

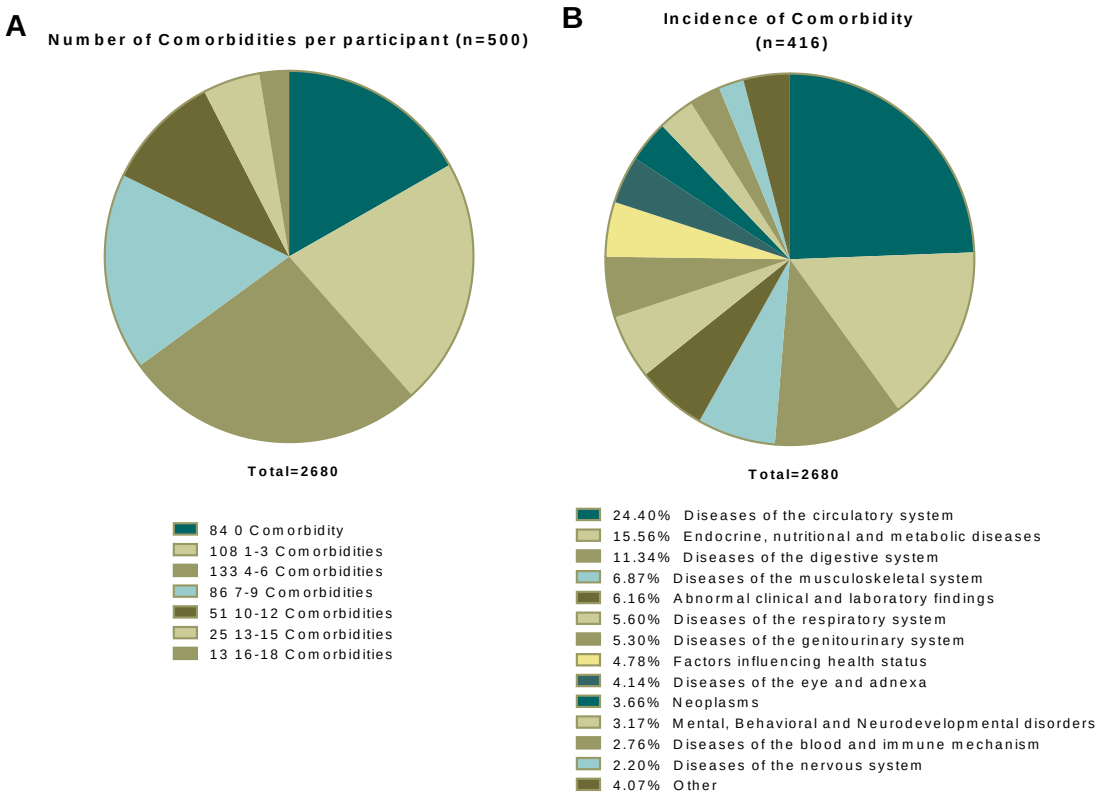


Figure 4.6 – **Incidence of comorbidity per participant and most common types of comorbidity in a Northern Irish T2D population.** (A) Total number of participants with 0, 1-3, 4-6, 7-9, 10-12, 13-15, 16-18 (n=500). (B) Percentage of comorbidity per ICD-10 coded biological system (n=416 participants, n=2680 recorded comorbidities). All health related information was obtained from Northern Ireland Electronic Care Record (NIECR). All comorbidities were classified using the 10th version of International Statistical Classification of Diseases and Related Health Problems (ICD). Other refers to any system that attributed to <1% of total recorded comorbidity.

Detailed analysis of the six most prevalent comorbidities are illustrated in Fig. 4.7. With respect to diseases of the circulatory system, hypertensive diseases were most common accounting for 48% (654, Fig. 4.7A), followed by ischemic heart disease (26%). Diseases of the endocrine system were the second most frequent comorbidity (417, Fig. 4.7B); hyperlipidaemia (50%) and obesity (17%) accounted for the majority. There were 303 incidences of digestive system disorders (Fig. 4.7C); 24% were associated with oesophagus, stomach and duodenum (including gastritis, acid reflux) and 24% were linked to intestinal disease. Ocular (111, Fig. 4.7D) and genitourinary (142, Fig. 4.7E) comorbidity are linked to microvascular complications. The most common ocular comorbidities were disorders of the lens, accounting for 44%, and disorders of the choroid and retina, 39%. Disorders of the retina most likely represent retinopathy and translate to 43 participants. Musculoskeletal disorders were the 4th most common type of comorbidity (184, Fig. 4.7F). Osteoarthritis was the most prevalent disorder constituting 35.9% of the total, followed by spinal disc abnormalities (15.8%), rheumatoid arthritis (10.9%) and bone density problems, such as osteoporosis (9.2%).

4.3.2.1 Relationship between currently available clinical data and cardiovascular comorbidity

Participants with cardiovascular disease (CVD), on average, have had diabetes for 5.6 years longer ($P<0.0001$) than those without CVD (Fig. 4.8A), and are 6.25 years older ($P<0.0001$, Fig. 4.8B). The mean BMI was 34.6 in CVD (+) patients compared to 32.4 in CVD (-) ($P<0.05$, Fig. 4.8C). C-peptide levels were significantly lower (1.4 ng/ml, $P<0.05$) in CVD (+) patients (Fig. 4.8D) compared to non-CVD patients (6.8 ± 6.1 ng/ml) . HbA_{1c} was significantly higher in CVD (+) (66 ± 12 mmol/mol, $P<0.05$) compared to CVD (-) (63 ± 15 mmol/mol, Fig. 4.8E). Interestingly, LDL and total cholesterol were significantly lower ($P<0.0001$) in CVD (+) participant; 1.7 ± 0.7 mmol/l (CVD(+) vs. 2.0 ± 0.8 mmol/l (CVD (-); 3.7 ± 0.9 mmol/l (CVD (+) vs. 4.1 ± 0.9 mmol/l (CVD (-),respectively (Fig. 4.8F).

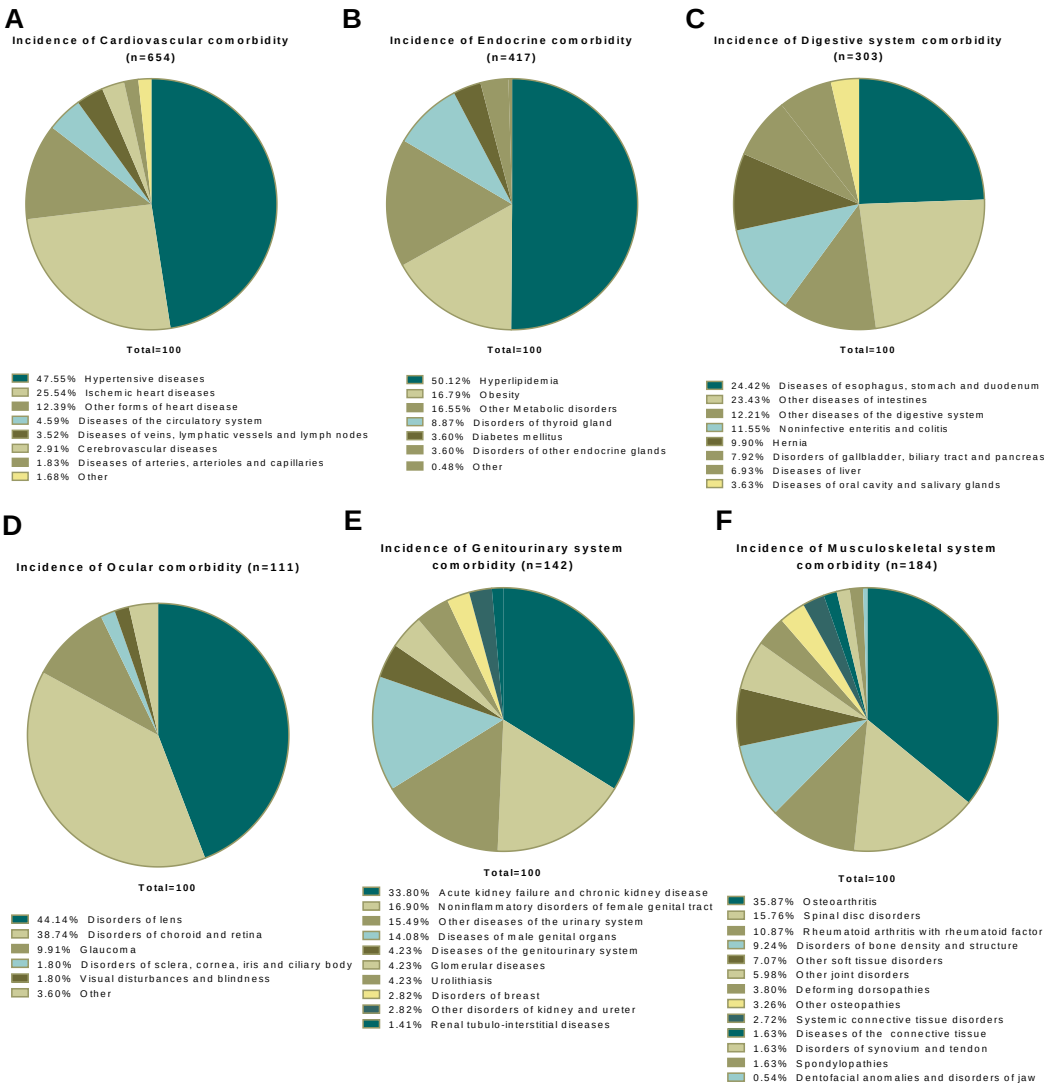


Figure 4.7 – **Detailed total incidence of six most prevalent comorbidities.** (A) Percentage of total number of recorded cardiovascular comorbidities (654 records, 316 participants). (B) Percentage of total number of recorded Endocrine comorbidities (417 records, 271 participants). (C) Percentage of total number of recorded Digestive system comorbidities (303 records, 162 participants). (D) Percentage of Ocular disorders (111 records, 83 participants). (E) Percentage of Genitourinary disorders (142 records, 107 participants). (F) Percentage of musculoskeletal disorders (184 records, 125 participants). All comorbidities were classified using the 10th version of International Statistical Classification of Diseases and Related Health Problems (ICD) and descriptors of level 2 coding system. Other represents any disorder that attributed to <1% of the total.

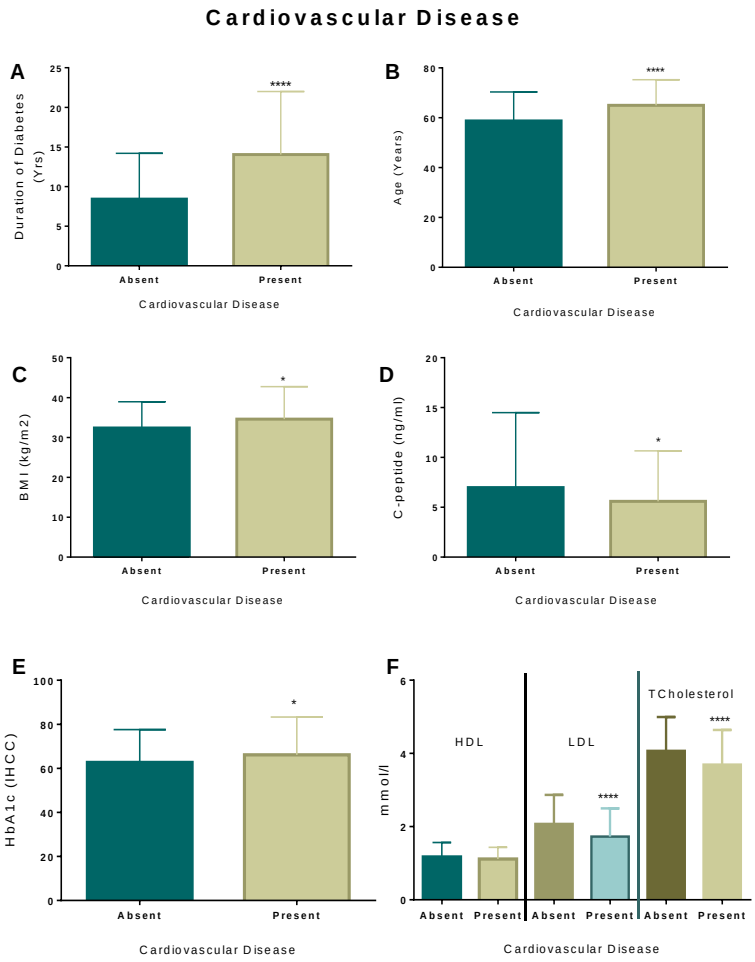


Figure 4.8 – **Relationship between currently available clinical data and cardiovascular comorbidity.** Participants were grouped dependant on presence or absence of CVD. (A) Duration of Diabetes (years): CVD absent n=120, CVD present n=273. (B) Age (years): CVD absent n=174, CVD present n=316. (C) BMI: CVD absent n=107, CVD present n=253. (D) C-peptide: CVD absent n=154, CVD present n=217. (E) HbA_{1c}: CVD absent n=154, CVD present n=299. (F) Lipid profile (HDL, LDL, Total Cholesterol): CVD absent n=152, CVD present n=294. Data presented as mean ± SD. Statistical significance was determined Student's t-test. *P<0.05, ****P<0.0001 vs. cardiovascular disease absent.

4.3.2.2 Relationship between currently available clinical data and diseases of the endocrine system

Diseases of the endocrine system were associated with a significantly longer duration of diabetes (P<0.0001, Fig. 4.9A), and older age (P<0.05, Fig. 4.9B). HbA_{1c} was significantly higher in those with endocrine disorders (68 ± 17 mmol/mol vs. 62 ± 18, P<0.001). C-peptide was significantly lower in patients with diseases of the endocrine system than in those without (4.8 ng/ml vs 7.2 ng/ml, P<0.001, Fig. 4.9D). LDL and total cholesterol were significantly lower in participants with endocrine

disorders, 1.7 ± 0.7 mmol/l vs 2.0 +0.8 mmol/l and 3.7 ± 0.9 mmol/l vs 3.9 ± 0.9 mmol/l respectively (Fig. 4.9E, F). There was no difference between in BMI (Fig. 4.9C) or HDL (Fig. 4.9F) between endocrine disease positive and negative patients.

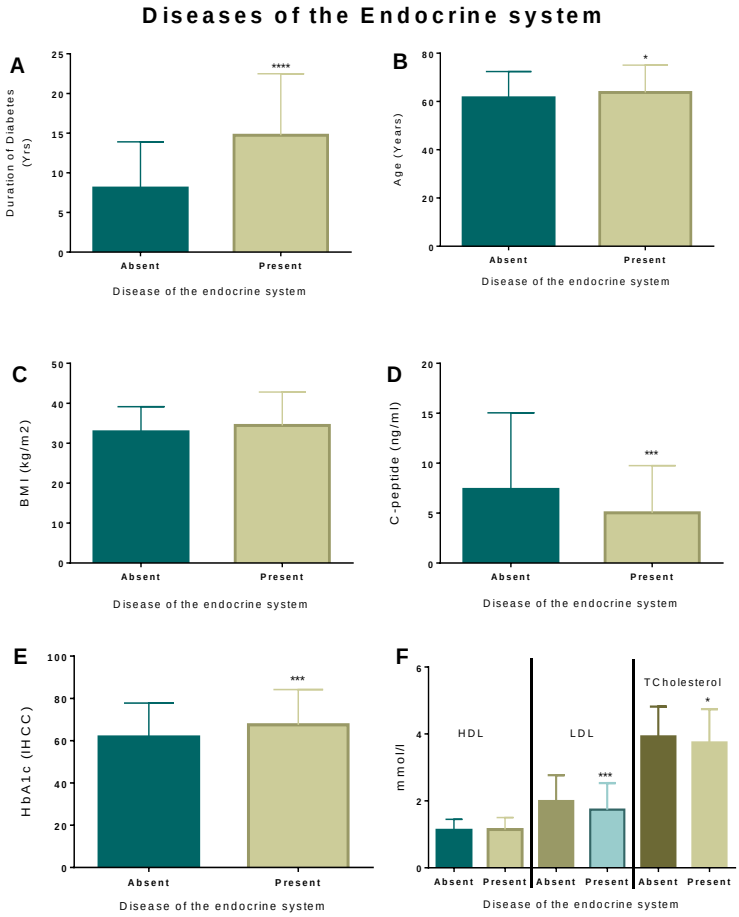


Figure 4.9 – **Relationship between currently available clinical data and diseases of the endocrine system.** Participants were grouped dependant on clinically recorded presence of Endocrine disease. (A) Duration of Diabetes (years): Endocrine disease absent n=142, Endocrine disease present n=251. (B) Age (years): Endocrine disease absent n=219, Endocrine disease present n=271. (C) BMI: Endocrine disease absent n=118, Endocrine disease present n=242. (D) C-peptide: Endocrine disease absent n=196, Endocrine disease present n=175. (E) $HbA_{1c:}$ Endocrine disease absent n=307, Endocrine disease present n=147. (F) Lipid profile: Endocrine disease absent n=302, Endocrine disease present n=144. Data presented as mean ± SD. Statistical significance was determined Student's t-test. *P<0.05, ***P<0.001, ****P<0.0001 vs. Endocrine disease absent

4.3.2.3 Relationship between currently available clinical data and diseases of the digestive system

As illustrated in Fig. 4.10, patients with digestive system disorders were not distinguished by diabetes duration (A), age (B), BMI (C), HbA_{1c} (E), LDL, HDL or total cholesterol (F). However, C-peptide was significantly lower (4.4 ng/ml vs. 6.9 ng/ml, P<0.001) in those with digestive system problems compared to those without (D).

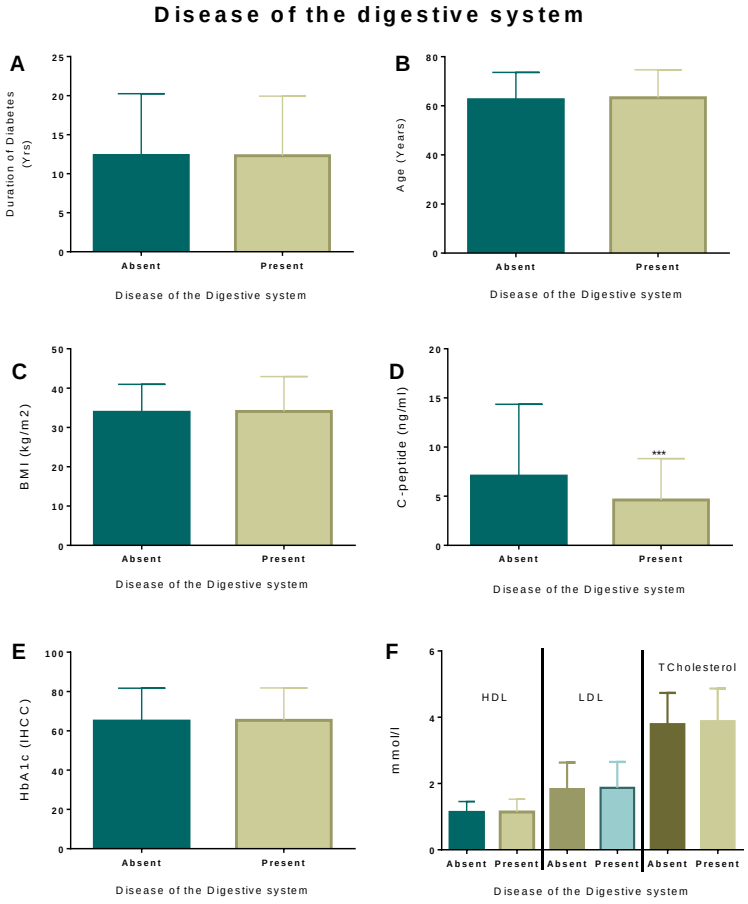


Figure 4.10 – **Relationship between currently available clinical data and diseases of the digestive system.** Participants were grouped dependant on clinically recorded presence or absence of digestive system disorders. (A) Duration of Diabetes (years): digestive disorders absent n=253, digestive disorders present n=141. (B) Age (years): digestive disorders absent n=328, digestive disorders present n=162. (C) BMI: digestive disorders absent n=230, digestive disorders present n=130. (D) C-peptide: digestive disorders absent n=255, digestive disorders present n=166. (E) HbA_{1c}: digestive disorders absent n=307, digestive disorders present n=147. (F) Lipid profile: digestive disorders absent n=302, digestive disorders present n=144. Data presented as mean ± SD. Statistical significance was determined Student's t-test. *P<0.05, ***P<0.001, ****P<0.0001 vs. digestive disorders absent

4.3.2.4 Relationship between currently available clinical data and disease of the musculoskeletal system

As illustrated in Fig. 4.11 diabetes duration (A), age (B), BMI (C), C-peptide (D), HbA_{1c} (E), HDL, LDL and total cholesterol (F) measurements were comparable in participants with and without musculoskeletal diseases.

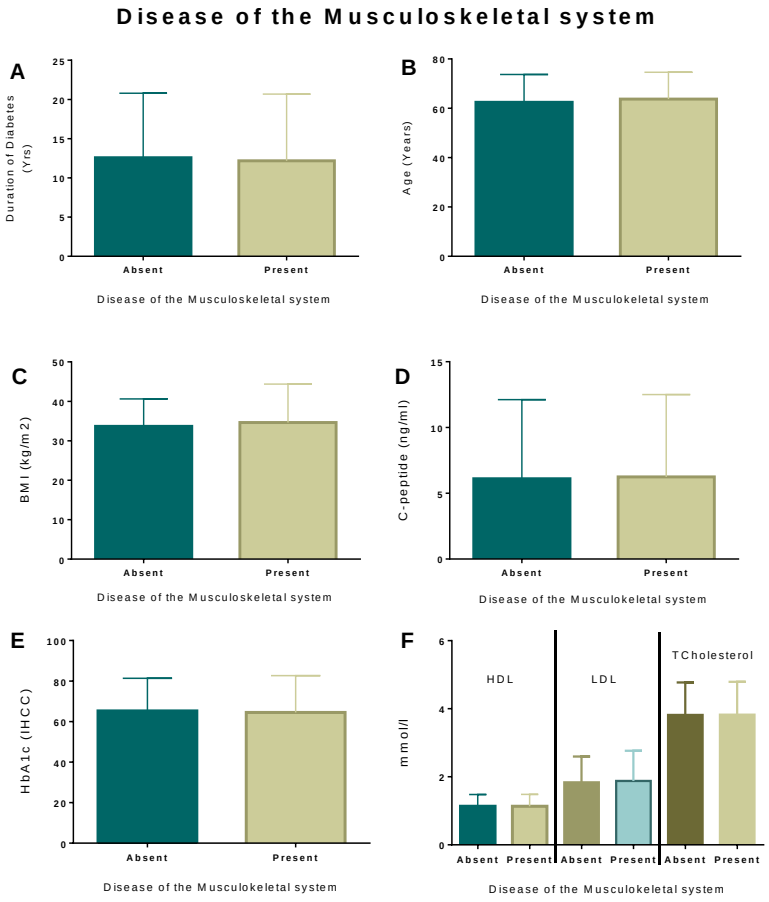


Figure 4.11 – **Relationship between currently available clinical data and diseases of the musculoskeletal system**. Participants were grouped dependant on clinically recorded presence or absence of musculoskeletal disease. (A) Duration of Diabetes (years): musculoskeletal disease absent n=288, musculoskeletal disease present n=106. (B) Age (years): musculoskeletal disease absent n=365, musculoskeletal disease present n=125. (C) BMI: musculoskeletal disease absent n=265, musculoskeletal disease present n=95. (D) C-peptide: musculoskeletal disease absent n=275, musculoskeletal disease present n=96. (E) HbA_{1c}: musculoskeletal disease absent n=336, musculoskeletal disease present n=118. (F) Lipid profile: musculoskeletal disease absent n=332, musculoskeletal disease present n=114. Data presented as mean ± SD.

4.3.2.5 Relationship between currently available clinical data and disease of the eye

Participants with disorders of the eye had significantly longer diabetes duration, 7.9 yrs, (P<0.0001, Fig. 4.12A) and were significantly older (P<0.0001, Fig. 4.12B) than individuals without eye disease. C-peptide was significantly reduced (6.01 ng/ml vs 4.02, P<0.05, Fig. 4.12D) in participants with eye disease. BMI (Fig. 4.12C), HbA_{1c}, HDL, LDL and total cholesterol (Fig. 4.12F) were not different between participants with and without disorders of the eye.

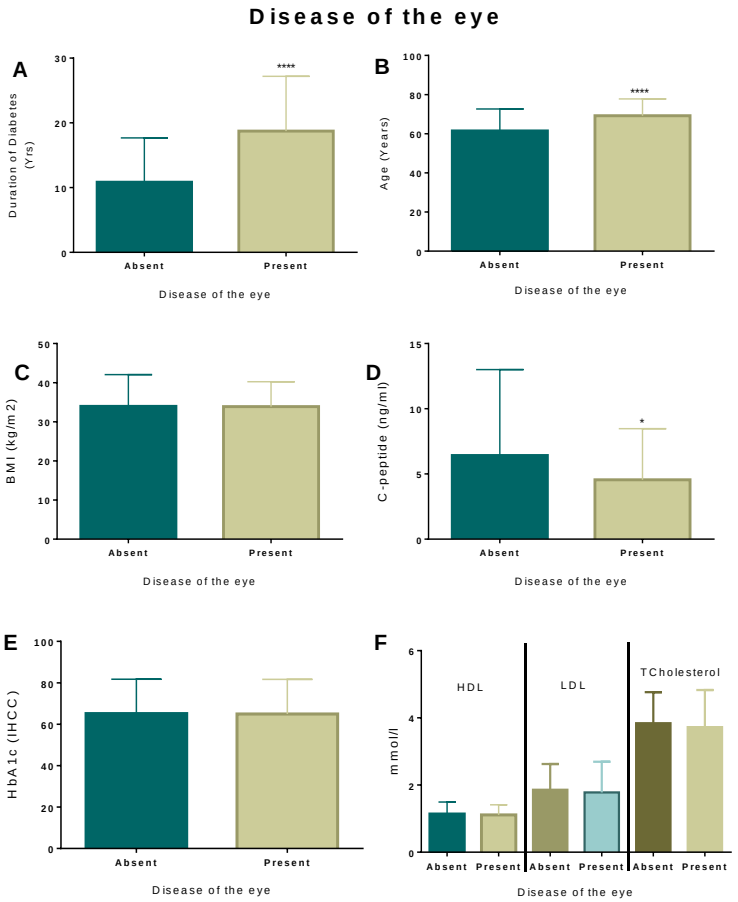


Figure 4.12 – **Relationship between currently available clinical data and diseases of the eye.** Participants were grouped dependant on clinically recorded eye disorders. (A) Duration of Diabetes (years): eye disorder absent n=319, eye disorder present n=74. (B) Age (years): eye disorder absent n=407, eye disorder present n=83. (C) BMI: eye disorder absent n=286, eye disorder present n=74. (D) C-peptide: eye disorder absent n=323, eye disorder present n=48. (E) HbA_{1c}: eye disorder absent n=374, eye disorder present n=80. (F) Lipid profile: eye disorder absent n=367, eye disorder present n=79. Data presented as mean ± SD. Statistical significance was determined using a Student's t-test. *P<0.05, ***P<0.001, ****P<0.0001 vs. eye disorder absent

4.3.2.6 Relationship between currently available clinical data and genitourinary disorders

Longer diabetes duration is a characteristic of genitourinary disease (Fig. 4.13A), with sufferers having diabetes for 3.1 yrs longer than those without (P<0.001). HDL was also significantly lower in the presence of genitourinary disease (1.0 ± 0.2 mmol/l vs. 1.2 ± 0.3 mmol/l, P<0.05, Fig. 4.13F). Age, BMI, C-peptide and HbA_{1c} were not significantly different between groups (Fig. 4.13 B, C, D, E).

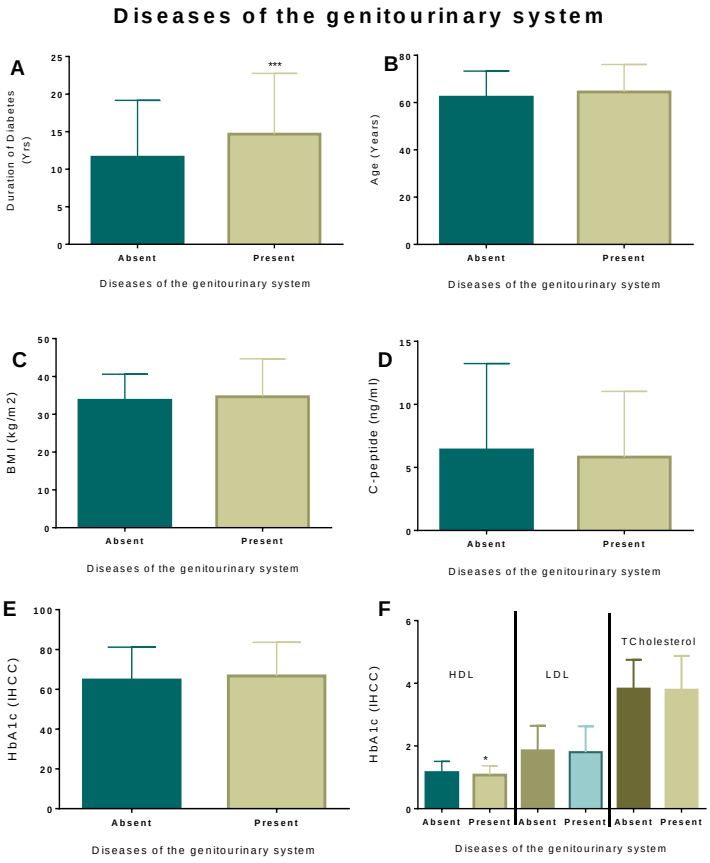


Figure 4.13 – **Relationship between currently available clinical data and genitourinary disorders.** Participants were grouped dependant on presence or absence of recorded genitourinary disorders. (A) Duration of Diabetes (years): genitourinary disorder absent n=299, genitourinary disorder present n=94. (B) Age (years): genitourinary disorder absent n=383, genitourinary disorder present n=107. (C) BMI: genitourinary disorder absent n=272, genitourinary disorder present n=88. (D) C-peptide: genitourinary disorder absent n=302, genitourinary disorder present n=69. (E) HbA_{1c}: genitourinary disorder absent n=354, genitourinary disorder present n=100. (F) Lipid profile: genitourinary disorder absent n=347, genitourinary disorder present n=99. Data presented as mean ± SD. Statistical significance was determined using a Student's t-test. *P<0.05, ***P<0.001, vs. genitourinary disease absent.

4.3.2.7 Relationship between currently available clinical data and clinically diagnosed depression

As illustrated in Fig 4.14, participants with clinically diagnosed depression, were indistinguishable from those without with respect to diabetes duration (A), age (B), BMI (C), C-peptide (D), HbA_{1c} (E), HDL, LDL and total cholesterol (F).

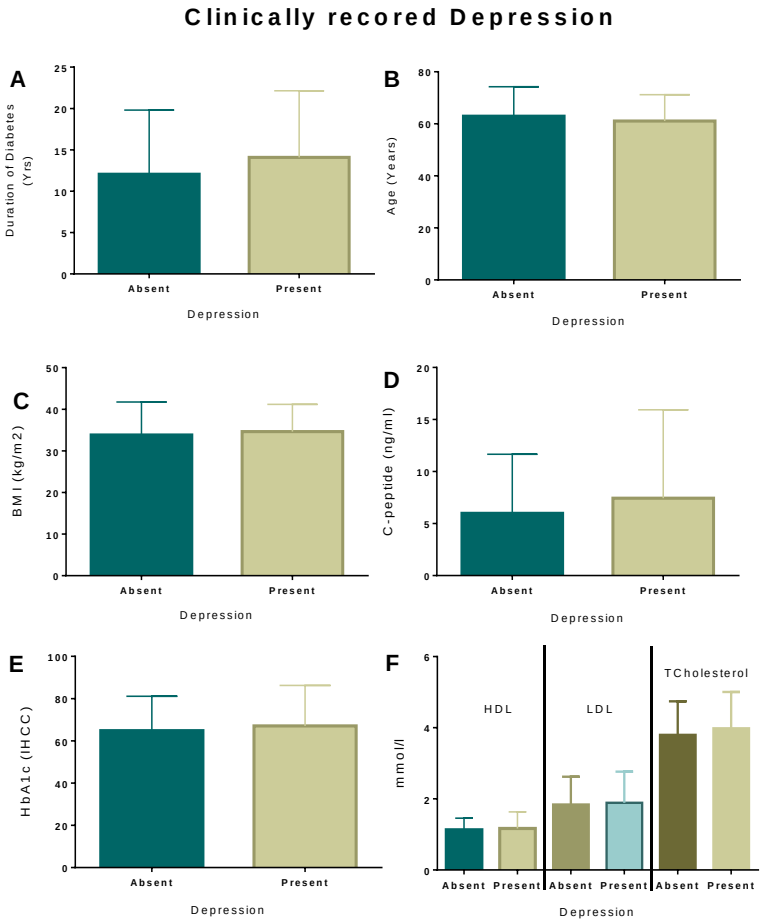


Figure 4.14 – **Relationship between currently available clinical data and clinically diagnosed depression.** Participants were grouped dependant on recorded presence or absence of clinically diagnosed depression. (A) Duration of Diabetes (years): depression absent n=346, depression present n=48. (B) Age (years): depression absent n=434, depression present n=55. (C) BMI: depression absent n=318, depression present n=43. (D) C-peptide: depression absent n=330, depression present n=42. (E) HbA_{1c}: depression absent n=399, depression present n=42. (F) Lipid profile: depression absent n=391, depression present n=55. Data presented as mean ± SD.

4.3.2.8 Relationship between currently available clinical data and participants prescribed an anti-depressant

From analysis of prescription medications we identified that there were significantly more participants prescribed antidepressant medications than with clinically diagnosed depression, therefore we investigated whether there were differences in currently available clinical data in those prescribed antidepressants compared with those not prescribed antidepressant medications. As illustrated in Fig. 4.15 participants prescribed an antidepressant were indistinguishable from those not in; diabetes duration, age, BMI, C-peptide, HbA_{1c}, HDL, LDL and total cholesterol.

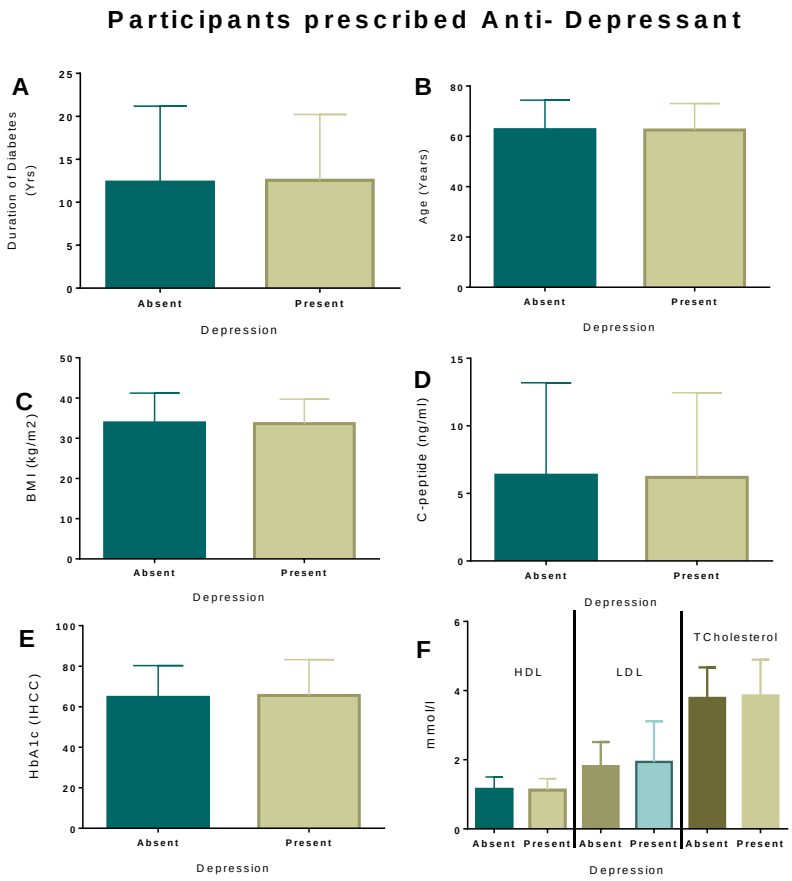


Figure 4.15 – **Relationship between currently available clinical data and participants prescribed an antidepressant.** Participants were grouped dependant on clinically recorded presence or absence of a prescribed antidepressant. (A) Duration of Diabetes (years): antidepressant absent n=208, antidepressant present n=187. (B) Age (years): antidepressant absent n=266 anti-depressant present n=224. (C) BMI: antidepressant absent n=191, antidepressant present n=169. (D) C-peptide: antidepressant absent n=203, antidepressant present n=168. (E) HbA_{1c}: antidepressant absent n=241, antidepressant present n=213. (F) Lipid profile: antidepressant absent n=236, antidepressant present n=210. Data presented as mean ± SD.

4.3.2.9 Most common comorbidity combinations in the DiaStrat cohort.

Given the alarming rate of comorbidity in the DiaStrat cohort we were interested in the combinations of comorbid disorders diagnosed. Interestingly there were no significant trends in terms of comorbidity profile in the cohort.

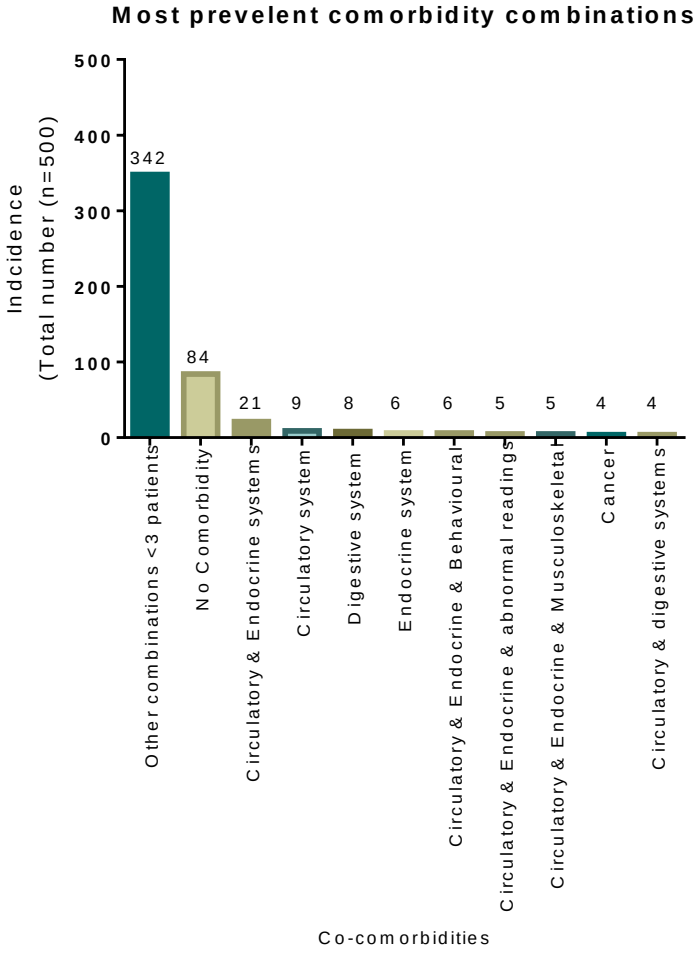


Figure 4.16 – **Most commonly co-diagnosed comorbidities in the DiaStrat cohort.** All data was obtained from Northern Ireland Electronic Care Record (NIECR), and coded using the ICD-10 classification system. Common comorbidities were clustered and sorted in Microsoft excel. Other combinations ≤3 includes all other combinations that had 3 or fewer patients in the cluster. The data shows, 342 participants have a unique combination of comorbidity.

The overwhelming majority, 342 participants, had a comorbidity profile affecting fewer than 3 individuals. Eighty four participants had no recorded comorbidity. The most prevalent combination was disease of the circulatory and endocrine systems, present in 21 participants. Disease of the circulatory (9), digestive (8) and endocrine (6) systems alone were the next most frequent comorbidities reported (Fig. 4.16). This highlights the complexity of comorbidity in clinical medical practice.

4.3.2.10 Effect GLP-1R agonist therapy on number of recorded comorbidities

The number of comorbidities present in GLP-1R agonist positive vs. GLP-1R agonist negative participants was assessed, and is illustrated in Fig. 4.17. This analysis is suggestive of a more severe clinical comorbidity profile in GLP-1Ra (+) participants than in GLP-1Ra (-) participants in line with GLP-1R agonists being a fourth line therapy, and normally prescribed in late stage T2D. GLP-1Ra (+) participants had 45% fewer individuals with 0 comorbidity ($Z= 2.08$, $P<0.05$) and 30% more individuals with 7-9 comorbidities than GLP-1Ra (-) participants. The remaining comorbidity groups were well matched. Evidence of a more severe phenotype in GLP-1Ra participants is further supported in Table 4.3, demonstrating that GLP-1Ra (+) participants are 19% more likely to have cardiovascular disease ($p=0.012$) and 26% more likely to suffer from endocrine disorders ($p= 0.0017$) than those not prescribed GLP-1R agonists.

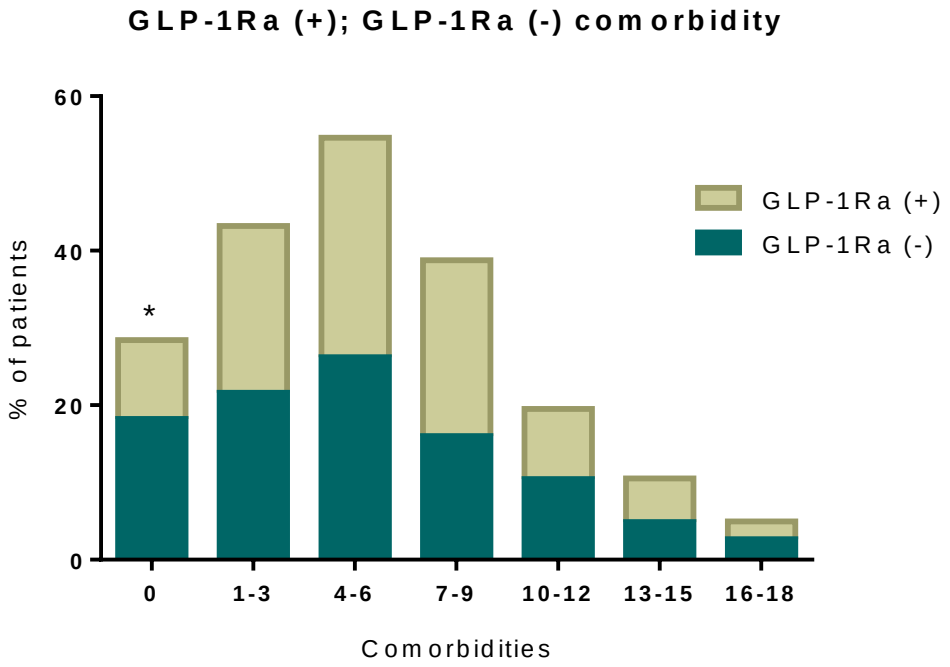


Figure 4.17 – **Effect of GLP-1R agonist therapy on number of recorded comorbidities.** Participants were divided into two groups dependant on the presence or absence of a GLP-1Ra in their anti-diabetes medication regime. GLP-1Ra (+) n=88, GLP-1Ra (-) n= 412. Data is presented as a percentage of the group total.

Comorbidity	Cardiovascular Disease		Disease of the Endocrine system		Disease of Digestive system		Disease of the skeletal system		Disease of the Genitourinary System		Disease of the Eye	
	Total	%	Total	%	Total	%	Total	%	Total	%	Total	%
GLP-1Ra (+) (**n=88**)	66	75*	61**	69	30	34	24	27	17	19	14	16
GLP-1Ra (-) (**n=412**)	250	61	210	51	132	32	101	25	90	22	69	17

Table 4.3 – **Major comorbidity in patients treated with and without GLP-1R agonists.** Data is presented as a percentage of total number of participants per group. A Z-test for the difference between two proportions demonstrated cardiovascular disease and diseases of the endocrine system were more common in participants receiving GLP-1 agonist therapy. *P<0.05 and P<0.01 vs. GLP-1 Ra (-) group.

4.3.3 Non-diabetes prescriptions in the DiaStrat cohort

Subsequent to analysis of comorbidity data from the Northern Ireland Electronic Care Record, prescribed non-diabetes medications were investigated. As illustrated in Fig. 4.18A most participants were prescribed 5-8 non-diabetes drugs (229) or 1-4 medications (130); 94% of the cohort were prescribed at least 1-4 non-diabetes medications, while two individuals were on 16 + medications.

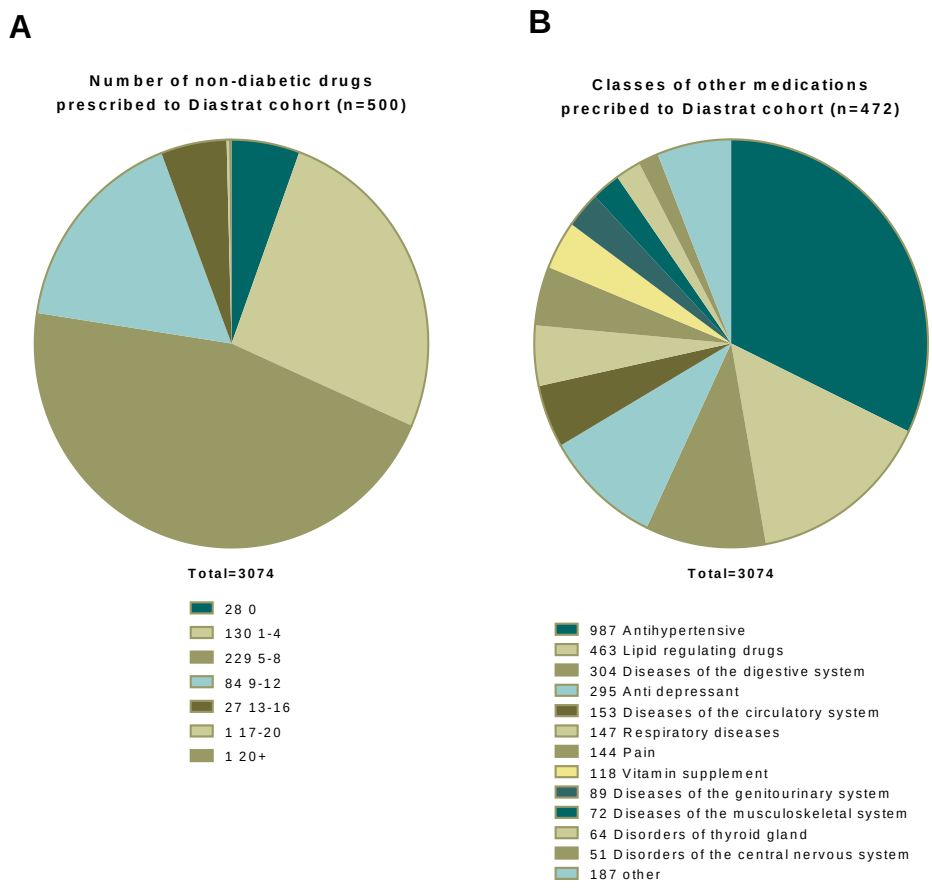


Figure 4.18 – **Number and class of non-diabetes drugs prescribed to the DiaStrat cohort.** (A) Total number of participants prescribed 0, 1-4, 5-8, 9-12, 13-16, 17-20, 20+ non-diabetes medications (n=500) (B) Total number of prescribed drugs per non-diabetes drug class (n=472 participants, n=3074 recorded medications). All drugs were classified according to the British National Formulary (BNF).

Antihypertensives were the most frequently prescribed class of non-diabetes medication, accounting for 987 prescriptions out of the 3074 total. This was followed by lipid regulating drugs, including statins (463), and drugs used to treat digestive disorders (304), such as proton pump inhibitors. Surprisingly anti-depressants were

the 4th most commonly prescribed drug class, accounting for 295 prescriptions (Fig. 4.18B), highlighting either an under reporting of depression within the ECR, or significant off-label prescribing of antidepressant medication (as only 55 participants had a diagnosis of depression ICD10 coded within their ECR) (Fig. 4.14).

Analysis of the number of drug classes prescribed to the DiaStrat cohort (Fig. 4.19) revealed that most participants were prescribed medications from 4 drug classes (19.2%, 96) followed closely by 3 drug classes (16.2%, 81). There were 5.4% (27) of the cohort on 0 non-diabetes medications, while 75.4% (377) are prescribed drugs from three or more drug classes.

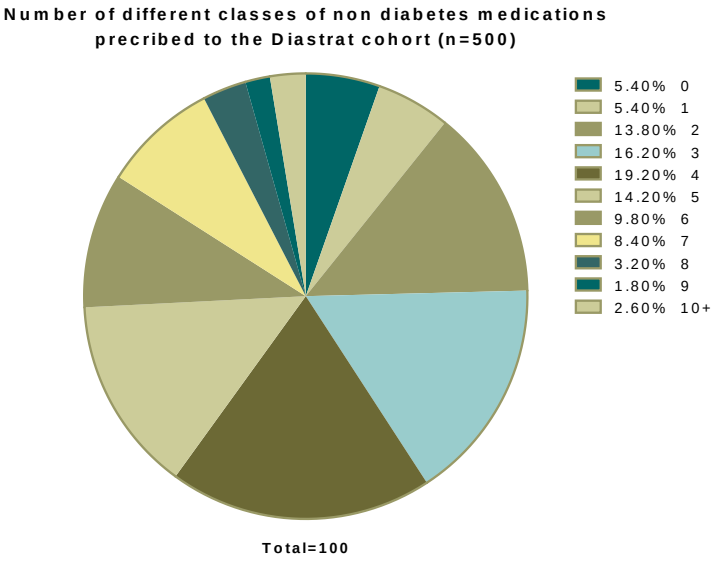


Figure 4.19 - **Number of non-diabetes drug classes prescribed per participant of the DiaStrat cohort (n=500).**

4.3.3.1 Disconnect between recorded comorbidity and prescribed medications

Following discovery of a disconnect between clinically recorded depression and antidepressive prescription we assessed other comorbidities and compared against prescribed medication. Results indicated that such a disconnect was not unique (Fig. 4.20A). Lipid abnormalities were clinically noted in 209 participants, however 428 were prescribed lipid regulating drugs (Z= -14.1, P= 0). A similar trend was observed with hypertension; 265 were clinically diagnosed while 392 were prescribed an anti-hypertensive therapy, suggestive of aggressive management (Z= -8.46, P= 0). Eye

disorders and cancer were clinically noted (83 and 73 respectively) but very few participants were on any form of medication (1, eye disorders (Z= 9.98, P= 0); 5, cancer (Z= 8.02, P= 1.1^{-15})).

Clinically noted incidence of common comorbidity was then compared to the participant's most recent bodyweight or biochemical read (Fig. 4.20B). Obesity was clinically noted in 72 individuals however 251 had a BMI > 30kg/m^2 (Z=-12.11, P=0). Interestingly hypertension was significantly over reported (265 diagnosed; 224 BP in NICE range (Z= 2.59, P= 0.009) while hyperlipidaemia diagnosis and laboratory measurement were well matched.

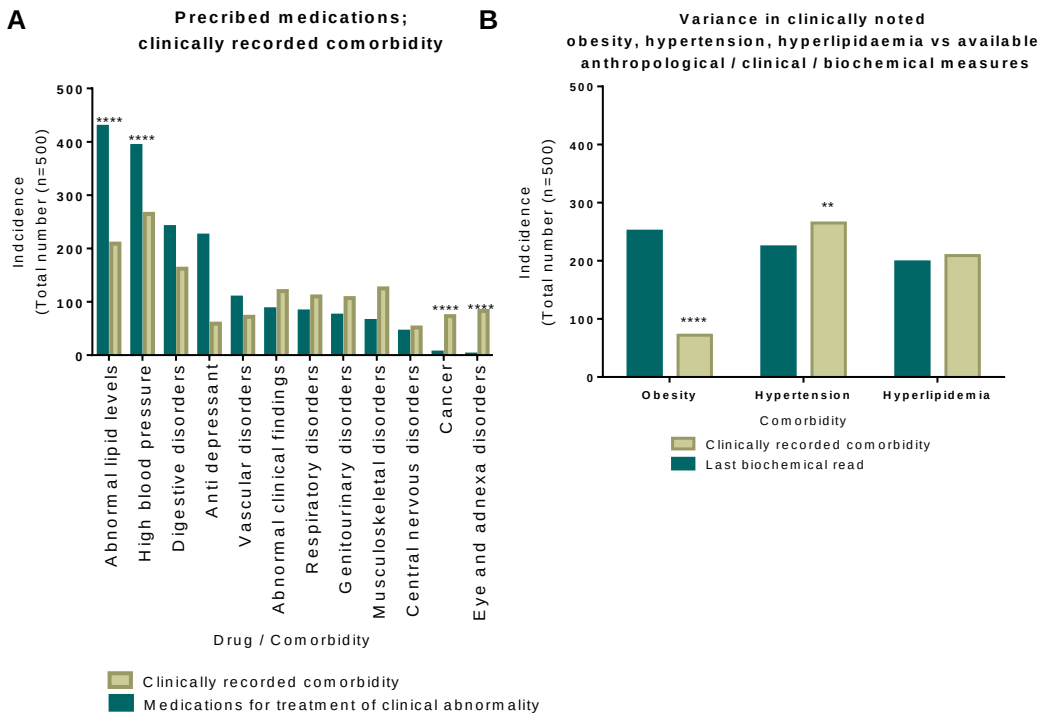


Figure 4.20 – **Difference in clinically diagnosed (noted) and treated conditions.** (A) Total number of participants that have a clinically diagnosed (ECR recorded) comorbidity versus total number of participants receiving treatment for the same comorbidity (n=500). (B) Total number of participants that have a clinically diagnosed comorbidity compared to total number of participants that are over normal guidelines as stipulated by NICE. Obesity BMI >30; Hypertension, either systolic >130 or diastolic >80; Hyperlipidaemia, either, LDL >2mmol/l or total cholesterol >4mmol/l. **P<0.05 and ****P<0.0001 vs. Clinically recorded comorbidity group.

4.3.3.2 Relationship between number of non-diabetes medications and common measures of diabetes management

Duration of diabetes was positively correlated with the number of non-diabetes medications ($P<0.05$, Fig. 4.21A). Participants on 0 non-diabetes medications had an average diabetes duration of 9 ± 6 yrs, while participants on more than 13 non-diabetes medications had a duration of 15 ± 10 yrs.

BMI was negatively correlated with the number of non-diabetes medications prescribed (Fig. 4.21B). Participants on 0 non-diabetes medications had a mean BMI of 36.1 ± 9 while participants on more than 13 non-diabetes medications had a mean BMI of 35.5 ± 7.

HbA_{1c} was not correlated with the number of non-diabetes medications (Fig. 4.21C). Average HbA_{1c} in participants on no non-diabetes medications was 59.5 ± 17.5 mmol/mol, which increased to 65.0 ± 15.3 mmol/mol in the 1-4 medication group and remained constant with increasing number of additional prescribed drugs.

LDL and total cholesterol were negatively correlated with the number of non-diabetes medications (Fig. 4.21D), $P<0.001$ and $P<0.05$ respectively. There was no correlation between HDL and number of medications. The mean LDL value for participants on no non diabetes medications was 2.3 ± 0.7 mmol/l, while in participants on 9-12 medications this value was significantly reduced to 1.7 ± 0.7 mmol/l ($P<0.05$) and further reduced after 13 + medications to 1.5 ± 0.7 mmol/l ($P<0.01$). Total cholesterol exhibited a similar trend. Average total cholesterol in participants on zero non-diabetes medications was 4.4 ± 0.7 mmol/l and was significantly reduced to 3.5 ± 0.7 mmol/l ($P<0.05$) in participants 13 + medications.

Plasma C-peptide levels were not correlated with the number of non-diabetes medications prescribed (Fig. 4.21E), nor was the number of diabetes medications (Fig. 4.21F). However post-hoc analysis revealed differences between the groups. Patients prescribed at least one non-diabetes medication were treated with significantly more diabetes drugs ($P<0.05$ - $P<0.01$).

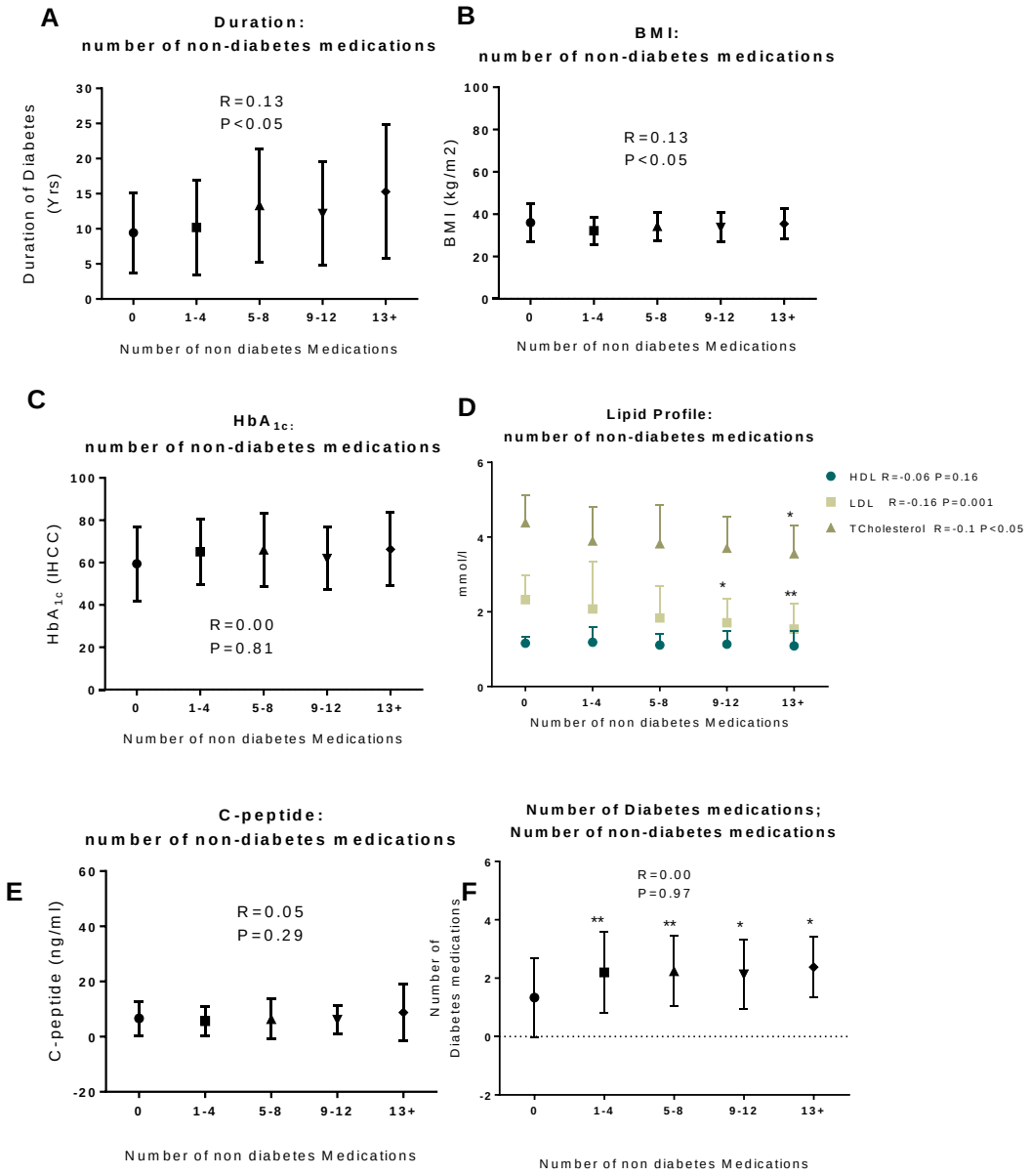


Figure 4.21 – **Relationship between number of non-diabetes medications and common measures of diabetes management.** (A) Duration of diabetes vs. number of non-diabetes medications, n= 395 (B) BMI vs. number of non-diabetes medications, n=360 (C) HbA_{1c} vs. number of non-diabetes medications, n=454 (D) Lipid profile vs. number of non-diabetes medications, n=443 (E) C-peptide vs. number of non-diabetes medications, n=371 (F) Number of Diabetes medications vs. number of non-diabetes medications, n=443. Participants were grouped dependant on number of non-diabetes medications prescribed, 0, 1-4, 5-8, 9-12 or 13+. Data presented as mean ± SD. Two tailed Spearman's correlation analysis between, number of non-diabetes medications and diabetes duration, BMI, HbA_{1c}, Lipids, C-peptide and number of diabetes medication was carried out using ungrouped data values. Statistical significance was determined using one-way ANOVA and Tukey's multiple comparisons test. *P<0.05, **P<0.01 vs. 0 non diabetes medications.

4.3.3.3 Most frequently prescribed lipid regulating, hypertensive, circulatory system, digestive system, respiratory system and anti-depressant medications.

Detailed analysis of the six most commonly prescribed non-diabetes medications (Fig. 4.22), highlighted commonality in each class. Statins were the most common type of lipid regulating drug accounting for over 95% of all prescriptions in this drug class (Fig. 4.22A). Antihypertensive medications were the most commonly prescribed class of drugs overall. Angiotensin Receptor Blockers (ARBs) and Angiotensin-converting enzyme (ACE) inhibitors the most frequently prescribed type (320 prescriptions), followed by anti-coagulant (252) and Diuretics (126) (Fig. 4.22B). There were 153 prescriptions related to treating disorders of the circulatory system; 51 were for anti-aggregants, 53 for anti-anginal, and 43 for anti-arrhythmia medications (Fig. 4.22C). There were 304 digestive system related medications prescribed to the DiaStrat cohort. The vast majority of these were proton pump inhibitors (216; Fig. 4.22D). There were 147 medications prescribed for the treatment of respiratory disorders (Fig. 4.22E), with beta-adrenoceptor agonists representing the vast majority of prescriptions. Anti-depressants were the 4th most frequently prescribed medication, accounting for 295 prescriptions. Amitriptyline accounted for the majority of prescriptions (154) (Fig. 4.22F),

The top ten prescribed non-diabetes drugs are listed in Table 4.4. Atorvastatin (lipid regulating drug) is the most commonly prescribed non-diabetes drug; 64% of participants on lipid regulating drugs are prescribed Atorvastatin. Aspirin is prescribed to 57% of participants on anti-hypertensive medications. Omeprazole and amitriptyline account for the largest percentage of prescriptions for digestive disorders and depression/nerve pain at 70% and 69% respectively. As a drug class, anti-hypertensive medications are the most commonly prescribed, accounting for 6 of the top 10 prescribed drugs (Table 4.4).

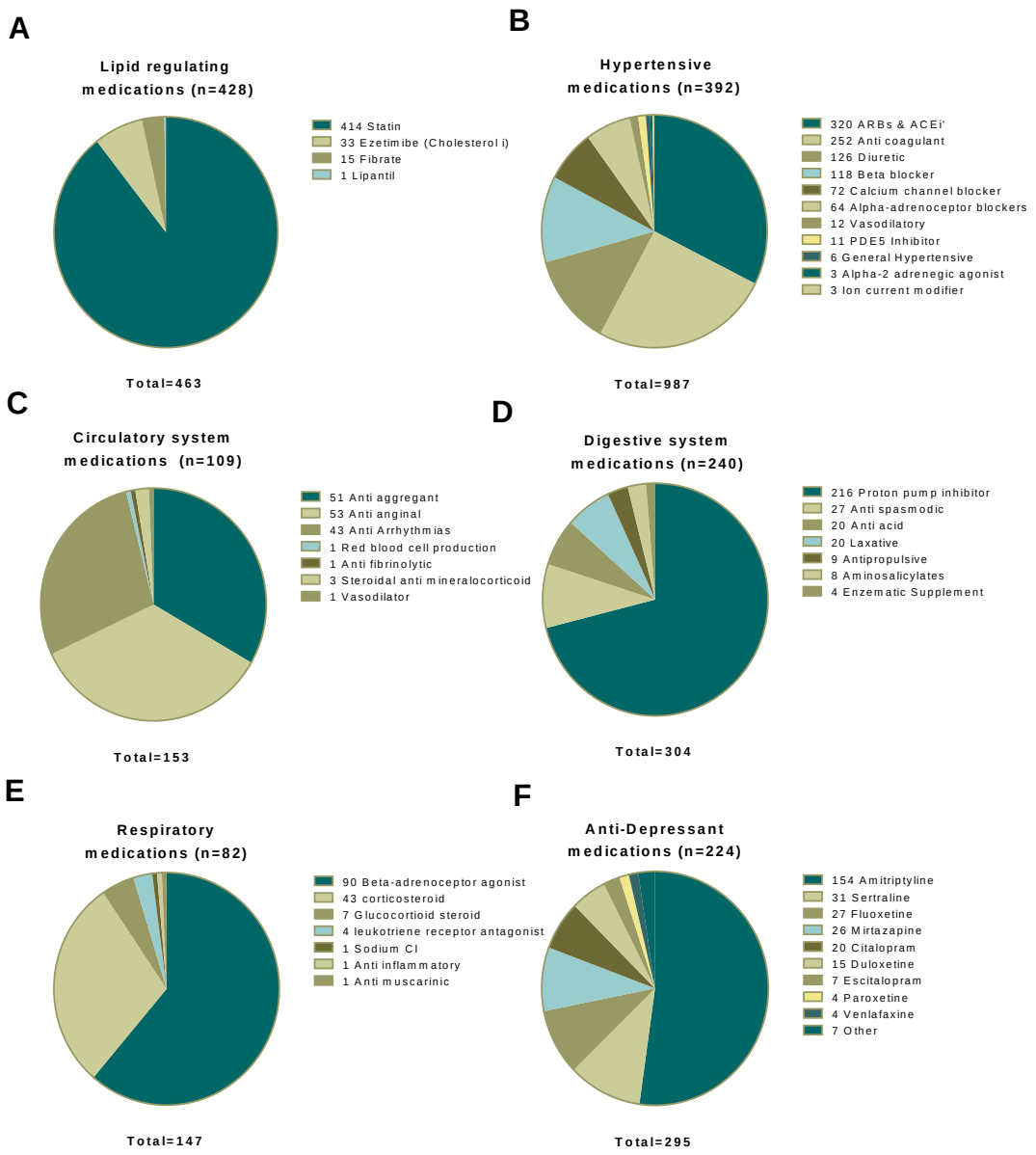


Figure 4.22 – **Sub classification of the most frequently prescribed lipid regulating, hypertensive, circulatory system, digestive system, respiratory system and anti-depressant medications prescribed in the DiaStrat cohort.** (A) Total number of Lipid regulating medications prescribed (n=463 records). (B) Total number of Hypertensive medications prescribed (987 records). (C) Total number of circulatory system medications prescribed (153 records). (D) Total number of digestive system medications prescribed (304 records). (E) Total number of respiratory medications prescribed (147 records). (F) Total number of anti-depressant medications (n=295). A-E classified by drug class, F classified by drug name. 'Other' accounts for single prescriptions.

Drug Name	Drug class	Purpose	Number of prescriptions	Number of participants on associated drug class	% of drug class sample receiving therapy
Atorvastatin	Statin	*Lipid regulating*	272	428	64
Aspirin	Anticoagulant	*Hypertension*	224	392	57
Omeprazole	Proton pump inhibitor	*Digestive disorders / Acid reflux*	167	240	70
Amitriptyline	Antidepressant	*Depression / Nerve pain*	154	224	69
Simvastatin	Statin	*Lipid regulating*	117	428	27
Ramipril	Angiotensin converting enzyme inhibitors (ACEi)	*Hypertension*	112	392	29
Bisoprolol	Beta blocker	*Hypertension*	99	392	25
Bendoflumethiazide	Diuretic	*Hypertension*	92	392	23
Perindopril	Angiotensin converting enzyme inhibitors (ACEi)	*Hypertension*	85	392	22
Doxazocin	Alpha-adrenoceptor blockers	*Hypertension*	80	392	20

Table 4.4 – **Most frequently prescribed non-diabetes drugs.** Data is presented as drug name, type and purpose as denoted in the BNF. Quantity prescribed is the total number of individual prescriptions. The percentage of cohort is the percentage of patients currently on the medication compared to non-diabetes medication class total, lipid regulating = 428, anti-hypertensive = 392, Digestive disorders = 240, anti-depressant = 224.

4.3.3.4 Prescription profile of participants receiving GLP-1R agonists compared to the remainder of the cohort

Participants not prescribed a GLP-1Ra were more frequently prescribed no non-diabetes medications (6.6% vs 1%) than GLP-1Ra-treated patients. The GLP-1Ra (+) group also had more individuals on more than 13 non-diabetes medications (8.0% vs. 5.3%) (Fig. 4.23).

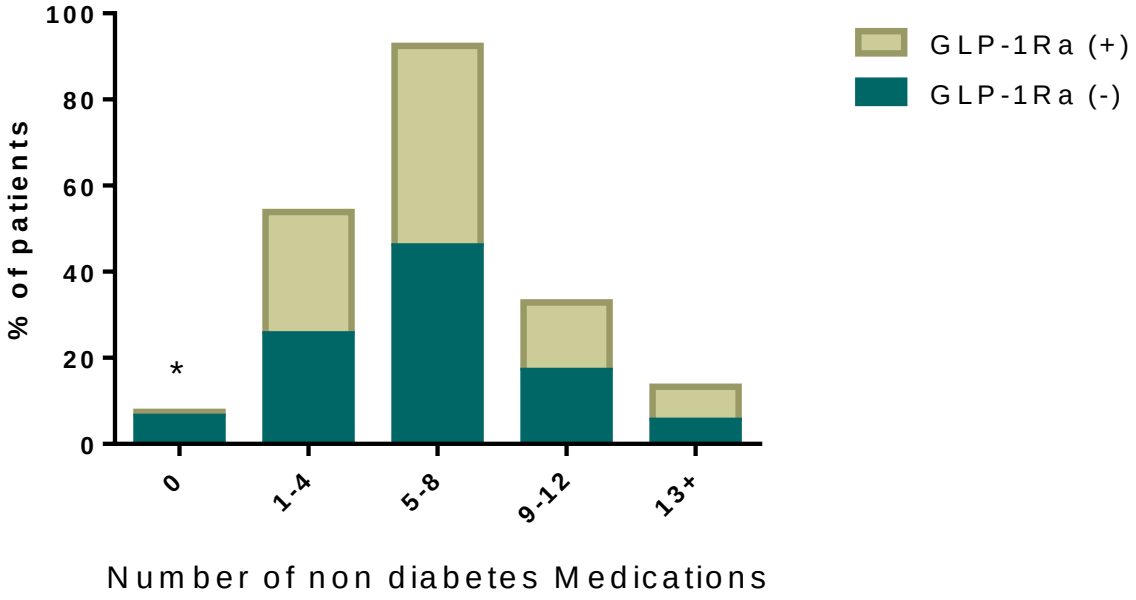


Figure 4.23 – **Comorbidity profile of participants prescribed GLP-1R agonists compared to the remainder of the cohort.** Participants were divided into two groups dependant on the presence or absence of a GLP-1Ra in their anti-diabetes medication regime. GLP-1Ra (+) (positive) n=88, GLP-1Ra (-)(negative) n=412. Data is presented as a percentage of each group. *P<0.05 vs. GLP-1 Ra (-) group.

Lipid regulating (94%, P=0.011) and anti-hypertensive (89%, P=0.009)) medications were more frequently prescribed to participants receiving GLP-1R agonist therapy, while no significant difference was determined in anti-depressant, digestive or vascular disorder (Table 4.5).

Drug	GLP-1Ra (+) (n=88)	GLP-1Ra (-) (n=412)
	%	%
Lipid regulating drugs	94*	84
Antihypertensive	89**	76
Antidepressant	52	43
Digestive disorders	42	50
Vascular disorders	18	23

Table 4.5 – **Prevalence of prescription of major drug classes in participants receiving GLP-1R agonists compared to those receiving other diabetes medications.** Participants were divided into two groups dependant on the presence or absence of a GLP-1Ra in their anti-diabetes medication regime. GLP-1Ra (+) n=88, GLP-1Ra (-) n=412. Data was analysed using a Z-test for 2 proportions. *P=0.0114 and **P=0.0017 vs. GLP-1Ra *(-) group.

4.3.4 Unscheduled care in the DiaStrat cohort and comparison of unscheduled care in GLP-1Ra treated participants compared with those treated with other diabetes drugs

Unscheduled care in the DiaStrat cohort was assessed using the Northern Ireland Electronic Care Record (NIECR). Attendance at the Accident and Emergency department (328) was the most frequent unscheduled care event and injuries such as fractures were the most common reason (27%), followed by abnormal clinical and laboratory findings (24%). Day care visits accounted for 221 incidences and in unscheduled inpatient admittances were recorded in 167 participants. Interestingly there were only 7 recorded out of hours visits, indicating unscheduled care in the T2D population sampled were for more serious complications (Table. 4.6)

Unscheduled Hospital attendance	Total
Day care (DC)	221
In patient (IP)	167
Out Of Hours (OOH)	7
Accident and Emergency (A&E)	328

Reason for A&E attendance	Total	%
Injury, poisoning and certain other consequences of external causes	88	27
Symptoms, signs and abnormal clinical and laboratory findings	80	24
Diseases of the musculoskeletal system and connective tissue	37	11
Diseases of the circulatory system	28	9
Diseases of the digestive system	19	6
Diseases of the respiratory system	19	6
Diseases of the skin and subcutaneous tissue	15	5
Factors influencing health status and contact with health services	12	4
Diseases of the eye and adnexa	7	2
Certain infectious and parasitic diseases	6	2
Diseases of the genitourinary system	6	2
Endocrine, nutritional and metabolic diseases	6	2
Mental, Behavioural and Neurodevelopmental disorders	4	1
External causes of morbidity	1	0

Table 4.6 – **Unscheduled care attendances and reasons for attending accident and emergency.** Data was obtained (723 records, n=231 participants) from the NIECR. All incidences ± 12months of sample date were recorded. Record type (Day care (DC), In patient (IP), Out of hours (OOH), Accident and Emergency (A&E)) was determined and coded by the Western Health and Social Care Trust. Reasons for A&E attendance were noted by clinical professionals using the ICD-10 system. Data is presented at total number and % of all records.

GLP-1Ra positive participants more frequently attended day care clinics (P=0.031) and out of hours services (P=0.025). Those not prescribed GLP-1Ra were more frequently admitted as inpatients, 34% GLP-1Ra (-) and at out of hours appointments, however differences were not statistically significant (Table 4.7).

Unscheduled Hospital attendance	GLP-1Ra(+) (n=88)		GLP-1Ra(-) (n=412)	
	Total	%	Total	%
Day care (DC)	48	55*	173	42
In patient (IP)	25	28	142	34
Out Of Hours (OOH)	2	2*	5	1
Accident and Emergency (A&E)	56	64	274	67

Table 4.7 – **Unscheduled care attendances in GLP-1R agonist treated and GLP-1R agonist naïve participants.** Groups were determined by the presence or absence of a GLP-1Ra. GLP-1Ra (+) n=88, GLP-1Ra (-) n=412. Data is presented as total ECR recorded attendances as a percentage of total group number. Data was obtained (723 records, n=231 participants) from NIECR and all unscheduled care ± 12 months of sample date were recorded and analysed using a Z-test for 2 proportions. *P<0.05 vs. GLP-1Ra *(-) group.

4.3.5 Noted pharmacological adverse reactions in the DiaStrat cohort

As illustrated in Fig. 4.24, within the DiaStrat cohort of 500 participants there were 420 records of adverse reactions noted in 185 participants. Hypertensive medications (37.1%) had the highest recorded incidence of adverse events, followed by anti-bacterial adverse events (17.6%). Interestingly there were only 12 participants (6.2%) with a recorded adverse event to anti-diabetes medications (Fig. 4.24A).

Angiotensin Receptor Blockers (ARBs) and Angiotensin-converting enzyme (ACE) inhibitors (35.9%) were the most commonly noted adverse reactions followed by statins (28.2%, Fig. 4.24B); these drugs are also the most commonly prescribed in the T2D cohort (Fig. 4.22).

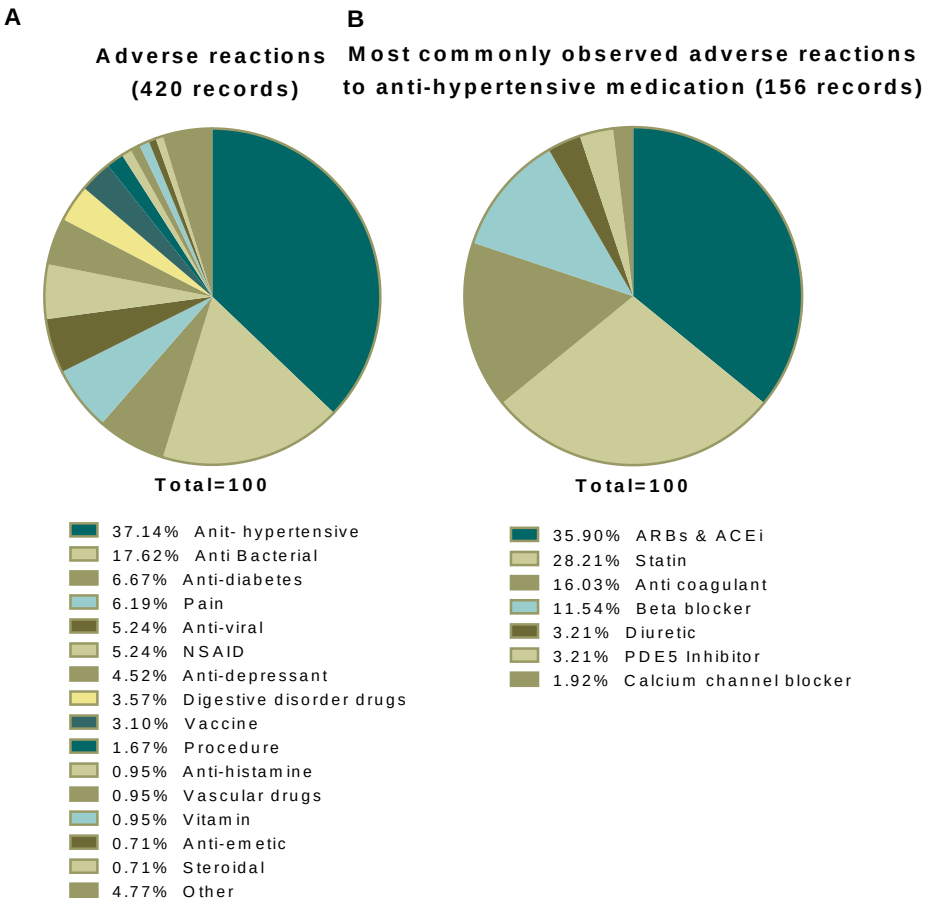


Figure 4.24 – **Pharmacological adverse reactions in the DiaStrat cohort.** (A) Percentage of clinically noted adverse reactions (420 records, n=185 participants). (B) Percentage of recorded adverse reactions to ICD-10 level 2 sub grouped anti-hypertensive medications (n=156 records). All adverse reaction data was obtained from Northern Ireland Electronic Care Record (NIECR) and drugs classified according to the British National Formulary (BNF).

4.4 Discussion

This descriptive study assessed trends in anti-diabetes and non-diabetes drug prescriptions and comorbidities in a T2D population in Northern Ireland. The relationship between currently available diabetes measures were evaluated against drug and morbidity progression, using electronic health records and primary data. All parameters were evaluated in GLP-1 analogue treated individuals vs. all other anti-diabetes drug classes.

Although the DiaStrat cohort was significantly male dominated, no sex differences were determined in key demographic characteristics including age, duration of diabetes, HbA_{1c}, blood pressure or blood lipid levels. Females were more obese and introduced to insulin at a younger age than males; well-documented sex differences (Kautzky-Willer et al., 2016). As key markers of diabetes management were comparable irrespective of gender, subsequent analysis was conducted on the cohort as a whole.

The mean age was 63 ± 11yrs and average diabetes duration was 12 ± 8 yrs, indicative of significant disease progression (Bansal et al., 2015). Seventy percent of participants were obese and ~93% were overweight, indicating body weight was poorly managed. HbA_{1c} levels in 86% of the cohort were significantly greater than the NICE recommended target of 48mmol/mol. This cohorts average HbA_{1c} was 65.08 ±17 mmol/mol, which is substantially greater than the NICE revised target of 53mmol/mol for individuals on multiple anti-diabetes drugs (NICE, 2015). In the UK, approximately 50% of patients achieve glycaemic targets (Mauricio et al., 2017), therefore this cohort may represent advanced or poorly managed disease. Historically, T2D patients managed with diet and oral anti-diabetes agents are treated in primary care, and patients requiring insulin therapy in secondary care (Branger et al., 1998). The participants in this study were predominantly recruited from secondary care and likely represent a severe T2D population. Average blood pressure and blood lipid levels in the DiaStrat cohort were within NICE recommended thresholds (NICE, 2016a). We observed significant therapeutic treatment of hyperlipidaemia and hypertension that may account for observed blood pressure and lipid profiles observed.

Anti-diabetes drug prescription patterns

Most participants in the DiaStrat cohort were prescribed 3 or more anti-diabetes medications. A meta-analysis using Electronic Health Records across Europe and the USA found the average number of anti-diabetes prescriptions per participant to be 2 in 2015 (Higgins et al., 2016), indicating DiaStrat may represent a more severe phenotype. The most frequently prescribed diabetes medications were Biguanides, Sulphonylureas, and DDP-IV inhibitors, while GLP-1R agonists (4^{th} line) made up 7% of prescriptions. Prescription patterns revealed approximately 40% of patients receive insulin therapy and few receive metformin only (20%). The global T2D monotherapy average is 35% (Higgins et al., 2016). In this cohort metformin is frequently prescribed with insulin as a dual therapy. GLP-1Ras are also commonly prescribed as a dual medication, despite being a fourth line therapy. Such prescription trends evidence a change in clinical attitudes (ADA, 2016). Clinicians are moving away from NICE stepwise intensification, towards individual approaches, as advised by the ADA (Chamberlain et al., 2017a). The national average for insulin inclusive regimes is ~30% (Higgins et al., 2016) while ~40% of the DiaStrat cohort were on a insulin therapy. There was an average lag of ~10 years until insulin introduction. Large clinical studies, DCCT and UKPDS have indicated this level of delay can blunt response and evidence suggests that insulin therapy should be initiated much sooner (American Association of Diabetes, 2002).

LDL was negatively correlated with number of diabetes medications. This effect is not likely to be due to diabetes specific medications, as only thiazolidinediones directly affect lipid metabolism (Griffis et al., 2004), and account for 2% of prescriptions. It is thought metformin can directly affect the therapeutic potential of atorvastatin (Kashi et al., 2016); 82% of this cohort were on a metformin positive regime and 55% on atorvastatin. It is possible that metformin exposure is implicated in the improved blood lipid profiles observed.

C-peptide is not currently measured during regular clinical visits but is useful as a measure of β-cell function and offers a surrogate marker of *de novo* insulin production for residual β-cells in diabetes (Leighton et al., 2017). Normal postprandial levels are 3-9 ng/ml in healthy individuals (Diabetes UK, 2017a). The average DiaStrat C-peptide was 6.6 ± 6.6 ng/ml. This suggests a wide range of insulin

production. Many participants were found to be secreting either low levels indicative of reduced β-cell function or high levels indicative of insulin resistance (Jones and Hattersley, 2013). BMI was not correlated with C-peptide. This is surprising as other studies have shown weight loss to improve insulin secretion and sensitivity and reduce HbA_{1c} (Holter et al., 2017). The significant duration of diabetes, is undoubtedly a factor in compromised β-cell function and insulin resistance observed (Khodabandehloo et al., 2016). C-peptide was inversely correlated with the number of diabetes medications, indicative of a reduced insulin secretory capacity with disease progression. Exogenous insulin therapy within the later treatment intensification groups may be responsible for the observed blunting of endogenous secretion from the β-cell (Jones and Hattersley, 2013). If C-peptide was routinely measured it would allow for a stronger prediction of response to non-insulin therapies, estimation of residual β-cell function and earlier indication of the need for exogenous insulin.

GLP-1Ra are recommended as a 4^{th} line medication (NICE, 2015), however, within the DiaStrat cohort, are observed as part of dual and triple therapies. Despite this, most patients prescribed GLP-1Ra will have advanced diabetes, and will generally have failed all non-insulin oral medications (NICE, 2017). In this work, HbA_{1c} was no different in patients prescribed a GLP-1Ra compared to those on other diabetes medications. Despite reports of prominent effects of GLP-1 agonists on weight loss (Davies et al., 2015), the present study indicates participants on GLP-1Ra are significantly heavier. Prospective studies in a general T2D population will assess the impact of GLP-1Ra on glycaemic control and weight loss.

Comorbidity in the DiaStrat cohort

Elderly T2D patients are thought to suffer from at least 2 chronic conditions alongside their diabetes (Hermans and Dath, 2017). Age, duration and HbA_{1c} are thought to be positively correlated with comorbidity (Pratley and Gilbert, 2012). Such observations translate directly to this cohort. Most patients were over 60 yrs old, have had diabetes for 12.5 yrs and 62% had at least 4 comorbidities. The most commonly recorded comorbidities were conditions associated with the circulatory

system, nutritional or metabolic disorders and diseases linked to the digestive system. This pattern corresponds with comorbidity trends in Asia, Europe and the US (Franch Nadal et al., 2016). Musculoskeletal disorders were the 4th most frequent comorbidity, and have been implicated as being directly linked to obesity and diabetes (Uhl et al., 2014). High BMI and poor glycaemic control, which are characteristic of the DiaStrat cohort, are known to increase joint load, articular cartilage breakdown, and inflammation, ultimately increasing osteopathies (Anandacoomarasamy et al., 2009). Musculoskeletal complications were more prevalent than all microvascular complications that are more widely considered to be secondary complications of diabetes.

Hypertension accounted for 50% of all cardiovascular comorbidity followed by heart disease. Hypertension rates were below the national average (Cicero and Ertek, 2011), presumably due to the aggressive pharmacological management observed. Within this cohort, CVD comorbidity was associated with increased duration of diabetes, advanced age and elevated HbA_{1c}, BMI and C-peptide, which are common CVD risk factors around the globe (Simmons et al., 2017). Participants with recorded CVD had reduced LDL and total cholesterol compared to those without a diagnosis of CVD. Aggressive pharmacological management of CVD risk follows current clinical guidance (NICE, 2016a). Statins have been shown to reduce atherosclerosis and cardiac events with fewer adverse events compared to anti-hypertensive medications (Mitchell and Simpson, 2012). The present data suggests that blood lipid levels are much more effectively managed than obesity and glycaemic control in this cohort.

Endocrine comorbidities encompass a wide range of conditions (Michels and Eisenbarth, 2010), but in this study predominantly reflect recorded incidence of hyperlipidaemia and obesity. DiaStrat patients with endocrine disorders had an increased duration of diabetes, were older and had higher HbA_{1c} and lower C-peptide than those without. BMI is not a distinguishing risk factor for endocrine abnormality, however, notably those with and without recorded endocrine disorders were obese. Other studies have indicated obesity is a major driving force in endocrine disorders (Kautzky-Willer et al., 2016). The disconnect between ECR recorded and observed obesity may explain the absence of impact of BMI on

endocrine comorbidity. Participants with recorded endocrine disorders had a lower LDL and cholesterol than participants with no recorded incidence, likely reflecting a large cross over in CVD and endocrine disorders.

Participants were exposed to significant polypharmacy, which tended to include multiple anti-hypertensives and statins. It is notable that obesity is not directly treated in the UK, and guidance is centred around education, exercise and regulation of secondary effects which include CVD and hyperlipidaemia (NICE, 2014b).

There is an indisputable link between obesity, diabetes and digestive system disorders (Fisher et al., 1999). Neuropathy causes the relaxation of the stomach allowing contents into the oesophagus (Koch, 1999) and obesity can cause compression of the stomach causing the same effect (Sugerman, 1998). There were a high degree of recorded digestive abnormalities throughout the upper and lower gastrointestinal (GI) tract, predominantly acid reflux. Western and eastern gastroesophageal reflux disease (GERD) prevalence differs due to diet and lifestyle but is thought to be 41% and 23% respectively (Mahadeva et al., 2005). Current clinical measures (Diabetes duration, age, HbA1c, BMI and blood lipids) were not different between patients with and without digestive disorders, however C-peptide was reduced.

Low C-peptide is a shared characteristic between the most common comorbidities and is indicative of β-cell failure (Jones et al., 2016). Controversially other studies have shown high C-peptide levels to be linked to CVD mortality (Leighton et al., 2017) and significant insulin resistance (Patel and Abate, 2013). This complicates the clinical utility of C-peptide in relation to comorbidity.

Microvascular complications were prevalent in the DiaStrat cohort; retinopathy made up 39% of ocular comorbidity and nephropathy made up 34% of genitourinary disorders. Recorded microvascular prevalence was low compared to macrovascular disease, only 9% of the cohort had retinopathy and 10% Chronic Kidney Disease. Such values are low when compared to the rest of Europe, where retinopathy accounts for ~25% (Lee et al., 2015a) and CKD ~30% (Gheith et al., 2016) of total comorbidity. Diseases of the eye and genitourinary system were linked to a longer duration of diabetes with retinopathy occurring at a later stage than CKD.

Patients with genitourinary disorders were further distinguished by a low HDL level. HDL has long been associated with reduced risk of CVD due to its role in reverse cholesterol transport (Lee-Rueckert et al., 2016), but it is also known that increasing HDL does not further reduce risk (Voight et al., 2012). HDL quality is now recognised as the key factor (Galvani and Hla, 2017). Patients with CKD have a lower quantity and quality of HDL due to impacted lipoprotein lipase activity, and increased endothelial dysfunction (Strauss et al., 2002).

Osteoarthritis was the most common musculoskeletal complication, followed by spinal disc disorders and inflammatory rheumatoid arthritis. There is growing evidence that arthritis and spinal disc disorders are closely linked to metabolic syndrome (Wearing et al., 2006). There is increased cartilage degeneration due to abnormal joint load (Anandacoomarasamy et al., 2008), and increased white adipose tissue due to obesity (Hui et al., 2012). This produces inflammatory cytokines (TNFα, IL-6, MMPs), contributing to systematic inflammation and chondrocyte dysregulation (Castillo-Hernandez et al., 2016). Global prevalence rates of arthritis in T2D were ~35% in 2012, and it is thought T2D patients are at 50% increased risk of developing a musculoskeletal condition (Vos et al., 2012b). In the present study there were no differences in duration of diabetes, age, HbA_{1c}, BMI or lipids.

According to NICE, people with chronic conditions such as obesity and diabetes are 3 times more likely to develop depression (NICE, 2016b). There were 55 ECR recorded incidences of depression in DiaStrat but 295 prescriptions for anti-depressants, to 224 patients. This raised questions regarding under reporting of conditions and off label prescribing. Amitriptyline constituted the majority of anti-depressant prescriptions and was the 4^{th} most common prescribed non-diabetes drug. Amitriptyline is widely prescribed to treat neuropathic pain associated with nerve disorders (Thompson and Brooks, 2015). This type of prescription could account for a portion of the recorded incidence, although only 2.2% of the Diastrat cohort had recorded nervous system disorders. Similar to musculoskeletal comorbidity, patients that are clinically diagnosed or treated for depression could not be distinguished by current diabetes measures. This again, is likely due to severity of disease in the DiaStrat cohort. Depression is common in diabetes

patients, due to comorbidity, pill burden and hospital requirements (Mut-Vitcu et al., 2016).

An assessment of patterns of multimorbidity revealed that there were not any significant comorbidity patterns within the cohort. A total of 342 participants had a unique comorbidity combination. The most common combination was a circulatory and endocrine complication, although this consisted of 21 individuals and both conditions are prevalent in many other combinations. It should be noted that for a comprehensive analysis of multimorbidity, a larger dataset is required. When considering 14 comorbid subgroups there are a possible 16,000 combinations (2^{14}). This data highlights the complexity facing clinicians for effective, personal management. The ADA have acknowledged this, and stressed how current patient management strategies can lead to gaps in patient care, due to a high expectation and difficulty in managing all aspects of comorbidity (American Diabetes Association, 2016). In recent years this resulted in primary and secondary care providers being utilised and prioritising different aspect of disease (Khunti et al., 2001). It is interesting to note that less than 15% of participants in this study are on HbA_{1C} target. The National Health and Nutrition Examination Surveys (NHANES) have reported that patients with multiple chronic conditions rarely meet glycaemic targets (Stark Casagrande et al., 2013). Many studies have correlated the 'pill burden' effect and increased unscheduled care as being significant factors (Bluher et al., 2015).

Cardiovascular and endocrine system disorders were both more prevalent in GLP-1Ra treated patients and associated with a longer duration of diabetes, older age, high HbA_{1c} and low C-peptide. GLP-1Ra positive patients were significantly younger and heavier than those not prescribed GLP-1Ra, perhaps evidencing moving away from NICE stepwise intensification, and towards a personalised medicine approach. This is likely due to the success this class of drugs has had in clinical trials as a weight loss therapy (Danne et al., 2017).

Polypharmacy

Treating multimorbidity adds another layer of complexity to patient treatment regimes and has a profound effect on a patient's ability to manage their diabetes.

Cardiovascular and musculoskeletal disorders have been associated with declining quality of life (UK Prospective Diabetes study group, 1998). Diabetes guidelines recognise quality of life as being linked with adverse outcomes in diabetes, such as response to therapy, disease progression and mortality (ADA, 2016).

In the DiaStrat cohort 68% of participants are prescribed more than 5 non-diabetes drugs, from ~3-5 individual drug classes, in line with recorded comorbidity. A recent European study assessed aggressive targeting of key diabetes measures (HbA_{1c} and BP) on specific patient populations, based on their comorbidity profile (Schmieder et al., 2015). High risk CVD patients benefited from loosening of glycaemic medications and intensifying hypertensive medications, and low risk CVD patients benefited from sole aggressive diabetes therapy and loosening of hypertensive medications. This alternative treatment strategy resulted in improved diabetes management, quality of life and CVD risk (Schmieder et al., 2015). This study was the first to assess different therapeutic approaches and treatment targets in line with ADA recommendations (Ott et al., 2009). It shows that treating patients with comprehensive polypharmacy may not be the most effective method.

Polypharmacy is routine in this cohort; there were a total of 3074 non-diabetes medications prescribed. The most common drugs were antihypertensives; 78% participants were prescribed at least one. Perhaps aligned with the frequency of prescription, antihypertensives, ARBs and ACEi, were also the most common adverse reaction noted in the DiaStrat population. Antihypertensive treatment exceeded noted hypertension, evidencing tight regulation of CVD risk. Studies have indicated that hypertensives are used as a long term management option to control CVD (Mukete et al., 2015), but there are significant adverse events associated with these drugs (McDowell et al., 2013). Unnecessary ‘pill burden’, and complicated regimes have been shown to obstruct effective diabetes management (Hauber et al., 2013).

Lipid regulating medications were the second most frequently prescribed medication. Similar to the antihypertensives, the high prevalence resulted in a high number of recorded adverse events. Lipid medications were also over prescribed, 209 participants had a clinically diagnosed lipid abnormality, but recent biochemical tests revealed 198 would be advised lipid treatment by NICE (NICE, 2016a). There were 428 patients on these medications, again posing a unnecessary ‘pill burden’

(Bluher et al., 2015). This cohort is an extremely high risk group, and recent studies have demonstrated a positive cost – risk benefit to over prescribing statin medications (Heller et al., 2017). Statin therapy is an inexpensive pre-emptive measure (Aarnio et al., 2015) but uniform global prescription may have other inherent risks, linked to adverse events such as, high creatine kinase, sleep abnormalities, glycaemia increases and haemorrhagic stroke (Chaipichit et al., 2015).

Medications to treat gastrointestinal problems were commonly prescribed in the DiaStrat cohort. Such conditions are recognised to have a significant impact quality of life (Lee et al., 2015b). Proton pump inhibitors, used to treat acid reflux (Tosetti and Nanni, 2017) represent 70% of digestive system prescriptions. Similar to CVD and endocrine medications, these drugs appear to be over prescribed. There are 162 patients with a clinically recorded digestion disorder but 240 are prescribed a medication.

In the present study duration of diabetes was associated with increased polypharmacy but this did not correlate with any improvement to HbA_{1c} or C-peptide. There was a weak negative correlation between BMI and the number of non-diabetes medications. The average BMI was over 35 in all groups. While 72 patients had obesity noted within the ECR as a comorbidity but anthropological measures suggest 251 (50%) of the cohort would be classified as obese by NICE (NICE, 2017). Obesity is the most underreported comorbidity in this cohort, as is the case in many studies in the US and Europe (Chiu et al., 2017).

LDL and total cholesterol were strongly correlated with the number of medications. NICE stipulate that for lipid regulating and hypertensive medications, after review if a patient is not responding the non-responsive medication should be removed and a different medication added (NICE, 2016a). This does not seem to be occurring, despite evidence that well informed simplification of prescriptions has a positive effect on patient outcome (Schmieder et al., 2015).

In line with comorbidity prevalence, anti-hypertensive and lipid regulating drugs were more frequently prescribed to those on GLP-1Ra therapy. Interestingly, GLP-1Ra prescribed patients also had increased anti-depressant prescription. There is a well-established link between diabetes severity, number of comorbidities and depression (Berge and Riise, 2015). Depression can cause unhealthy behaviour, low

exercise, motivation and energy, leading to progressive worsening of diabetes (Chowdhury, 2004). Our results indicate that participants exposed to four or more anti-diabetes medications do not respond well to therapy, which may be due to increased levels of depression (Iglay et al., 2017).

Unscheduled care attendances

Due to the complexity of diabetes, research has shown patients are more likely to use hospital services (Steiner et al., 2006) and this has a significant impact on the NHS (Hex et al., 2012). A large proportion of hospital visits have been shown to be readmissions (Yam et al., 2010) but population studies on readmission rates and risk factors are poorly described. There were 231 participants and 723 records of participants admitted to hospital for unscheduled care ± 1yr of their sample date. That translates to 46% of the cohort and ~3 visits per patient. Research in the US indicated that the average number of hospital visits per year for a healthy individual is 1.5, indicating a high rate of unscheduled care for diabetes patients (Steiner et al., 2006). Accident and Emergency (A&E) visits were the most common unscheduled care accounting for 328 records. This cohort is elderly and it is well known that elderly diabetes patients are prone to falls (Vinik et al., 2015). Injury is the most frequent reason for A&E attendance, followed by abnormal clinical readings, both of which are heavily influenced by age (Mayne et al., 2010) and most likely not directly linked to diabetes. The remainder of recorded A&E visits most likely result from diabetes related conditions including circulatory, digestive and respiratory problems (American Association of Diabetes, 2002). There were 221 admissions to day care (DC) and 167 inpatient (IP) admissions. Studies have shown that repeat admission whether it A&E, DC or IP is correlated with deficiencies in overall healthcare delivery (Steiner et al., 2006).

Limitations

This analysis has several limitations, the most significant being the modest sample size and absence of age and sex-matched controls to allow for comparisons of prescribing practice and comorbidity in the absence of diabetes. Vulnerabilities in accurate reporting of comorbidity due to collation of data from a number of healthcare providers by various professionals within the ECR cannot be ignored. DiaStrat participants were recruited from secondary care and likely represent a severe diabetes phenotype. Few participants had a low or normal BMI or HbA_{1c} therefore changes associated with treatment and comorbidity were minimal. Analysis of a primary care sample would allow assessment of prescribing practice and comorbidity earlier in the disease timecourse, which may allow appropriate interventions to be identified and tested in prospective studies. The NIECR has a wealth of data but does not contain any financial information related to the treatment of disease. Future studies should integrate this information to identify the most expensive comorbidities and co-comorbidity clusters, enabling rational targeting of disease management.

Conclusion

This study identified diabetes prescription trends in Northern Ireland which differed from proposed stepwise NICE guidance (NICE, 2017). Significant polypharmacy was present and reflective of the diverse clinical profiles recorded. Majority of patients had a unique comorbidity cluster highlighting the complexities associated with effective disease management. Blood pressure and lipids were well managed, with evidence of aggressive treatment of CVD risk, however most patients were poorly managed with respect to glycaemia and bodyweight. The prescription and comorbidity trends identified in this study did not correlate strongly with any of the current biochemical measures assessed in the clinic (HbA_{1c}, lipid profile). The data is suggestive that insulin and C-peptide would be a beneficial addition in the assessment of diabetes management. We hope to further this work using proteomics to better stratify and risk assess specific groups of patients to better inform clinical practice.

Chapter V

Proteomic markers of T2D, glycaemic control, comorbidity and response to therapy in the DiaStrat cohort

Abstract

Abnormal glucose levels and poor control is a significant clinical problem that affects millions of type 2 diabetes (T2D) patients in the United Kingdom (UK). This study aimed to identify proteins associated with T2D, glycaemic control, comorbidity and response to therapy to develop better understanding of T2D pathogenesis. OLink proteomics, high throughput protein assays (4x 92 panels), were utilised to screen 374 T2D patients and 20 non-diabetes controls. Quality control measures were met by 96-99% of all samples across panels.

T2D and non-diabetes control patient demographics were significantly different with respect to age, BMI, HbA_{1c}, lipid profile and number of chronic conditions (P<0.0001). A total of 55 proteins were indicative of T2D, and most strongly associated with syndecan-1-mediated signalling, according to Enrichr. The top 8 proteins could distinguish T2D well (~AUC 0.75). Unique protein panels were identified for obesity (n=10), poor glycaemic control (n= 30), and abnormal lipid levels (n=143). SORT, CASP3 and CD84 correlated negatively with diabetes regime intensification (R= -0.15, P=0.01), and CTRC was the only protein to correlate with recorded comorbidity number (R= -0.62, P<0.0001), although 10 proteins uniquely changed in patients with 1-3 comorbidities, and 21 in patients with more than 13 comorbidities.

Poor glycaemic control in response to GLP-1Ra was linked with 17 proteins in participants with HbA_{1c} >53 mmol/mol, and 13 proteins were associated with obesity in GLP-1Ra treated individuals. Only changes to ITGA11 (P<0.01-P<0.001), and Ep-CAM, (P<0.05-P<0.01) were specific for GLP-1Ra prescribed patients for glycaemic and weight related changes respectively. Using online enrichment software (Enrichr), pathways involved in vascular endothelial damage were identified as being a common feature between the most significant proteomic markers for all measured variables. The proposed markers need to be validated in secondary cohorts to determine their utility for clinical practice.

5.1 Introduction

Type 2 diabetes (T2D) is a multifaceted disease with many treatment options and numerous long-term complications and comorbidities (NICE, 2017). All of these aspects severely impact a patients quality of life (World Health Organisation, 2017). The development of micro and macrovascular complications are positively correlated with hyperglycaemia (Choby, 2017) and thought to be due to endothelial dysfunction. Endothelial dysfunction has long been associated with type 2 diabetes (Sena et al., 2013) and is thought to be caused by hyperglycemia and insulin resistance (Steinberg et al., 1996). It is classically characterised by reduced angiogenesis (Kolluru et al., 2012) which affects vascular endothelial cell recruitment, remodelling and sprouting (Tonnesen et al., 2000). If these processes fail there is increased vasodilation, oedema and inflammation (Avogaro et al., 2011), which has been shown to damage tissues such as the retina, kidney and brain (Sutton et al., 2002).

Integrin signalling also plays an important role in vascular endothelial integrity (Finney et al., 2017). Integrins are transmembrane receptors that can activate a number of signal transduction pathways mediating various cell signals involved in the cell cycle (Borghesan and O'Loghlen, 2017), cellular organelle organisation, and receptor localisation (Giancotti, 1997). Integrins enable rapid, flexible responses to events at the cell surface (Hynes, 2002). Studies have demonstrated that high glucose affects expression of integrins and has a negative effect on the reendothelialization process, contributing to microvascular occlusion (Roth et al., 1993). Subsequent work implicated integrin alpha-v beta-3 (αvβ3) as a key protein associated with IGF-1 mediated atherosclerotic lesions and macrovascular disease (Cascella et al., 2010). Interestingly, GLP-1Ras have been shown to directly reduce this effect (Song et al., 2015) but whether this class of drugs interacts with integrins directly is unknown. Overall, proteins involved in the vascular endothelium have a significant impact on various aspects of macro and microvascular conditions (Finney et al., 2017) and are likely implicated in comorbidity in T2D.

Biomarkers indicative of endothelial change exist, and include plasminogen activator inhibitor-1 (PAI-1) for peripheral artery disease (Aso, 2007), vascular endothelial growth factor (VEGF) for heart disease (Taimeh et al., 2013) and various cell adhesion molecules for diabetic nephropathy (Sabbisetti et al., 2014). These are generally not adopted in the clinic because they are also indicative of other conditions (Carmeliet, 2005), and therefore not specific. Novel and specific biomarkers associated with comorbidity may have significant value.

Currently, in clinical practice glycosylated haemoglobin (HbA_{1c}) is the most frequently used measure of glycaemic control (Metcalf et al., 2017). Most T2D patients have a target of 53 mmol/mol (7%) but very few achieve this (NICE, 2015). HbA_{1c} is a useful biomarker for monitoring long term glycaemia in established type 2 diabetes patients, but is limited in terms of its prognostic and predictive capabilities (Reynolds et al., 2006). Prevention, and management of early symptoms of comorbidity are the most effective ways of treating diabetes (Jelinek et al., 2017). Many current tests such as a lipid, glucose and creatinine blood examinations, as well as electrocardiograms (ECG) and foot and eye screenings are used in stereo by different clinicians and medical staff to diagnose and manage each aspect of comorbidity (Piette and Kerr, 2006). This involves significant patient and clinical time commitments (Baxter et al., 2016).

The development of sensitive and specific markers that can accurately and specifically (Thambisetty and Lovestone., 2010) diagnose pre-diabetes, inform effective prescription and improve clinicopathological prediction, are highly sought after (Dorcely et al., 2017). Clinical utility is of great importance (Cramer et al., 2011). Discoveries must be detectable from non-invasive samples, such as blood, urine or saliva (Yao et al., 2017). In recent years blood is more routinely collected and regarded as being most effective for biomarker identification (Figueras-Roca et al., 2017), irrespective of its complex proteome (Jambunathan and Galande, 2014). Thousands of proteins are present in plasma and serum and many are secreted into the blood under various disease conditions (Tu et al., 2010).

New peripheral biomarkers have been developed in recent years. This has highlighted the scope for new discoveries (Yao et al., 2017). Low plasma adiponectin has been found to be a strong predictor of T2D advancement (Jiang et al., 2016). It is

thought that if used in conjunction with current anthropometric measurements, insulin sensitivity indexes and other insulin or non-glucose related biomarkers it may increase the accuracy of patient risk stratification and reduce clinical appointment times (Steiner et al., 2017). The glycoprotein, GlycA, has been reported to have utility as a predictor of incident T2D (Connelly et al., 2016a). Metabolites such as dimethylglycine (DMG) (Magnusson et al., 2015), 2-amino adipic acid (Wang et al., 2013) and glycine (Yan-Do and MacDonald, 2017), have also been recognised as reliable markers of onset of diabetes and have shed light on mechanisms outside of glucose metabolism (Molnos et al., 2017).

Biomarker development consists of four stages; discovery, verification, validation and product development (Pepe et al., 2001). Most biomarkers fail at clinical verification or validation (Diamandis, 2012), but recent multiplex technologies allow for large scale, non-targeted, proteomics (Olink, 2017). This approach facilitates high throughput, accurate measurement of hundreds of candidate proteins in large clinical cohorts.

In this study we integrated 368 proteomic targets associated with the immune and inflammatory response, cell adhesion and differentiation, catabolic and apoptotic processes and gene regulation, with real world data obtained from ECRs. All quantified proteins were chosen based on previously published work (Ebtehaj et al., 2017). The protein panel was assessed to identify proteins associated with T2D onset, glycaemic control (HbA_{1c}), BMI, blood lipids, diabetes therapy, C-peptide, comorbidities and GLP-1Ra response within the DiaStrat cohort.

5.2 Methods

5.2.1 Participant recruitment

T2D participants were recruited from the Altnagelvin Hospital diabetes clinic, NI. Healthy non-diabetes controls were identified and sampled at the University of the Third Age (U3a). All recruitment was in accordance with ORECNI ethical recommendations, described in *Chapter 2, Section 2.7*.

5.2.2 Sample collection

5.2.2.1 Blood sampling

Blood samples were obtained using 21 G Vacuette® safety needle (Greiner Bio-One, Stonehouse, UK; Cat no. 450091), as described in *Chapter 2, Section 2.8.1.2.* Approximately 26 ml of blood were extracted into 2 x 9 ml EDTA (K3E K3EDTA) coated Vacuette® tubes (Greiner Bio-One, Stonehouse, UK; Cat no. 455036) and 1 x 8ml Serum (Z Serum Sep Clot Activator) tube (Greiner Bio-One, Stonehouse, UK; Cat no. 455071). All blood samples were processed immediately.

5.2.3 Blood processing

Polypropylene tubes were pre-labelled using Item Tracker© software (ItemTracker Software Ltd, Suffolk, UK), prior to processing for serum, plasma, protein, RNA and whole blood. Full protocol described in *Chapter 2, section 2.8.1.3*. All samples were then frozen at -80^{o} C.

5.2.4 Clinical database construction

Relevant clinical information for all participants (n= 500) was obtained from Western Health and Social Care Trusts, Hicom Diamond.NET diabetes management system (Hitcom, Surrey, UK) and Orion Health technologies, Northern Ireland Electronic Care Record (NIECR) (Orion health, Hammersmith, UK). All data was used in compliance with research ethics and the Data Protection Act, 1998. Fields of data are described in *Chapter 2, Section 2.11.1.*

5.2.5 Multiplex proteomics

Proteomic quantification was out conducted at OLink proteomics (OLink, SW). EDTA plasma from 374 T2D patients and 20 non-diabetes controls was analysed. A total of 5 plates were shipped on dry ice (CO^2, - 76^o C). All data is presented as Normalised Protein Expression (NPX), OLink Proteomics' arbitrary unit on a log2 scale. Full description in *Chapter 2, Section 2.12.*

5.2.6 Statistical analysis

All statistical analysis was conducted using Graphpad Prism software (Graphpad Software Inc., La Jolla, CA, USA; v6.0h). Student's *t* tests and one way ANOVA with Tukey's post-hoc test, and Receiver Operator Characteristic (ROC) curves were used as described in *Chapter 2; Section 2.15.*

5.3 Results

Assay performance

Samples were examined on four Proseek multiplex immunoassays (CVD II, CVD III, Inflammation, immune response; 92 analytes each). Quality control (QC) criteria were implemented and 96-99% of all samples met QC across all panels. Cardiovascular panels II & III exhibited a 99% overall protein detection rate, immune response and inflammation panels were 88% and 80%, respectively. Due to the fact that there were 14 duplicate proteins between panels and some analytes failed to meet QC, a total of 337 unique proteins were reportable. A list of all 368 proteins is included in Supplementary Table 1, and a list of proteins that failed QC is included in Supplementary Table 2.

Cohort characteristics

Cohort characteristics are described in Table 5.1. Control participants were significantly older than the T2D cohort, had a significantly lower BMI and HbA_{1c} and significantly higher HDL, LDL and total cholesterol levels, as well as a significantly reduced number of recorded chronic conditions.

DiaStrat	Diabetes Cohort			Non-Diabetes Cohort		
	Total	Mean (SD)	%	Total	Mean (SD)	%
Number of Participants	374		100	20		100
Males	225		60	9		45
Age *(yrs)*	368	61.70 (10.6)	98	20	68.37 (9.1) ****	100
Age at diagnosis *(yrs)*	280	50.63 (10.5)	75	~	~	~
Duration *(yrs)*	297	11.03 (7.9)	79	~	~	~
C-peptide *(ng/ml)*	365	6.33 (6.6)	98	~	~	~
BMI *(Kg/m2)*	242	34.00 (8.3)	65	18	25.40 (3.2) ****	90
HbA_{1c} *(mmol/mol)*	363	63.00 (16.7)	97	18	36.22 (2.2) ****	90
HDL *(mmol/l)*	356	1.15 (0.3)	95	16	1.70 (0.5) ****	80
LDL *(mmol/l)*	356	1.87 (0.9)	95	16	2.58 (0.8) ***	80
Total Cholesterol *(mmol/l)*	356	3.85 (0.9)	95	16	4.80 (1.1) ***	80
Number of Diabetes Medications	321	2.10 (1.3)	86	~	~	~
Number of Chronic Conditions	298	4.84 (4.2)	80	13	0.95 (0.9) ****	65

Table 5.1 - **Cohort characteristics.** Significance determined using Student's T-test. ***P<0.001, ****P<0.0001 vs. Diabetes cohort.

5.3.1 Proteins distinguishing between T2D and non-diabetes controls

As illustrated in Table 5.2, a total of 55 proteins were significantly different in the T2D cohort compared to controls, and are listed from highest to lowest level of significance, with PRSS8 and MMP7 most significantly different (P= 0.00001) and LAMP3 and TREM1 just below the P<0.05 threshold. Pathway analysis revealed that this protein signature was associated with Syndecan-1-mediated signalling. Related pathways of the eight most significantly altered proteins are listed in Table 5.3 and trend analysis in Figure 5.1.

DiaStrat v Control					
Number	Protein	P-value	**Number**	Protein	P-value
1	*PRSS8*	0.00001	**28**	*LIF-R*	0.02266
2	*MMP7*	0.00001	**29**	*LPL*	0.02394
3	*CDCP1*	0.00016	**30**	*PSGL-1*	0.02401
4	*HSP 27*	0.00141	**31**	*ITGB1BP2*	0.02534
5	*PAI*	0.00143	**32**	*PAR-1*	0.02605
6	*HGF*	0.00252	**33**	*Ep-CAM*	0.03011
7	*Gal-4*	0.00303	**34**	*FAM3B*	0.03094
8	*VSIG2*	0.00370	**35**	*CXCL5*	0.03102
9	*IDUA*	0.00397	**36**	*HO-1*	0.03394
10	*IL-1RT1*	0.00518	**37**	*MMP12*	0.03470
11	*HAOX1*	0.00540	**38**	*TNFRSF11A*	0.03588
12	*ANG-1*	0.00692	**39**	*KIM1*	0.03609
13	*TFF3*	0.00727	**40**	*FGF-21*	0.03729
14	*CCL17*	0.00790	**41**	*PDGF subunit B*	0.03788
15	*IL-27*	0.00800	**42**	*TRAIL-R2*	0.03863
16	*PDGF subunit A*	0.00876	**43**	*U-PAR*	0.03923
17	*PD-L1*	0.00914	**44**	*REN*	0.03931
18	*IL-18R1*	0.00931	**45**	*CXCL6*	0.03935
19	*PON3*	0.01080	**46**	*CLEC4D*	0.04095
20	*SRC*	0.01324	**47**	*CASP-3*	0.04209
21	*ITGA11*	0.01429	**48**	*DCBLD2*	0.04219
22	*GALNT3*	0.01472	**49**	*JAM-A*	0.04298
23	*KLRD1*	0.01737	**50**	*SLAMF1*	0.04325
24	*CCL4*	0.01740	**51**	*NTF4*	0.04380
25	*AREG*	0.01741	**52**	*SOD2*	0.04622
26	*CXCL1*	0.01766	**53**	*MPO*	0.04830
27	*CXCL1*	0.01766	**54**	*LAMP3*	0.04914
			55	*TREM1*	0.04968

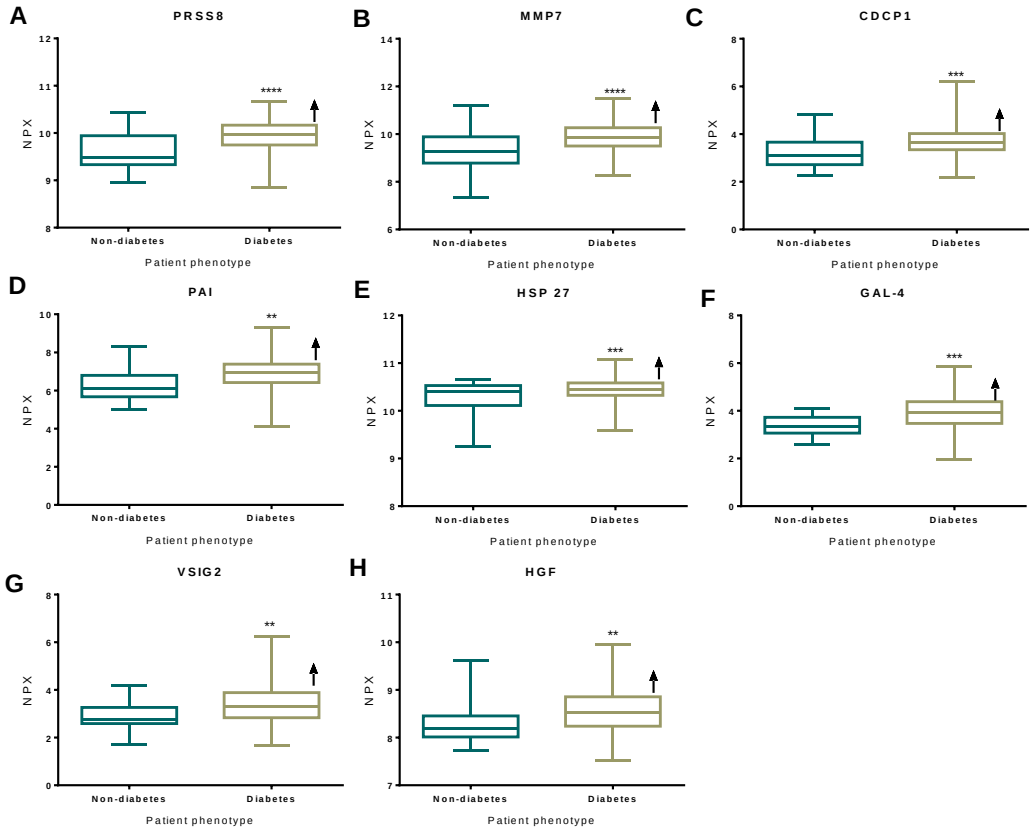
Table 5.2 - **Proteins distinguishing type 2 diabetes patients and controls. N=374 T2D, N=20 non-diabetes controls.**

Protein	Abbreviation	P-value	Function	Ref	Pathway
Prostasin	*PRSS8*	0.0000078	Epithelial sodium channel regulation	(Koda et al., 2009)	CASP/PAK2-p34/actin
Matrix metalloproteinase-7	*MMP7*	0.0000105	Breakdown of extracellular matrix / wound healing	(Puthenedam et al., 2011)	IR / IGF
CUB domain-containing protein 1	*CDCP1*	0.0001606	Cell adhesion / glycoprotein action	(Spassov et al., 2011)	EGF/EGFR
Heat shock protein 27	*HSP 27*	0.0014145	Supports cell survival under stress conditions	(Sharp et al., 2013)	TNF / NfKb
Plasminogen activator inhibitor-1	*PAI*	0.0014335	Increases fibrinolysis	(Aso, 2007)	tPA / uPA / Plasmin
Hepatocyte growth factor	*HGF*	0.0025194	Myogenesis, organ regeneration, wound healing	(Gallagher, 2006)	HGF-cMET
Regulatory protein GAL4	*Gal-4*	0.0030250	Positive regulator of galactose-induced genes	(Klar and Halvorson, 1974)	GAL4 - UAS system
V-set and immunoglobulin domain containing 2	VSIG2	0.0037027	Potent inhibitor of the alternative complement pathway convertases	(Yu et al., 2009)	Immune system / Tcells

Table 5.3 – **Top 8 T2D associated proteins and related pathways**

5.3.2 Most significantly altered proteins in diabetes

The protein concentration in the plasma of 374 diabetes patients was compared to 20 non-diabetes controls using a Student T-Test. Fifty five proteins (Table 5.2) appeared to be associated with diabetes (P<0.05). The 8 most significant (A) PRSS8; P<0.0001, (B) MMP7; P<0.0001, (C) CDCP1; P<0.001, (D) PAI; P<0.01, (E) HSP 27; p<0.001, (F) GAL-4; P<0.001, (G) VSIG; P<0.01, (H) HGF; P<0.01 are illustrated in Figure 5.1.



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Pathway	Source	P-Value
Syndecan-1-mediated signalling	*NCI-Nature 2016*	0.007

Figure 5.1 - **Proteomic Profile of 8 top proteins altered in T2D vs. non-diabetes controls.** (A) PRSS8, (B) MMP7 (C) CDCP1, (D) HSP 27, (E) PAI, (F) HGF, (G) Gal-4, (H) VSIG2, (I) Enrichment analysis. N= 374 diabetes, N=20 non-diabetes. All data presented as box and whisker plots (min, mean, max) and showed all proteins of interest were increased in diabetes. Data enrichment was conducted on integrative web based software application Enrichr (Chen et al., 2013). All data was inputted and ranked for level of membership according to Enrichr protocol (N=55 proteins). All significantly associated pathways are shown (P<0.05). Significance was determined using Student's T-test. **P<0.01, P<0.001, ****P<0.0001 vs. non-diabetes controls.

Receiver Operator Characteristic (ROC) curve for discrimination between non-diabetes controls and T2D demonstrated that PRSS8 (AUC 0.75), MMP7 (AUC 0.74), CDCP1 (0.74), PAI (AUC 0.72), GAL-4 (AUC 0.71), VSIG2 (AUC 0.70), HGF (AUC 0.73) were average (fair) at distinguishing disease. HSP 27 (AUC 0.64) was poor (Fig. 5.2), suggestive that a combination of proteins would be most informative.

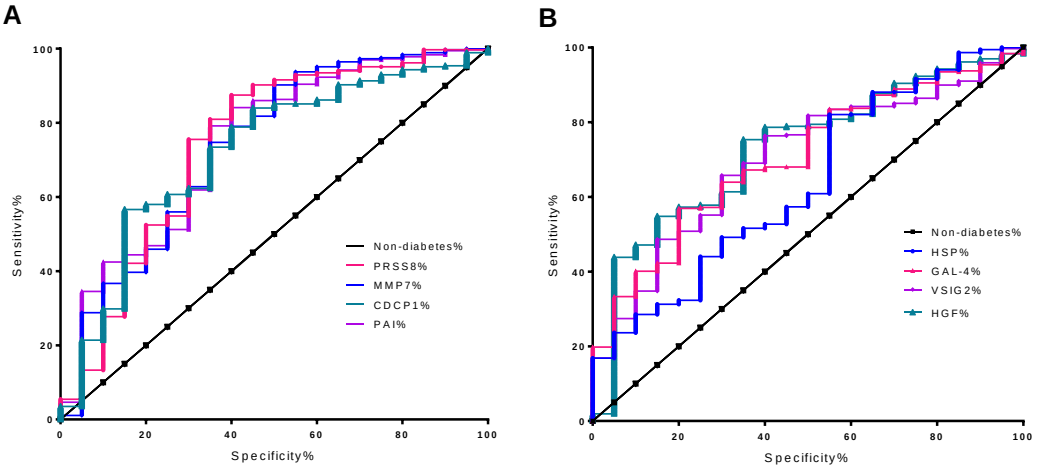
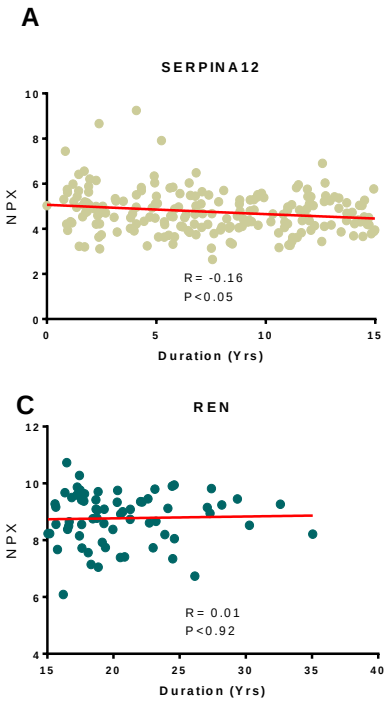


Figure 5.2 - **ROC curves for most significantly altered proteins between non-diabetes controls and T2D.** (A) ROC curves for PRSS8, MMP7, CDCP1, PAI. (B) ROC curves for HSP27, GAL-4, VSIG2, HGF. All proteins quantified from plasma and identified by in graph by colour key. Non-diabetes control protein concentrations represented by black line.

5.3.3 Proteins associated with increasing diabetes duration

When looking at proteins altered with increasing duration of diabetes, there were 0 proteins significantly changed in all groups (5-10, 10-15, 15-20, 20+ years) when comparing to patients with newly diagnosed (0-5 yrs) diabetes. Serpin Family A Member 12 (SERPINA12, Fig. 5.3A) declined from 0-15 years ($P<0.05$, $R= - 0.15$) and Thioredoxin reductase (TR, Fig. 5.3B) increased from 0-15 years ($P<0.05$, $R= -0.15$). These were the only common significantly changed proteins associated with diabetes progression between 0-15years. Renin (REN) expression was significantly increased from 15 yrs (Fig. 5.3C, D), although linear regression analysis could not determine any correlation.



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vs. 0-5 years duration				
Protein	5-10yrs	10-15yrs	15-20yrs	20+yrs
SERPINA12	0.024	0.007		
TR	0.028	0.008		
REN			0.002	0.024

Figure 5.3 - **Protein markers linked to diabetes duration.** (A) SERPINA12, linear regression. (B) TR, linear regression. (C) REN, linear regression. (D) Table illustrating significant changes as diabetes duration increases vs. newly diagnosed diabetes (0-5yrs). N=374 T2D patients. All trends expressed as OLink NPX values, and R values reported on graphs.

There were 22 proteins associated with over 20 year's diabetes duration (Table 5.4). Fibroblast growth factor 5 (FGF-5, Fig. 5.4A) and Decorin (DCN, Fig. 5.4B) were both solely increased (P<0.001, P<0.01 respectively) in patients that had been suffering from diabetes for more than 20 years and not in other groups.

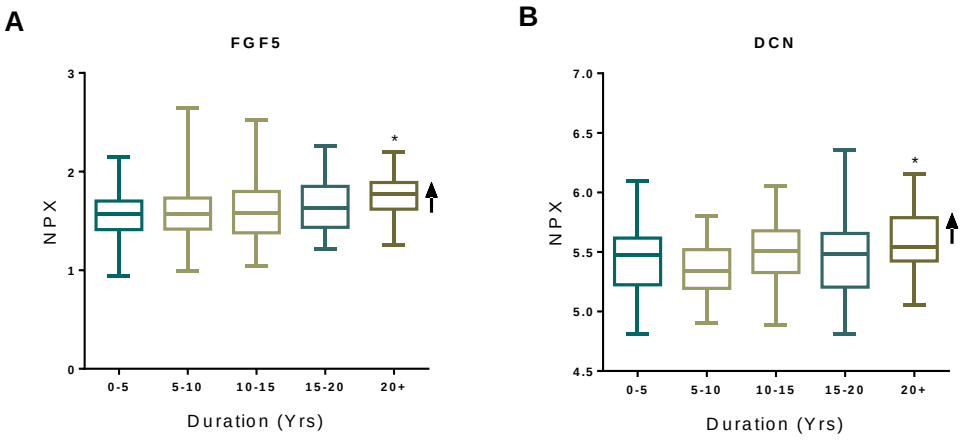


Figure 5.4 - **Proteins indicative of 20 + years diabetes.** (A) FGF5. (B) DCN. Protein expression was determined using OLink proteomics and normalised to in house OLink control. N= 374 T2D patents. All data presented as box and whisker plots (min, mean, max). Data showed unique time dependent upregulation of proteins of interest. Significance was determined using One-Way ANOVA and Tukey's post hoc test. *P<0.05 vs. 0-5 yrs diabetes duration.

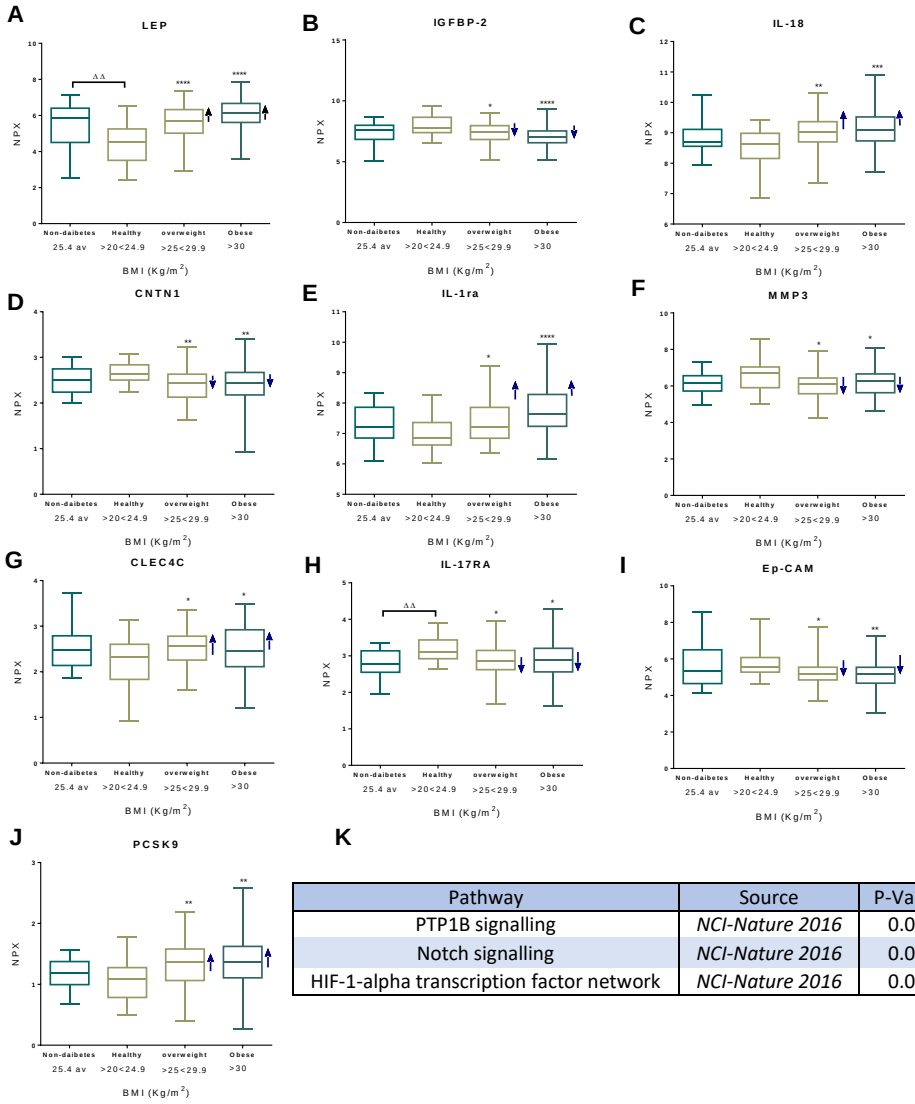
0-5 Y							
5-10y		10-15y		15-20y		20+y	
Protein	P-value	Protein	P-value	Protein	P-value	Protein	P-value
LAG3	0.021	*SERPINA12*	0.008	*IL2*	0.000	*FGF-5*	0.003
SERPINA12	0.024	*TR*	0.008	*FCRL3*	0.002	*DCN*	0.005
CXCL10	0.024	*GLO1*	0.019	*ACE2*	0.014	*SCF*	0.005
MCP-2	0.025	*uPA*	0.024	*TRANCE*	0.021	*SCF*	0.007
TR	0.029	*HO-1*	0.032	*TGM2*	0.028	*NFATC3*	0.009
LPL	0.029	*ITGB2*	0.033	*GLO1*	0.036	*PRELP*	0.009
hOSCAR	0.036	*GH*	0.033	*IL18*	0.040	*EGFR*	0.013
PD-L1	0.037	*GIF*	0.038	*PAPPA*	0.041	*TFF3*	0.015
NFATC3	0.040	*GDF-15*	0.038	*ITGB6*	0.042	*TIMP4*	0.019
CXCL9	0.043	*ALCAM*	0.042	*FAS*	0.050	*IL-17A*	0.020
		t-PA	0.043	*REN*	0.050	*ST2*	0.022
		CCL4	0.047	*CXCL10*	0.050	*AREG*	0.024
		NFATC3	0.047	*ACE2*	0.014	*REN*	0.024
		IL-6RA	0.047	*TRANCE*	0.021	*CDSN*	0.026
		MERTK	0.048	*TGM2*	0.028	*IGFBP-2*	0.026
				GLO1	0.036	*CCL28*	0.028
						CCL25	0.028
						IGFBP-1	0.031
						TNFRSF11A	0.033
						MASP1	0.035
						CKAP4	0.036
						GDF-15	0.037
						AMBP	0.039
						TNFRSF9	0.040
						TF	0.046

Table 5.4 – **Proteins associated with increasing diabetes duration**. Proteins significantly altered in patients with diabetes duration of 5-10, 10-15, 15-20 and 20+ years compared to those within 0-5 years of diagnosis. Significance determined with Student's T-Test of each grouping compared to a group with 0-5 year's diabetes duration.

5.3.4 Proteins associated with poor weight control in diabetes

Participants were clustered according to BMI and disease state. There were ten common proteins between overweight and obese patients, compared to healthy weight diabetes patients. Leptin (LEP, Fig. 5.5A) was most significantly affected by BMI in diabetes and data indicated a progressive increase in leptin as BMI increased (overweight P=0.000005745, obese P= 0.00000000000001). It should be noted that leptin was also significantly increased in non-diabetes controls compared to diabetes patients with a healthy BMI (P<0.01). The non-diabetes controls had an average BMI of 25.4 Kg/m^2, therefore leptin may be considered to be sensitive to BMI rather than disease state. Insulin-like growth factor binding protein 2 (IGFBP-2, Fig. 5.5B) was reduced in diabetes patients with a BMI above 24.9 Kg/m^2 and not in non-diabetes controls. Interleukin-18 (IL-18, Fig. 5.5C), the interleukin-1 receptor antagonist (IL-1 ra, Fig. 5.5E), C-Type Lectin Domain Family 4 Member C (CLEC4C, Fig. 5.5G) and Proprotein convertase subtilisin/kexin type 9 (PCSK9, Fig. 5.5J) were all increased in overweight and obese diabetes patients compared to diabetes patients with a healthy BMI. IGFBP-2 (Fig. 5.5B), Contactin 1 (CNTN1, Fig. 5.5D), Matrix metalloproteinase-3 (MMP3, Fig. 5F) and Epithelial cell adhesion molecule (EpCAM, Fig. 5.5I) were all significantly

reduced in overweight and obese diabetes patients compared to diabetes patients with a healthy BMI. Interleukin 17 Receptor A (IL-17RA, Fig. 5.5H) was significantly reduced in overweight diabetes patients and was also reduced in the non-diabetes controls compared to diabetes patients with a healthy BMI. Weighted Enricher pathway analysis revealed that the 10 proteins were associated with PTP1B and Notch homolog 1 (NOTCH) signaling as well as the Hypoxia-Inducible Factor (HIF) 1 alpha transcription factor network (P<0.03, Fig. 5.5K).



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Pathway	Source	P-Value
PTP1B signalling	*NCI-Nature 2016*	0.02
Notch signalling	*NCI-Nature 2016*	0.02
HIF-1-alpha transcription factor network	*NCI-Nature 2016*	0.03

Figure 5.5 - **Proteomic markers indicative of overweight and obesity in T2D.** (A) LEP, (B) IGFBP-2, (C) IL-18, (D) CNTN1, (E) IL-1ra, (F) MMP3, (G) CLEC4C, (H) IL-17RA, (I) Ep-CAM, (J) PCSK9 (K) Enricher pathway analysis. Protein expression was determined using OLink proteomics and normalised to in house OLink control. N=20 non-diabetes controls, N=18 diabetes patients with a healthy BMI (BMI 20-24.9 Kg/m^2), N= 55 overweight diabetes patients (BMI 25-29.9 Kg/m^2) and N=169 obese diabetes patients (BMI 30+ Kg/m^2). Data shows mean protein levels for 10 proteins unique to overweight and obesity. Data enrichment was conducted on integrative web based software application Enrichr (Chen et al., 2013). All data was inputted and ranked for level of membership according to Enrichr protocol (N=10 proteins), and significantly associated pathways shown (P<0.05). Significance was determined using one- way ANOVA and Tukey's post hoc test. *P<0.05, **P<0.01, *** P<0.001, ****P<0.0001, vs. diabetes patients with a healthy BMI (20-24.9 Kg/m^2). ΔΔ P<0.01 vs. non-diabetes controls.

5.3.5 Proteins associated with obesity

There were a total of 14 proteins that were exclusively increased in patients with a BMI greater than 30 Kg/m^2. Fatty acid binding protein 4 (FABP4) was significantly increased (Fig. 5.6A, P<0.001) and Paraoxonase 3 (PON3) was reduced (Fig. 5.6B, P<0.01) in participants with a BMI >30 Kg/m^2.

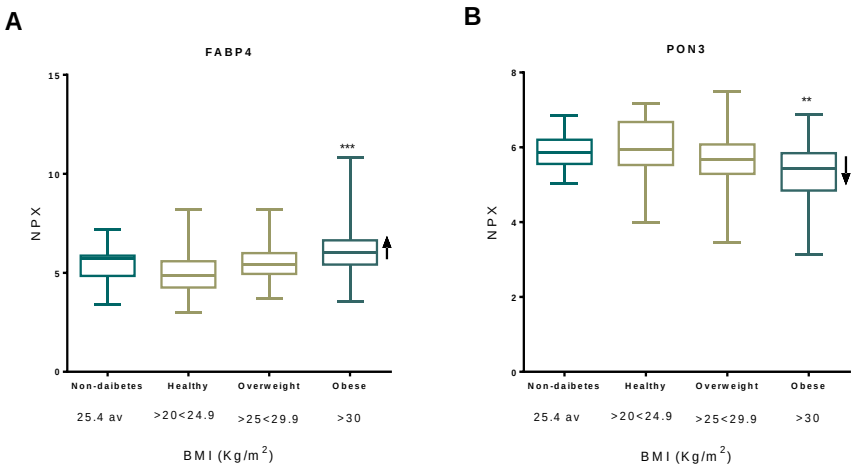


Figure 5.6 - **Proteins indicative of BMI >30 Kg/m^2**. (A) FABP4, (B) PON3. All data presented as OLink NPX values. N=20 non-diabetes controls, N=18 diabetes patients with a healthy BMI (BMI 20-24.9 Kg/m^2), N=55 overweight diabetes patients (BMI 25-29.9 Kg/m^2) and N=169 obese diabetes patients (BMI >30 Kg/m^2). Significance was determined using one-way ANOVA and Tukey's post hoc test. **P<0.01, *** P<0.001 vs. diabetes patients with a healthy BMI (20-24.9 Kg/m^2).

To further assess the specificity of markers associated with BMI in T2D, patients were grouped into non-diabetes controls and diabetes and then sub-grouped into healthy BMI (<24.9 Kg/m^2) and overweight (>25 Kg/m^2). Data showed that LEP (P<0.0001, Fig. 5.7A) IL-1ra (P<0.0001, Fig. 5.7C) and IL-18 (P<0.001, Fig. 5.7D) were increased and IGFBP-2 (P<0.0001, Fig. 5.7B) decreased, exclusively in overweight diabetes patients.

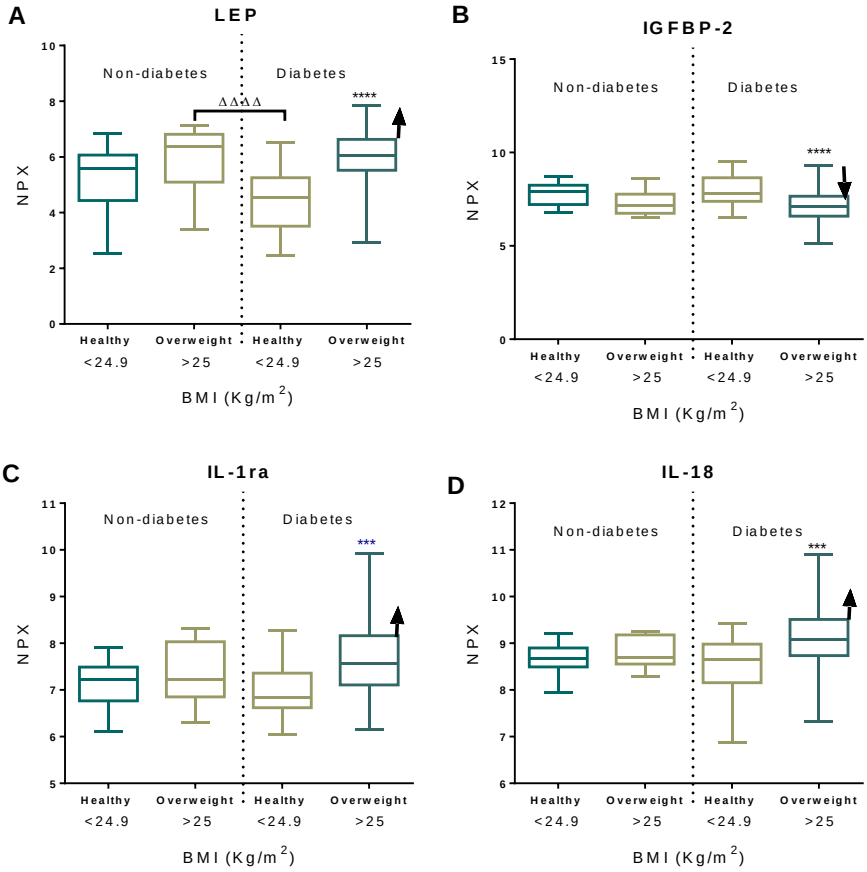


Figure 5.7 – **Specific proteomic markers of overweight in T2D**. (A) LEP, (B) IGFBP-2, (C) IL1ra, (D) IL-18. All data presented as OLink NPX values. N=20 non-diabetes controls, N=18 diabetes patients with a healthy BMI (20-24.9 Kg/m^2), N= 224 diabetes patients with a BMI>25. Significance was determined using one way ANOVA and Tukey's post hoc test. **P<0.01, *** P<0.001, ΔΔΔΔ v diabetes patients with a healthy BMI (20-24.9 Kg/m^2).

5.3.6 Proteins associated with poor glycemic control in T2D

Participants were clustered according to NICE specified HbA_{1c} guidelines non-diabetes controls, on target (53 mmol/mol), poor (>53 mmol/mol) or extremely poor (>80 mmol/mol). A total of 30 proteins were significantly changed in diabetes patients with a HbA_{1c} greater than 53 mmol/mol and 80 mmol/mol compared to on target patients <53 mmol/mol HbA_{1c} (Table 5.5). Seven proteins were characteristic of poor and extremely poor glycaemic control in diabetes. Interleukin 6 (IL-6) was reduced (Fig. 5.8A, P<0.05) in patents with a HbA_{1c} >53 mmol/mol (7%) <80 mmol/mol (9.5%) and increased when HbA_{1c} was above 80 mmol/mol. Integrin Subunit Beta 8 (ITGB8, Fig. 5.8B), Kidney Injury Molecule-1 (KIM1, Fig. 5.8C),

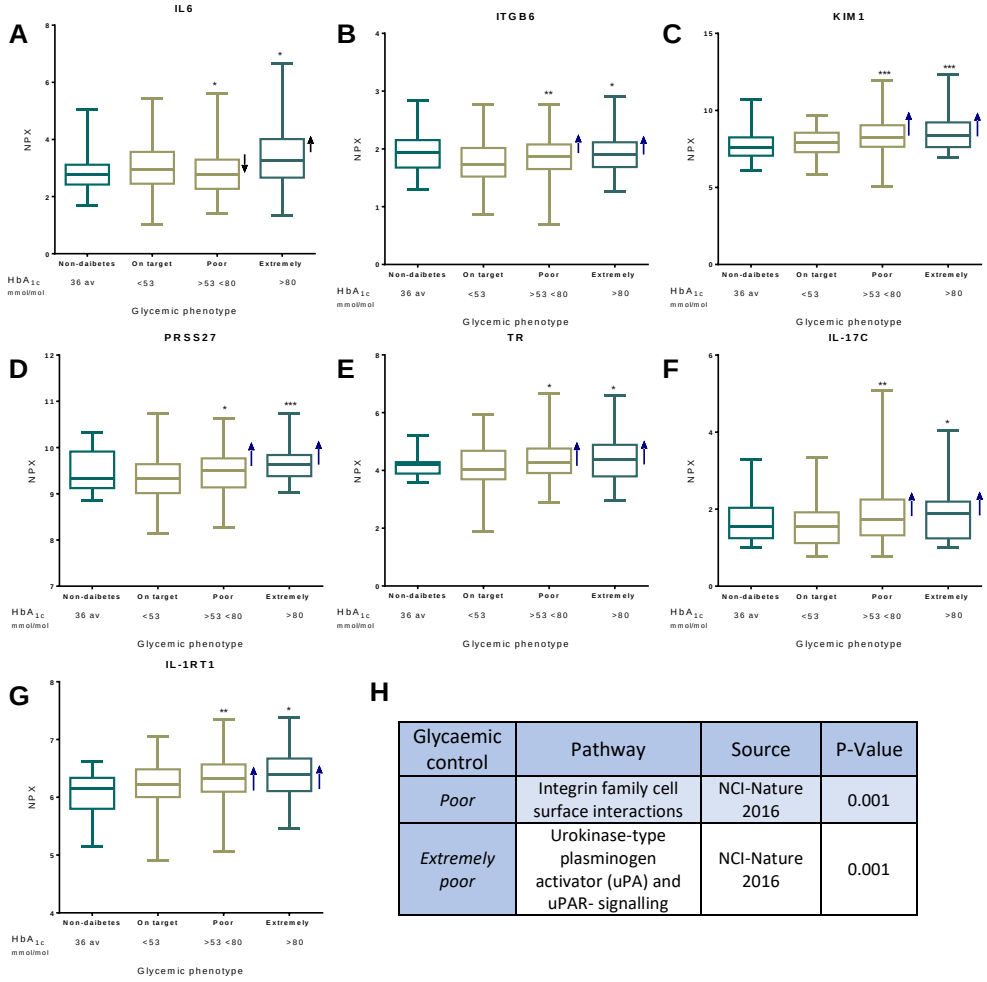
Protease, Serine 27 (PRSS27, Fig. 5.8D), Transferrin receptor protein 1 (TfR1, Fig. 5.8E), Interleukin 17C (IL-17C, Fig. 5.8F) and Interleukin-1 receptor type 1 (IL-1RT1, Fig. 5.8G) were increased in diabetes and at both stages of HbA_{1c} severity (P<0.05-P<0.001). There were no changes in these proteins when comparing non-diabetes controls to on target diabetes patients.

Pathway enrichment analysis (Enrichr) (Chen et al., 2013) did not identify any common signaling pathways between the 7 dysregulated proteins common between poor and extremely poor glycaemic control. The protein signature associated with poorly controlled glycaemia (>53 mmol/mol, 7%) was associated with altered Integrin family cell surface interactions, while that associated with extremely poor glycaemic control was linked to uPA and uPAR- signalling (Fig. 5.8H).

On target vs. Poor glycaemic control >53 mmol/mol	
Protein	P-Value
FABP2	0.002
CCL11	0.003
MMP-10	0.004
KIM1	0.008
VEGFD	0.008
REN	0.010
HO-1	0.010
SPON1	0.012
uPA	0.012
ITGB6	0.013
GH	0.014
PRSS27	0.015
TR	0.015
TNFSF14	0.016
IL-17C	0.016
ITGA6	0.017
PRELP	0.018
BTN3A2	0.019
IL-1RT1	0.019
GDF-15	0.021
PRDX3	0.024
IL6	0.026
IL6	0.029
DCBLD2	0.030
VSIG2	0.032
CCL25	0.034
ITGB2	0.042
ITGA11	0.045
FAM3B	0.048

On target vs. extremely poor glycaemic control >80 mmol/mol	
Protein	P-Value
PRSS27	0.000
CTSD	0.001
EGFR	0.002
IL-1ra	0.002
ITGB2	0.002
KIM1	0.006
HSD11B1	0.007
FGF-19	0.008
CCL20	0.011
FABP4	0.019
CPB1	0.022
PCSK9	0.024
CXCL16	0.027
TFPI	0.027
SCF	0.028
IL6	0.028
TNF-R1	0.029
IL6	0.030
TR	0.032
CXCL9	0.033
MB	0.035
CLEC4C	0.036
IL6	0.038
TWEAK	0.041
DPP10	0.041
IL-1RT1	0.042
IL-17C	0.043
CTRC	0.044
MCP-1	0.048

Table 5.5 – **Proteins associated with poor and extremely poor glycaemic control (HbA_{1c}) in T2D.** N= 121 on target <53, n=206 >54<85, n=37 >86. Significance determined using Student T test vs. on target (<53 mmol/mol HbA_{1c}).



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Glycaemic control	Pathway	Source	P-Value
Poor	Integrin family cell surface interactions	NCI-Nature 2016	0.001
Extremely poor	Urokinase-type plasminogen activator (uPA) and uPAR- signalling	NCI-Nature 2016	0.001

Figure 5.8 - **Proteomic markers indicative of poor glycemic control.** (A) IL6, (B) ITGB6, (C) KIM1, (D) PRSS27, (E) TR, (F) IL-17C, (G) IL-1RT1 (H) Enricher pathway analysis. Protein expression was determined using OLink assays and normalised to in house OLink control. N=20 non-diabetes controls (HbA_{1c} 36 mmol/mol av), N=121 on target diabetes (HbA_{1c} <53 mmol/mol), N= 206 poor control (HbA_{1c} >53 <80 mmol/mol) and N=37 extremely poor control (HbA_{1c} >80 mmol/mol). Data shows levels of 7 proteins specific to poor glycemic control in diabetes. Data enrichment was conducted on the integrative web based software application Enrichr (Chen et al., 2013). All data was inputted and ranked for level of membership according to the Enrichr protocol (30 proteins were identified for poor and extremely poor glycaemic control groups). All significantly associated pathways are shown (P<0.05). Full list of proteins illustrated in Table 5. Significance was determined using one-way ANOVA and Tukey's post hoc test. *P<0.05, **P<0.01 and *** P<0.001 vs. on target diabetes (HbA_{1c} <53 mmol/mol).

There were 21 proteins exclusively associated with extremely poor glycaemic control >80 mmol/mol HbA_{1c} (Diagram 5.1) only 2 were significant to P<0.01 and unique to diabetes. Cathepsin D (CTSD, Fig. 5.9A) and IL-1RA (Fig. 5.9B) were both increased in the extremely poorly managed group compared to the on target group. There were 22 proteins unique to patients with a poor glycaemic control (HbA_{1c} >53 <80 mmol/mol), many of which were subject to significant outliers that impacted upon trends. The proteins with the highest P-values were chosen for further analysis. C-C motif chemokine 11 (CCL11, Fig. 5.10A, P<0.05) and Fatty Acid-Binding Protein 2 (FABP2, Fig. 5.10B, P<0.01) were increased only in diabetes patients with a HbA_{1c} >53<80 mmol/mol.

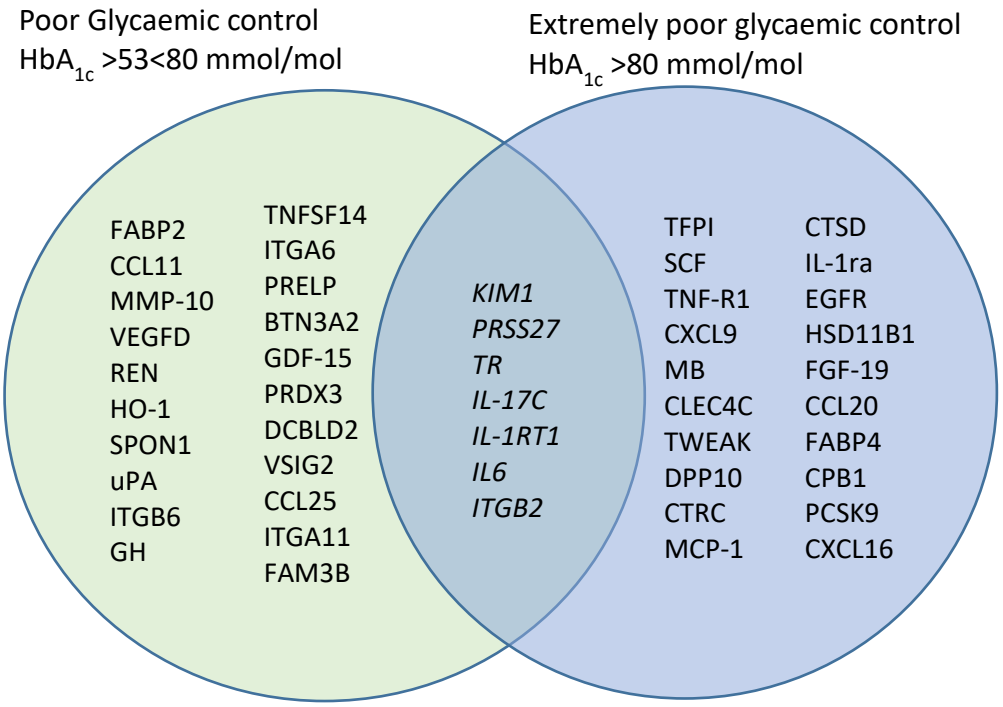


Diagram 5.1 – **Venn diagram illustrating proteins significantly altered in patients with poor and extremely poor glycaemic control compared to on target diabetes patients.**

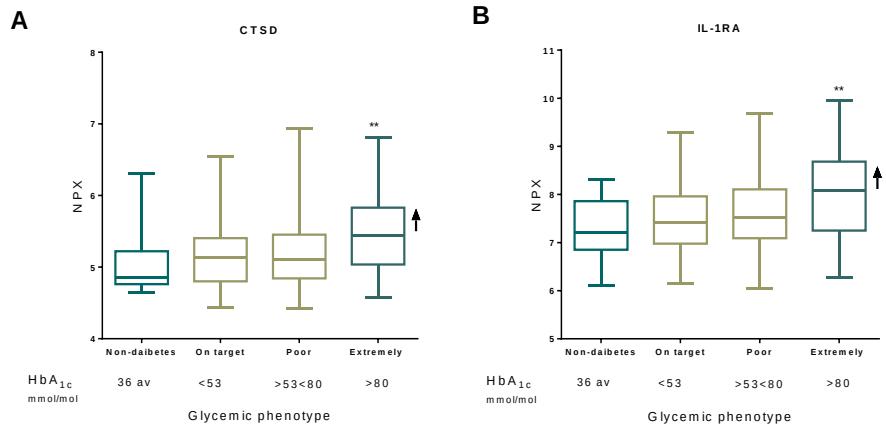


Figure 5.9 – **Most significant proteins indicative of HbA_{1c} >80 mmol/mol**. (A) CTSD, (B) IL-1ra. N=121 on target diabetes (HbA_{1c} <53 mmol/mol), N= 206 poor glycaemic control (HbA_{1c} >53 <80 mmol/mol) and N=37 extremely poor glycaemic control (HbA_{1c} >80mmol/mol). Significance was determined using one-way ANOVA and Tukey's post hoc test. **P<0.01 and *** P<0.001 vs. on target diabetes (HbA_{1c} <53 mmol/mol).

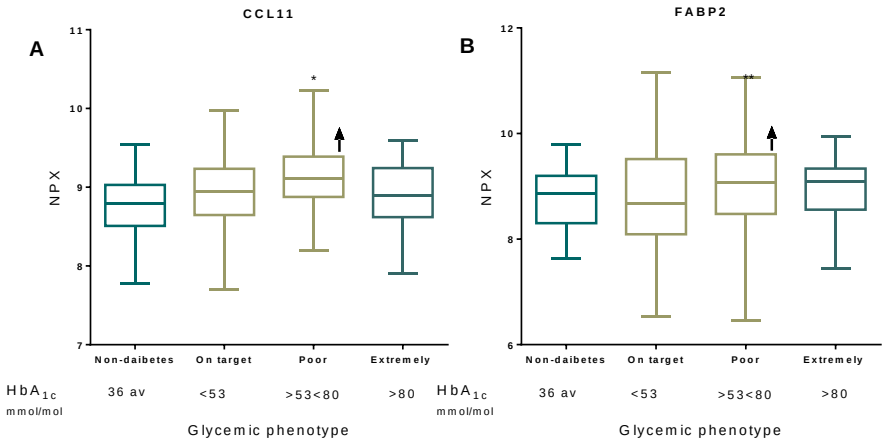


Figure 5.10 – **Most significant proteins indicative of poor glycaemic control (HbA_{1c} >53 <80 mmol/mol)**. (A) CCL11, (B) FABP2. N=121 on target diabetes (HbA_{1c} <53mmol/mol), N= 206 poor control (HbA_{1c} >53<80 mmol/mol) and N=37 extremely poor control (HbA_{1c} >80 mmol/mol). Significance was determined using one-way ANOVA and Tukey's post hoc test. **P<0.01 and *** P<0.001 on target diabetes (HbA_{1c} <53 mmol/mol).

5.3.7 Proteins associated with poorly controlled blood lipids in diabetes

Two proteins, stem cell factor (SCF, Fig. 5.11A) and Galactose 4 (GAL-4, Fig. 5.11B) were universally implicated in changes in HDL, LDL and cholesterol. In patients with a healthy HDL level (>1 mmol/l) SCF was increased (P< 0.0001), while in patients with high LDL (>2 mmol/l) and total cholesterol (>4 mmol/l) this protein was significantly reduced, P<0.05 and P<0.05 respectively. GAL-4 levels were reduced in high HDL (P<0.01), LDL (P<0.05) and total cholesterol (P< 0.05).

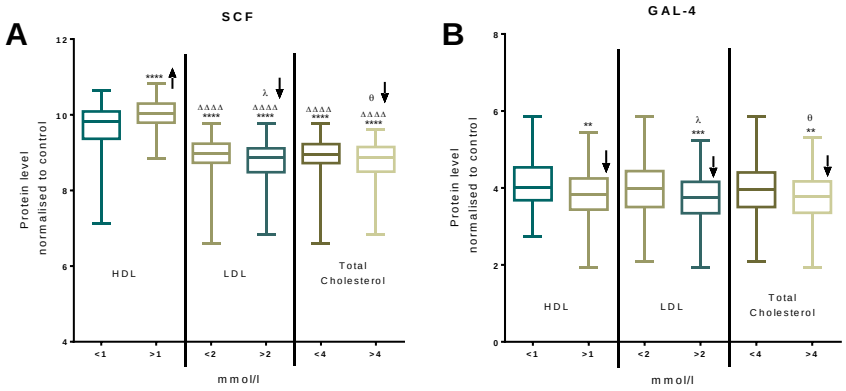


Figure 5.11 - **Common proteomic changes in patients with poorly controlled blood lipids.** (A) SCF (B) GAL-4 was measured in plasma of diabetes participants and controls. Protein expression was determined using OLink assays and normalised to in house OLink control. N= 108 HDL <1 mmol/l, N=248 HDL > 1mmol/l. N=233 LDL <2 mmol/l, N= 132 LDL >2 mmol/l, N= 233 total cholesterol <4 mmol/l, N= 122 total cholesterol >4 mmol/l. All data presented as box and whisker plots (min, mean, max) Significance was determined using one-way ANOVA and Tukey's post hoc test. **P<0.01 and ***P<0.001, ****P<0.0001 vs. <1 mmol/l HDL. ΔΔΔΔ vs >1 mmol/l HDL. ʎ vs <2 mmol/l LDL. ϴ vs <4mmol/l total cholesterol.

	Pathway	Source	P-Value
HDL	Direct p53 effectors	*NCI-Nature 2016*	0.01
	Beta1 integrin cell surface interactions	*NCI-Nature 2016*	0.02
	HIF-1-alpha transcription factor network	*NCI-Nature 2016*	0.02
HDL	CXCR3-mediated signalling events	*NCI-Nature 2016*	0.001
	IL4-mediated signalling	*NCI-Nature 2016*	0.003
Total Cholesterol	Syndecan-4-mediated signalling events	*NCI-Nature 2016*	0.0008

Table 5.6 – **Pathway enrichment analysis for blood lipid proteins.** Data enrichment was conducted on integrative web based software application Enrichr (Chen et al., 2013). All data was inputted and ranked for level of membership according to Enrichr protocol. N=81 proteins HDL, N=32 proteins LDL, N=30 proteins total cholesterol. Pathways identified via Nature Pathway Interaction Database. All significantly associated pathways are shown (P<0.05).

Pathway enrichment analysis revealed plasma proteome profiles for HDL, LDL and total Cholesterol all had different primary pathway activation (Table 5.6), but interestingly were all linked to integrin signaling or integrin mediated targets.

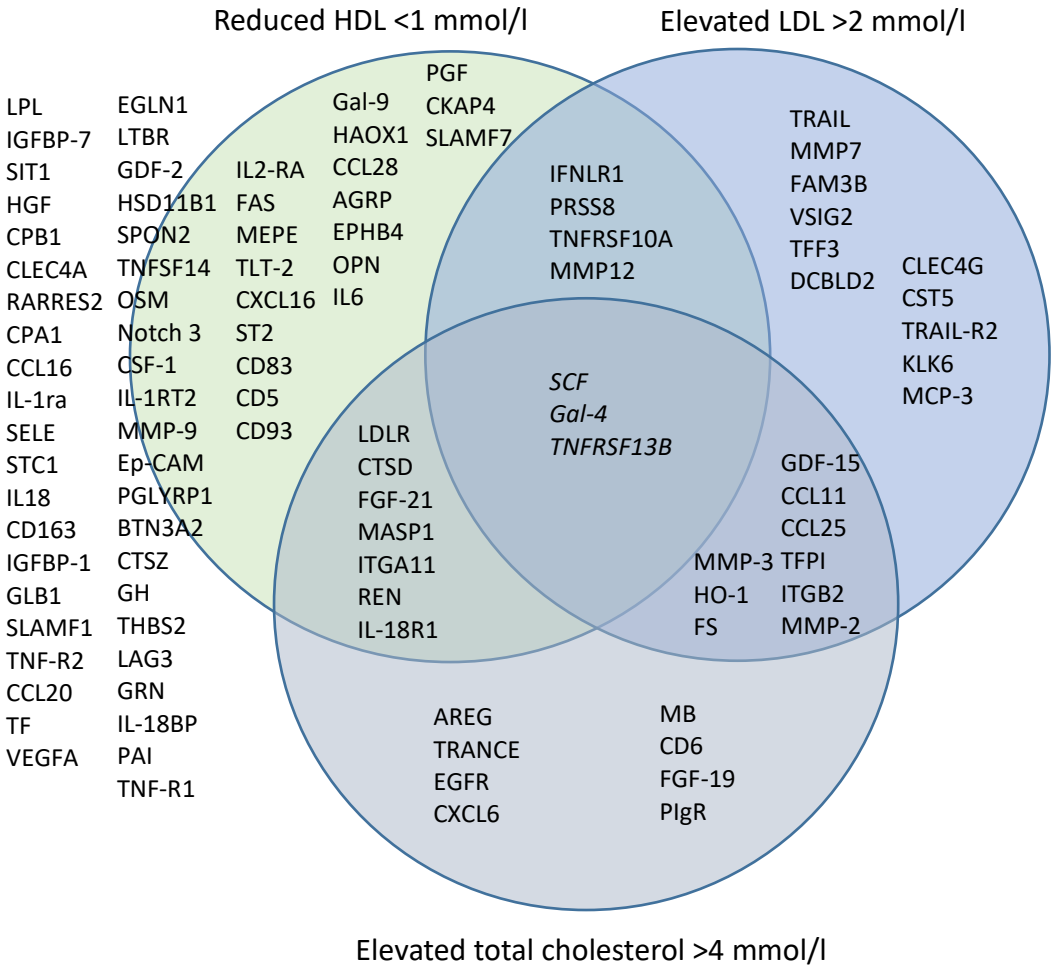


Diagram 5.2 – **Venn diagram of proteins significantly associated with alterations in blood lipids; clusters are dependent NICE guidance for hyperlipidaemia. Significance was determined from on target diabetes patients.**

5.3.7.1 Proteomic signature of reduced HDL

There were a total of 81 significantly altered proteins in patients with reduced HDL. The top four were selected for regression analysis. SCF (Fig. 5.12A) displayed a non-linear, positive regression (R= 0.48, P< 0.0001). PON3 (Fig. 5.12B) had a positive linear relationship (R= 0.36, P< 0.0001). Interestingly LPL expression and LDL receptor expression were inversely related. Lipoprotein lipase (LPL, Fig. 5.12C) had a positive non-linear relationship (R= 0.36, P< 0.0001) to plasma HDL concentration, while LDL receptor expression had a negative non-linear (R= -0.29, P< 0.0001, Fig. 5.12D) relationship.

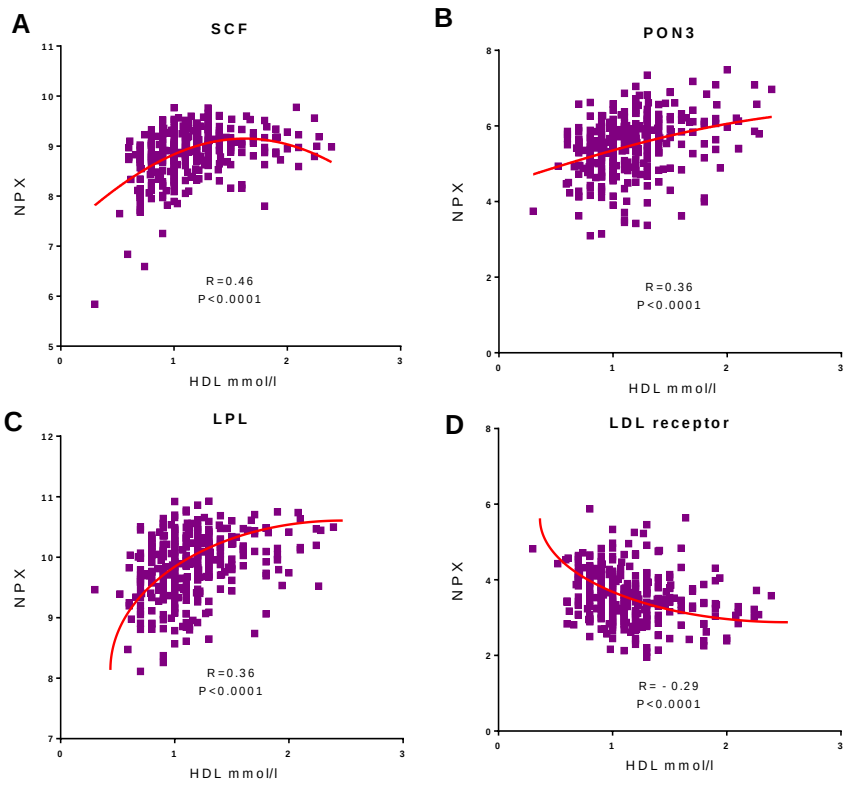


Figure 5.12 - **Plasma protein correlated with HDL concentration.** (A) SCF (B) PON3 (C) LPL (D) LDL receptor. N=356 diabetes patients for all HDL correlations. Data presented as scatter plots with non-linear, Gaussian regression, fitted with least squares (ordinary) fit. R value representative of relationship.

5.3.7.2 Proteomic signature of elevated LDL

A total of 32 proteins were changed in patients with elevated LDL (>2 mmol/l). The top four were selected for regression analysis. Growth Differentiation Factor 15 (GDF15, R=-0.23, P<0.0001, Fig. 5.13A), CCL11 (R= -0.22, P<0.0001, Fig. 5.13B) and C-C motif chemokine 25 (CCL25, R= -0.24, P<0.0001, Fig. 5.13C) displayed a linear negative regression. TNF-related apoptosis-inducing ligand (TRAIL, Fig. 5.13D) displayed a positive correlation against LDL concentration (R= 0.24, P<0.0001).

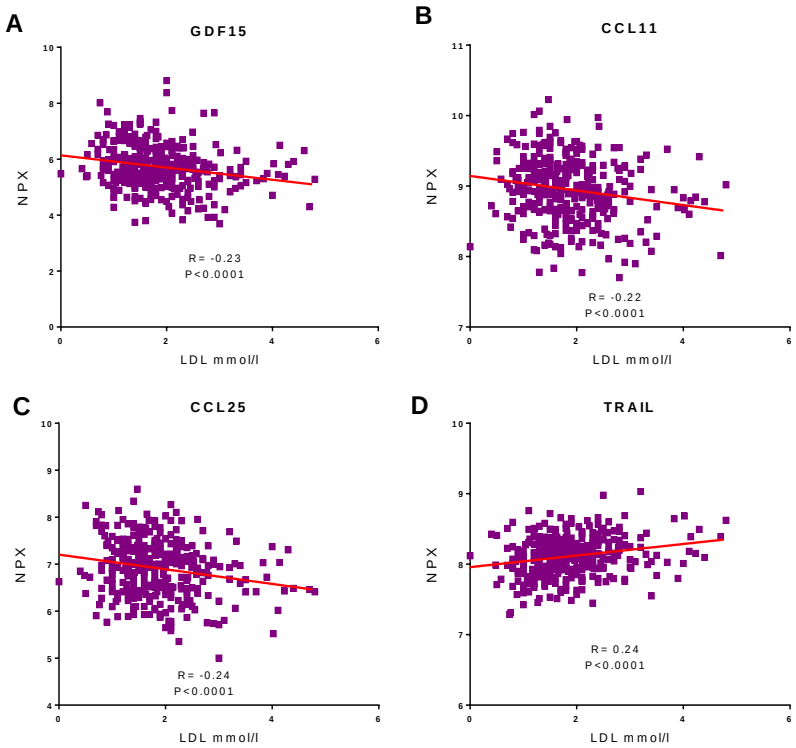


Figure 5.13 - **Plasma protein correlated with LDL concentration.** (A) GDF15 (B) CCL11 (C) CCL25 (D) TRAIL. N= 354 diabetes patients for all LDL correlations. Data presented as scatter plots with linear regression best line fitted. R value representative of relationship.

5.3.7.3 Proteomic signature of elevated total cholesterol

Elevated total cholesterol (>4 mmol/l) was correlated with 2 proteins, Tissue factor pathway inhibitor (TFP1, Fig. 5.14A) and LDL receptor (Fig. 5.14B). A total of 30 proteins (Diagram 5.2) were increased in patients with elevated cholesterol although most changes were small and did not correlate with total cholesterol concentration. TFP1 and LDL receptor protein levels were positively correlated with total cholesterol (R=0.31, P<0.0001, and R=0.24, P<0.0001 respectively).

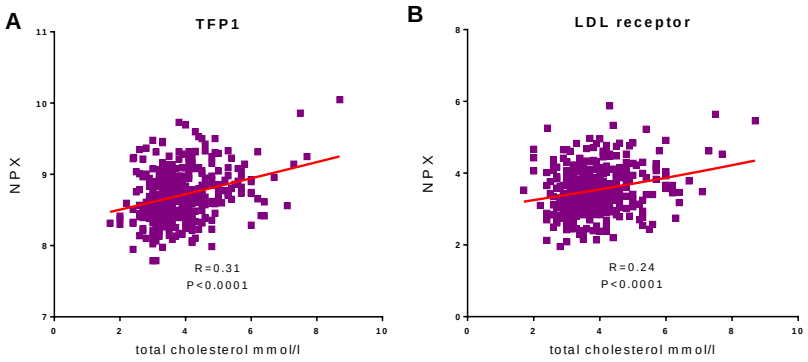


Figure 5.14 - **Plasma proteins correlated with total cholesterol concentration.** (A) TFP1 (B) LDL receptor. N= 354 diabetes patients for all total cholesterol correlations. All data presented as scatter plots with linear regression best line fitted. R value representative of relationship.

5.3.8 Proteins associated with diabetes regime intensification and drug class

There were 6 overlapping proteins (Diagram 5.3) in patients on 2, 3, and 4 + diabetes medications compared to medication naïve patients, however only Sortilin (SORT, R= - 0.15, P<0.01, Fig. 5.15A, B), Caspase 3 (CASP-3, R= -0.11, P<0.05, Fig. 5.15C, D) and CD84 (R= - 0.15, P<0.01, Fig. 5.15E, F) were correlated with regime intensification.

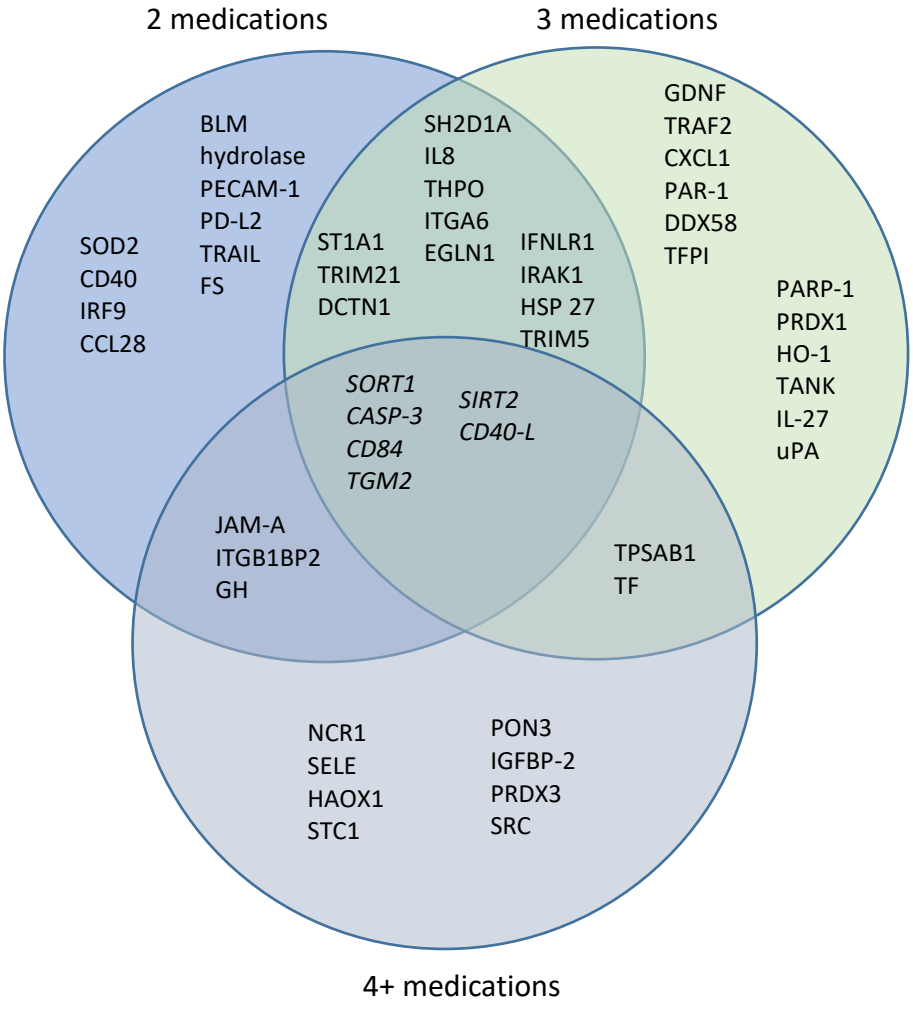


Diagram 5.3 – **Illustration of significantly changed proteins associated with diabetes regime intensification. Clusters are dependent on the number of diabetes drugs a participant is prescribed. Significantly changed proteins are compared against average level of protein in medication naïve participants.**

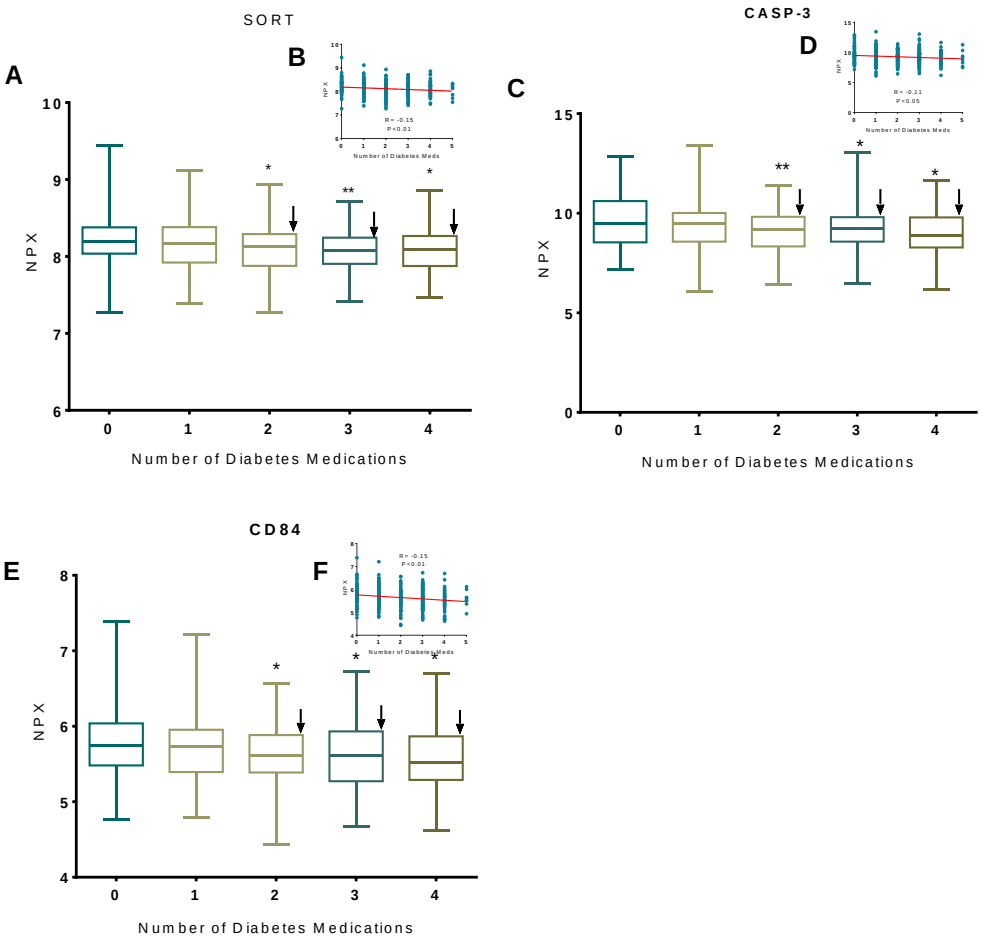


Figure 5.15 – **Proteins associated with diabetes regime intensification.** (A) SORT box and whisker plot (B) SORT linear regression (C) CASP-3 box and whisker plot (D) CASP-3 linear regression (E) CD84 box and whisker plots (F) CD84 linear regression. N= 52; 0 medications. N= 85; 1 medication. N= 83 2 medications. N= 104; 3 medications. N= 50; 4 + medications. Data presented as box and whisker plots (min, mean, max) showing significance level compared to 0 medications. Linear regression analysis shows all proteins have a negative relationship with regime intensification. Significance was determined using one-way ANOVA and Tukey's post hoc test. *P<0.05, **P<0.01 vs. 0 medications

5.3.8.1 Proteins associated with metformin therapy

Metformin monotherapy is associated with initial treatment of T2D followed by addition of other oral medications and injectables, GLP-1Ras and insulin (NICE, 2017). Thirty two proteins were significantly altered in metformin monotherapy patients. Twenty six proteins were specific to patients on injectables, and 77 proteins linked with any other oral type of diabetes medication (Diagram 5.4). The six most significant are shown in Table 5.7, with trend analysis.

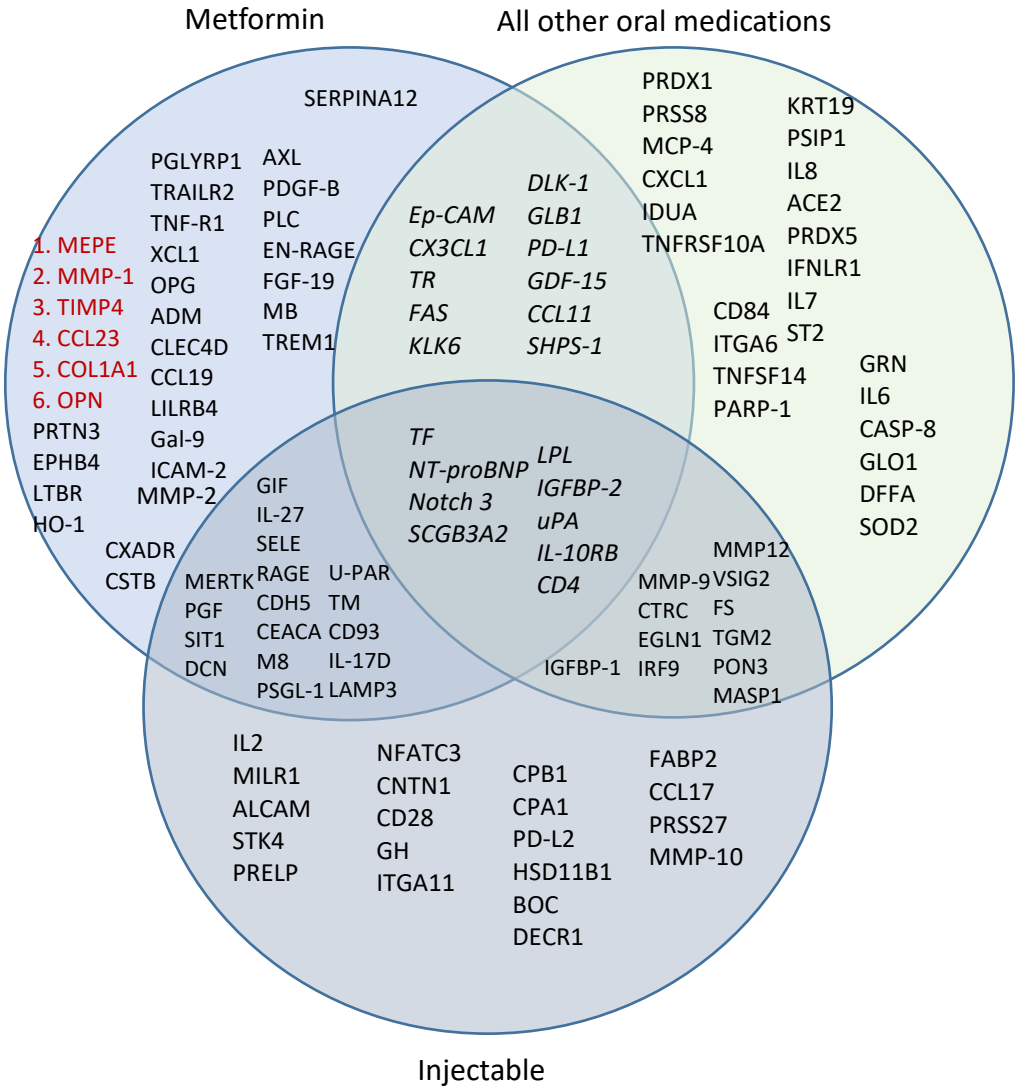


Diagram 5.4 – **Illustration of significantly changed proteins associated with metformin, all other oral medications and injectable diabetes medications.** 55 further proteins were identified in the other oral medication group, a full list can be found in Supplementary Table 3.

Metformin Monotherapy		
Protein	P-value	Trend
MEPE	0.0000	↑
MMP-1	0.0001	↓
TIMP4	0.0002	↓
CCL23	0.0004	↑
COL1A1	0.0007	↓
OPN	0.0008	↑

Table 5.7 – **Most significantly changed proteins in metformin only prescribed patients.** N= 66 metformin (only), N= 163 all other prescription combinations, Significance determined using Student's t test vs. metformin negative patients.

5.3.9 Proteins associated with other oral diabetes medications

There were 27 significant proteins (P<0.05, Table 5.8), unique to DDP-IVi prescribed patients. Lymphocyte-activation protein 3 (LAG3, P<0.01), Interleukin-18-binding protein (IL-18BP, P<0.01) and C-X-C motif chemokine 10 (CXCL10, P<0.01) were the most significant, and all were increased. SGLT2i prescribed patients had 11 significant proteins specific to the class (Table 8). Hydroxyacid oxidase 1 (HAOX1, increased, P<0.01), IL-1RA (increased, P<0.05,) and Fms-related tyrosine kinase 3 ligand (Flt3L, decreased, P<0.05) were most significant.

DDP-IVi			SGLT2i		
Protein	P value	Trend	Protein	P value	Trend
LAG3	0.0017	↑	*HAOX1*	0.0041	↑
IL-18BP	0.0028	↑	*IL-1rα*	0.0137	↑
CDCP1	0.0033	↑	*Flt3L*	0.0145	↓
CXCL10	0.0062	↓	*MMP7*	0.0150	↓
TRANCE	0.0064	↓	*THBS2*	0.0168	↑
CST5	0.0077	↑	*BTN3A2*	0.0193	↓
CCL28	0.0079	↑	*SCF*	0.0197	↓
TNFRSF13B	0.0155	↑	*hOSCAR*	0.0206	↓
NCR1	0.0228	↑	*AMBP*	0.0238	↓
NT-3	0.0250	↑	*IL18*	0.0240	↑
CXCL16	0.0254	↑	*OSM*	0.0351	↑
TNF-R2	0.0281	↑			
CD83	0.0323	↑			
FAM3B	0.0328	↑			
IL-17C	0.0330	↑			
CCL22	0.0336	↓			
CLEC7A	0.0373	↑			
PAI	0.0373	↓			
CXCL9	0.0407	↑			
SLAMF1	0.0423	↑			
CCL15	0.0439	↑			
CD163	0.0447	↑			
LY75	0.0450	↑			
CD5	0.0455	↑			
ITGB6	0.0457	↑			
IGFBP-7	0.0465	↑			
Gal-4	0.0483	↑			

Table 5.8 - **Proteins associated with DDP-IVi and SGLT2i therapy.** N= 307 DDP-IVi (-), N= 67 DDP-IVi (+). N= 303 SGLT2i (-), N= 70 SGLT2i (+). Significance determined using Student's t test vs. drug negative patients.

SU			TZD		
Protein	P value	Trend	Protein	P value	Trend
CLEC4A	0.0062	↓	*TRAF2*	0.0025	↓
CD84	0.0062	↓	*CA5A*	0.0031	↓
TNFSF14	0.0199	↓	*PRDX1*	0.0034	↓
GRN	0.0364	↑	*PRSS8*	0.0074	↓
IL6	0.0397	↑	*MCP-4*	0.0119	↓
CTSZ	0.0424	↑	*CXCL1*	0.0119	↓
			IDUA	0.0168	↓
			TNFRSF10A	0.0174	↓
			CASP-8	0.0177	↓
			GLO1	0.0186	↓
			DFFA	0.0234	↓
			SOD2	0.0273	↓
			KRT19	0.0311	↓
			PSIP1	0.0386	↓
			IL8	0.0393	↓
			ACE2	0.0414	↓
			PRDX5	0.0428	↓
			IFNLR1	0.0436	↓
			IL7	0.0445	↓
			ST2	0.0466	↓

Table 5.9 **- Significantly changed proteins associated with SU and TZD therapy.** N= 251 SU (-), N= 123 SU (+). N= 362 TZD (-), N= 12 TZD (+). Significance determined using Student-T test vs. drug negative patients.

Sulphonylurea treatment was associated with six unique protein changes (Table 5.9). CD84 (P<0.01,) CLEC4A (P<0.01,) and Tumor Necrosis Factor Superfamily Member 14 (TNFSF14, P<0.05) were the most significant and all were decreased. Interestingly, TZDs were associated with 20 significantly altered proteins all of which were reduced (Table 5.9). The most significant were TNF receptor-associated factor 2 (TRAF2), Carbonic anhydrase 5A (CA5A) and Peroxiredoxin-1 (PRDX1) (P<0.01).

5.3.10 Proteins associated with injectable diabetes medications

There were 14 significantly (P<0.05) proteins specifically changed in GLP-1Ra treated patients, and 11 proteins altered specifically in insulin treated patients. There were two proteins altered in both GLP-1Ra and insulin treated patients (Diagram 5.5). The top two proteins unique to GLP-1Ra prescribed patients were Programmed cell death ligand 2 (PD-L2) and Hydroxysteroid 11-Beta Dehydrogenase 1 (HSD11B1), both were decreased (P<0.01). Growth Hormone (GH, P<0.01) and Integrin alpha-11 (ITGA11, P<0.05) were exclusively increased in insulin prescribed patients. CPA1 and

CPB1 were increased in GLP-1Ra prescribed patients and decreased in insulin prescribed patients (P<0.0001) (Table 5.10).

GLP-1Ra			Insulin			GLP-1Ra & Insulin		
Protein	P-value	Trend	Protein	P-value	Trend	Protein	P-value	Trend
PD-L2	0.002	↓	GH	0.004	↑	CPA1	0.000	Opposite (GLP-1Ra >)
HSD11B1	0.008	↓	ITGA11	0.014	↑	CPB1	0.000	Opposite (GLP-1Ra >)
BOC	0.013	↓	FABP2	0.036	↑			
DECR1	0.014	↑	CCL17	0.045	↓			
IL2	0.014	↑	PRSS27	0.046	↑			
MILR1	0.019	↓	MMP10	0.047	↑			

Table 5.10 – **Top significantly changed proteins associated with injectable medications only**. N= 310 GLP1-Ra (-), N= 64 GLP1-Ra (+). N= 261 Insulin (-), N= 113 Insulin (+). Significance determined using Student-T test vs. drug negative patients.

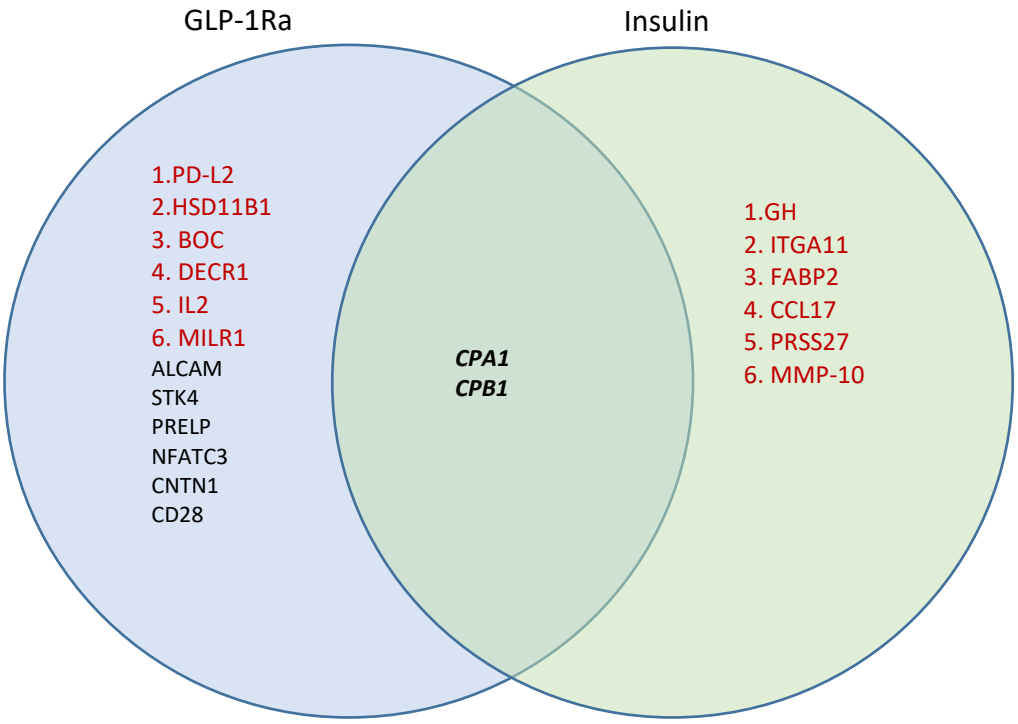


Diagram 5.5 - **Venn diagram of significantly changed proteins associated injectable medications, GLP-1Ra and insulin.**

5.3.11 Proteins associated β-cell function (C-peptide)

There were 4 proteins associated with all concentrations (0-5, 5-10, 10-15, 20+ ng/ml) of plasma C-peptide. Angiotensin converting enzyme 2 (ACE2, R=0.26, P<0.0001, Fig. 5.16A), IL-18 (R= 0.25, p< 0.0001, Fig. 16B) and LEP (R=0.24, p<0.0001, Fig. 16C) were positively correlated with plasma C-peptide levels and in all cases patients with 20 + ng/ml C-peptide had significantly more of the target protein than those with 0-5 ng/ml (P<0.05). ITGA11 (R= -0.24, P<0.0001, Fig. 16D) was negatively correlated with plasma C-peptide levels (P< 0.05).

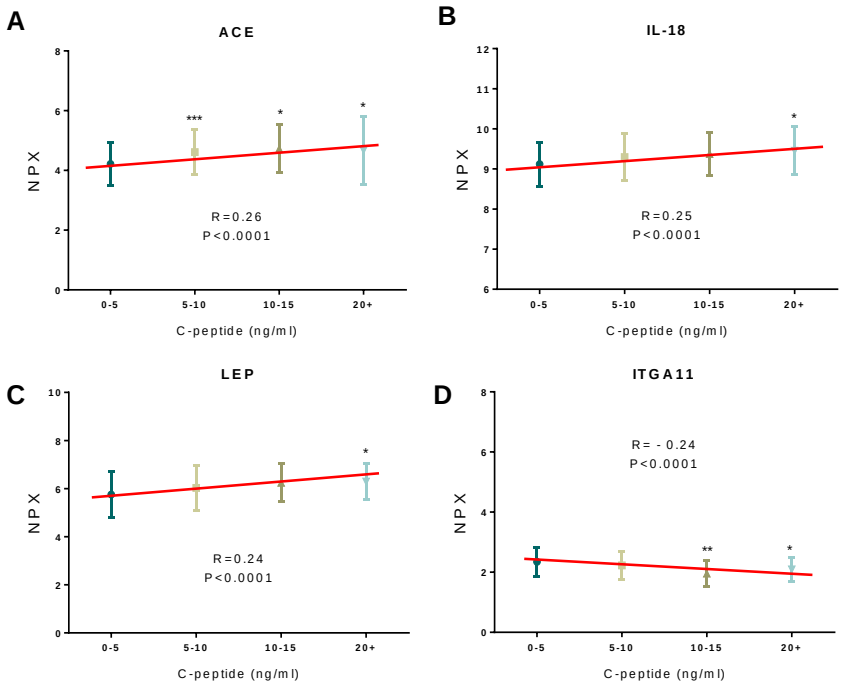


Figure 5.16 – **Proteomic markers indicative of alterations in plasma C-peptide concentrations.** (A) ACE correlation analysis. (B) IL-18 correlation analysis (C) LEP correlation analysis (D) ITGA11 correlation analysis. N= 219; 0-5 ng/ml C-peptide, N= 93; 5-10ng/ml C-peptide, N= 21; 10-15ng/ml C-peptide, N= 31; 20+ng/ml C-peptide. Data identified 4 proteins correlated with plasma C-peptide concentration. Significance was determined using one way ANOVA with Tukey's post hoc test. **P<0.01, ***P<0.001, ****P<0.0001 vs. 0-5ng/ml C-peptide.

Elevated C-peptide (>9 ng/ml) is characteristic of severe insulin resistance. There were a total of 32 proteins capable of distinguishing patients with a plasma C-peptide of >9 ng/ml. Reduced C-peptide (<3 ng/ml) is indicative of severe β-cell

dysfunction and 68 proteins were characteristic of such individuals (Diagram 5.6). There were only 2 proteins highly significantly associated with a C-Peptide greater than 9 ng/ml (P< 0.01); MGMT (P< 0.001) and Zinc Finger and BTB Domain Containing protein 16 (ZBTB16, P<0.01). There were many highly significant proteins changed in patients with C-Peptide lower than 3 ng/ml; GH (P<0.0001) and IGFBP-2 (P<0.0001) were the most significant (Table 5.11).

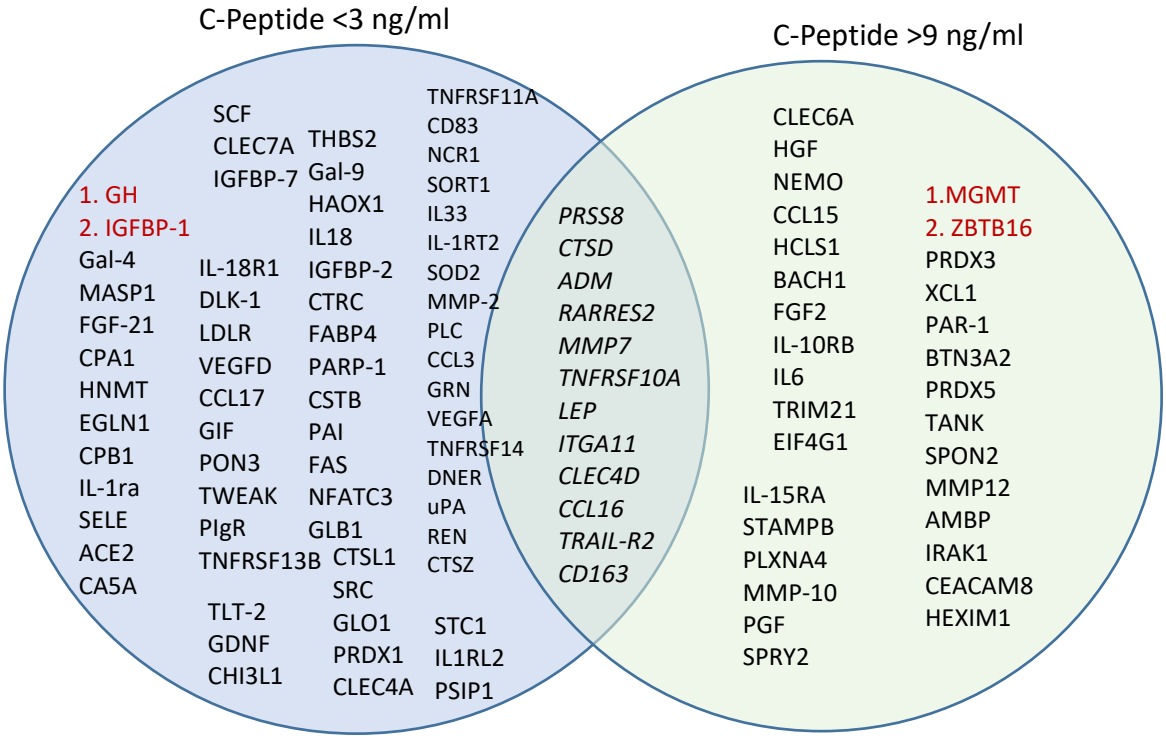


Diagram 5.6 – **Venn diagram of significantly changed proteins associated low and high plasma C-peptide levels. Top two are in red.**

C-Peptide <3 ng/ml			C-Peptide >9 ng/ml		
Protein	P-value	Trend	Protein	P-value	Trend
GH	0.0001	↑	*MGMT*	0.001	↑
IGFBP-1	0.0001	↑	*ZBTB16*	0.01	↑

Table 5.11 – **Top significantly changed proteins associated with high and low C-Peptide levels.** N= 101 C-peptide <3ng/ml, N= 204 C-Peptide >3<9ng/ml. N= 59 C-Peptide >9ng/ml. Significance determined using Student-T test vs. healthy range (3><9ng/ml) plasma C-Peptide.

5.3.12 Proteins associated comorbidity and comorbidity type

Chymotrypsin C (CTRC) was the only assayed protein significantly associated with the presence of any comorbidity (Fig. 5.17A, P<0.05-P<0.001). Linear regression analysis showed CTRC negatively correlated (R=-0.62, P0.001, Fig. 5.17B) with comorbidity number (P<0.05 – P<0.001).

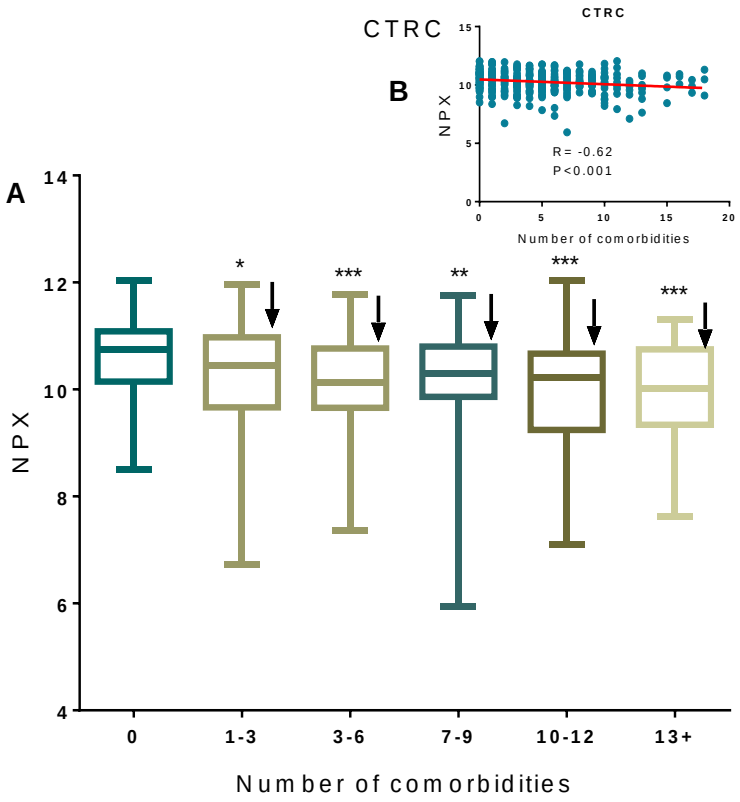


Figure 5.17 – **CTRC protein reduced in the presence of comorbidity.** (A) CTRC box and whisker plots (B) CTRC linear regression. N= 75; 0 comorbidities. N= 88; 1-3 comorbidities. N= 98; 3-6 comorbidities. N=56; 7-9 comorbidities. N=35; 10-12 comorbidities. N=22 13+ comorbidities. Significance was determined using one-way ANOVA with Tukey's post hoc test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 v 0 comorbidities.

There were nine proteins (Table 5.12) associated uniquely with 1-3 comorbidities in T2D patients. CDSN (P<0.05) and TGF-alpha (P<0.05) were most significant. There were 17 proteins significantly changed in patients with 13 + comorbidities (Table 5.12), TNF Receptor Superfamily Member 10a (TNFRSF10A, P<0.01) and HGF (P<0.01) were the most significant. Both were increased, although HGF was much more abundant in plasma (average NPX 8.5).

1-3 Comorbidity			13+ Comorbidity		
Protein	P-value	Trend	Protein	P-value	Trend
CDSN	0.011	↑	*TNFRSF10A*	0.004	↓
TGF-alpha	0.015	↑	*HGF*	0.005	↓
MMP-10	0.016	↓	*IL12RB1*	0.008	↓
REN	0.021	↓	*KIM1*	0.012	↓
ADAM-TS13	0.024	↓	*LPL*	0.021	↓
CLEC4C	0.024	↑	*IL-17A*	0.023	↓
SCF	0.035	↑	*CLEC4D*	0.028	↓
PRSS8	0.040	↓	*IL-18R1*	0.028	↓
CXCL12	0.042	↓	*CD28*	0.031	↓
			CTSZ	0.033	↑
			AMBP	0.037	↓
			VSIG2	0.038	↓
			THBS2	0.040	↓
			TRAF2	0.041	↑
			AGRP	0.042	↑
			OPG	0.046	↓
			PGF	0.048	↓

Table 5.12 – **Proteins associated with 1-3 (few) and 13+ (many) Comorbidities.** N=75 0 comorbidities. N=88 1-3 comorbidities. N= 22 13+ comorbidities. Significance determined using Student-T test vs. 0 comorbidities.

5.3.12.1 Clustering of comorbidity in diabetes

Patients were clustered by 3 comorbidity groups. The first was classic secondary diabetes complications, which included cardiovascular disease, Endocrine and nutritional disorders, disorders of the eye, genitourinary disorders. The second was other diabetes related conditions which included digestive and musculoskeletal disorders. The final group was patients that have clinically diagnosed depression. There were 118 proteins unique to micro and macrovascular disease patients. The top 20 are shown in Diagram 5.7. There were 20 proteins specific to 'other diabetes' related conditions and 5 proteins indicative of depression (Diagram 5.7).

The six most significant proteins for each group are shown in table 5.13. pro-Brain Natriuretic Peptide (NT-proBNP) and NCR1 were most strongly and uniquely associated with classic diabetes complications ($P<0.0001$), both were increased in the presence of disease. Glyoxalase I (GLO1) and Osteoclast-associated immunoglobulin-like receptor (hOSCAR) were specially increased in 'other diabetes related conditions' (digestive & musculoskeletal) ($P<0.05$) and TNFSF14 and HGF were increased in depressed patients ($P<0.05$).

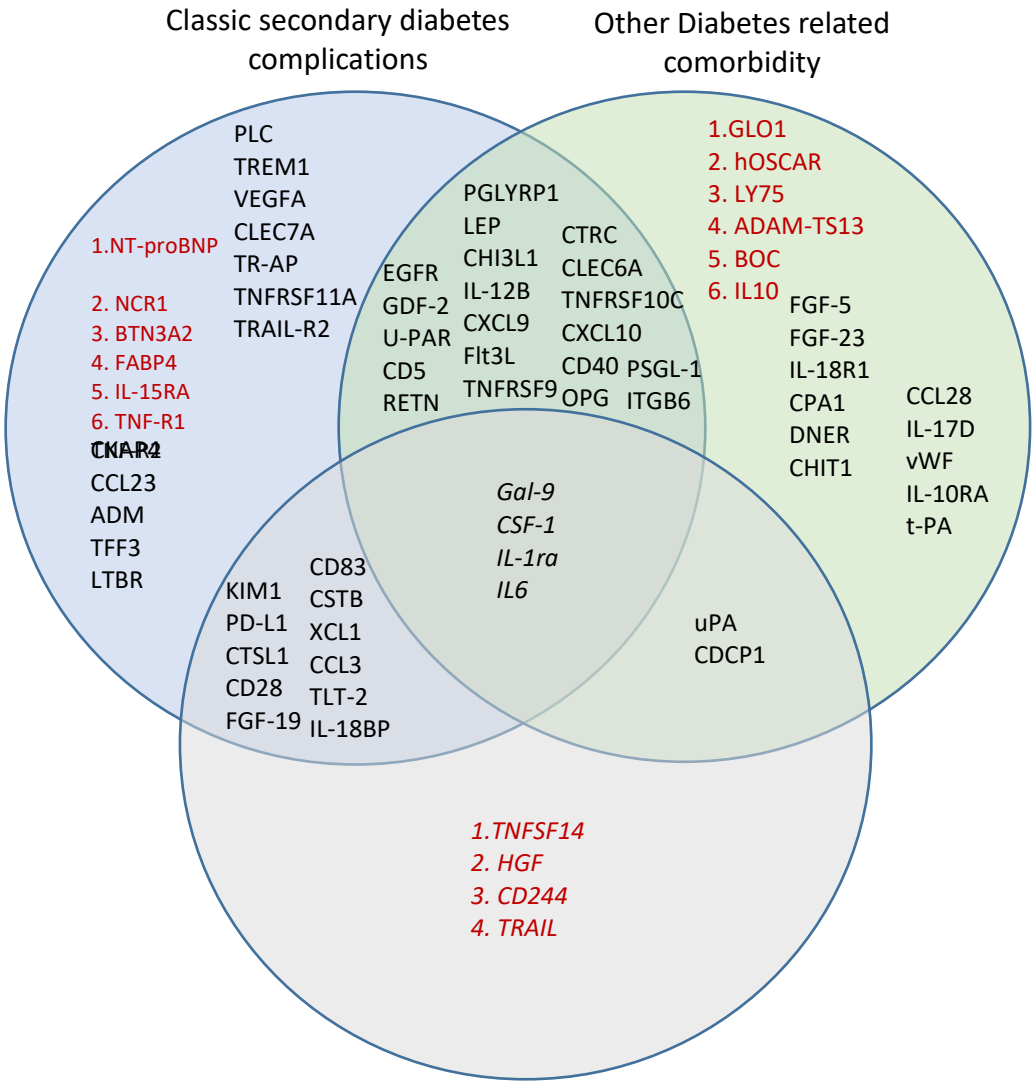


Diagram 5.7 – **Venn diagram of proteins associated with classic diabetes complications (Micro & Macrovascular comorbidity, other diabetes comorbidities (digestive and musculoskeletal disorders) and clinically diagnosed depression**; top 20 proteins associated with classic secondary diabetes complications included, remaining 98 included in Supplementary Table 3).

Comorbidity								
Micro & Macrovascular			Other diabetes related			Clinically diagnosed depression		
Protein	P-value	Trend	Protein	P-value	Trend	Protein	P-value	Trend
NT-proBNP	0.000	↑	*GLO1*	0.050	↑	*TNFSF14*	0.050	↑
NCR1	0.000	↑	*hOSCAR*	0.050	↑	*HGF*	0.050	↑
BTN3A2	0.000	↑	*LY75*	0.050	↑	*CD244*	0.050	↑
FABP4	0.000	↓	*BOC*	0.050	↓	*TRAIL*	0.050	↓
IL-15RA	0.000	↑	*IL10*	0.050	↓			
TNF-R1	0.000	↑	*ADAM-TS13*	0.050	↑			

Table 5.13 – **Top six proteins associated with micro & macrovascular disease, other diabetes related comorbidity and clinically diagnosed depression.** N=154 micro & macrovascular disease (-), N=220 micro & macrovascular disease (+). N=259 other diabetes related comorbidity (-), N=115 other diabetes related comorbidity. (+). N= 331 depression (-), N=43 depression (+). Significance determined using Student-T test vs. participants without related comorbidity

5.3.13 Proteins associated GLP-1 analogue treatment response with respect to glycemic control and body weight

There were 17 proteins unique to GLP-1Ra prescribed patients with poor response defined in this case as those with elevated HbA_{1c} (>54 mmol/mol). ITGA11 was the most significant and was the only protein uniquely changed (reduced) when compared to GLP-1Ra (-) patients (Fig. 5.18, P<0.01-P<0.001)

There were 13 proteins associated obesity (>30 Kg/m^2) in GLP-1Ra prescribed patients, again taken as an indicator of poor response. Ep-CAM was the most significant and the only protein specifically changed (increased) in GLP-1Ra (+) patients compared to GLP-1Ra (-) patients (Fig. 5.19, P<0.05-P<0.01).

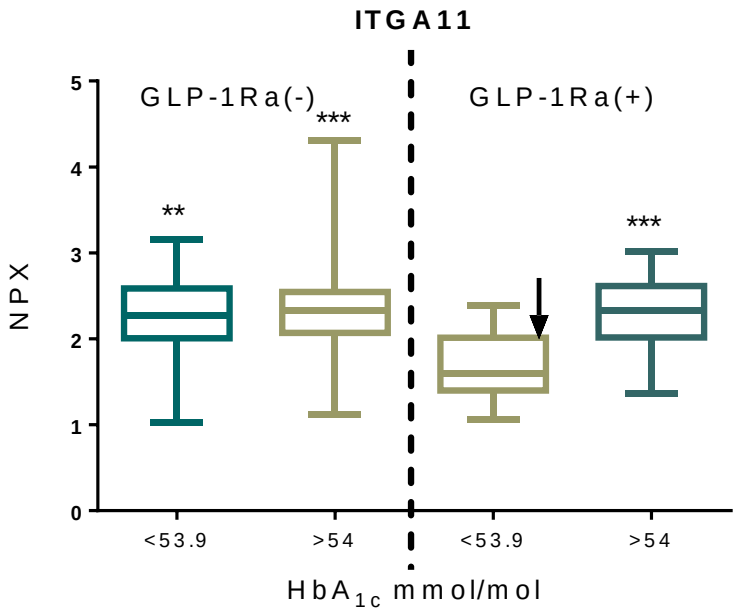


Figure 5.18 – **ITGA11 is associated with good glycaemic control in GLP-1Ra positive patients.** Protein expression was determined using OLink assays and normalised to in house OLink control. N= 112 GLP-1RA (-) HbA1c < 53.9. N= 191 GLP-1Ra (-) HbA1c >54. N= 9 GLP-1RA (+) HbA1c < 53.9. N= 50 GLP-1Ra (+) HbA1c >54. Data presented as box and whisker plots (min, mean, max). Significance was determined using one-way ANOVA with Tukey's post hoc test. **P<0.01, ***P<0.001, vs. GPL-1(+) HbA1c <53.9.

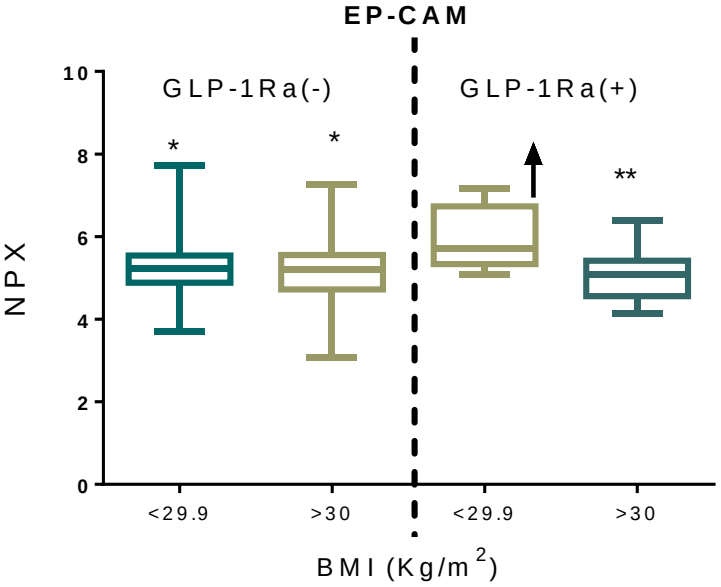


Figure 5.19 – **EP-CAM is associated with lower BMI in GLP-1Ra positive patients.** Protein expression was determined using OLink assays and normalised to in house OLink control. N= 63 GLP-1RA (-) BMI < 29.9. N= 131 GLP-1Ra (-) BMI >30. N= 9 GLP-1RA (+) BMI < 29.9. N= 50 GLP-1Ra (+) BMI >30. All data presented as box and whisker plots (min, mean, max). Significance was determined using one-way ANOVA with Tukey's post hoc test. *P<0.05, **P<0.01, v GLP-1 (+) BMI <29.9.

Complication	Pathway	Source	P-Value
GLP-1Ra *Glycaemia*	HIF-1-alpha transcription factor network	*NCI-Nature 2016*	0.04
GLP-1Ra *Obesity*	Integrin family cell surface interactions	*NCI-Nature 2016*	0.0001

Table 5.14 – **Pathway enrichment analysis for proteins associated with poor HbA_{1c} and obesity control in GLP-1Ra prescribed patients.** Data enrichment was conducted on integrative web based software application Enrichr (Chen et al., 2013). All data was inputted and ranked for level of membership according to Enrichr protocol (N= 17 proteins). Pathways identified via Nature Pathway Interaction Database. All significantly associated pathways are shown ($P<0.05$).

Pathway enrichment analysis (Enrichr) (Chen et al., 2013) identified GLP-1Ra prescribed patients with poor glycemic control had altered Integrin family cell surface interactions (Table 5.14) and obese GLP-1Ra prescribed patients had dysregulation HIF-1-alpha transcription factor network (Table 5.14).

5.4 Discussion

In this study we used a non-targeted proteomic approach to discover new risk markers associated with T2D, HbA_{1c}, BMI, blood lipids, anti-diabetes drug prescription, C-peptide and 6 comorbid conditions previously identified in the DiaStrat cohort (*Chapter 4*). Pathway enrichment analysis was also conducted. Previous research using this group of patients (*Chapter 4*) and other meta-analysis have emphasised the importance of our chosen variables in T2D management and stratification (Wilding, 2014). This analysis has been designed to efficiently identify proteomic targets in areas that could inform best treatment and management practices.

There were 55 proteins that differentiated T2D patients compared to non-diabetes controls; the top 8 proteins were each capable of distinguishing between disease with a reasonable accuracy (AUC ~71%). PRSS8, Hsp27 and MMP7 are involved in the inflammatory response (Uchimura et al., 2014, Nahomi et al., 2014, Ayuk et al., 2016) and CDCP1 is involved in cell adhesion and endothelial dysfunction (Law et al., 2016). These processes can be impaired by PAI and HGF, both of which are catabolic proteins involved in angiopathy and cardiovascular disease (Lyon and Hsueh, 2003, Konya et al., 2014), and were altered in T2D in the present study.

Enrichment analysis using all 55 proteins revealed Syndecan-1-mediated signalling as a common factor. Previous research has found syndecan-1 to be a reliable marker for endothelial glycocalyx degradation (Johansson et al., 2011). Protein levels have been found to correlate directly with endothelial damage, hyperfibrinolysis and inflammation (IL-6 and IL-10) (Stensballe et al., 2009). Other studies have proposed degradation of endothelial glycocalyx advances clot formation and protein C depletion (Rahbar et al., 2015), resulting in increased hyperfibrinolysis (Davenport et al., 2017) and ultimately endothelial cell death (Zouaoui Boudjeltia et al., 2012). This process would have a significant impact on a diabetes patient, resulting in loss of vascular integrity and ultimately organ damage (Lin et al., 2007). Syndecan-1 signalling has not been explored in the context of diabetes but could be a good marker for vascular damage (Clua-Espuny et al., 2017). Interestingly, Fibroblast growth factor 5 (FGF5) was the most significantly altered protein in long suffering diabetes patients with disease duration in excess of 20

years. This growth factor is involved in angiogenesis and would healing (Seo et al., 2016). A number of studies have implicated FGF5 pathogenic variants in diabetes, hypertension (Xi et al., 2014) and dyslipidaemia (Li et al., 2015b). This study supports findings from other studies that have identified FGFs as being directly linked to diabetes duration (Pena et al., 2016). Decorin was the only other protein associated with an long duration diabetes, and interestingly is also involved in endothelial integrity (Davies et al., 2001). Decorin is a proteoglycan that interacts with VEGFR2 to increase Paternally expressed 3 (Peg3) (Buraschi et al., 2013). This increases endothelial cell autophagy and inhibits angiogenesis (Neill et al., 2017). Other research has linked increased levels of Decorin to obesity and CVD onset (Bolton et al., 2008). Measuring this protein, together with other with syndecan-1 signalling components, could provide novel vascular endothelial cell markers indicative of vascular integrity and diabetes progression.

Increased BMI (>24.9Kg/m^2) in diabetes was found to be associated with 10 proteins. As expected plasma leptin levels and PTPB1 signalling are most significantly affected (Feng et al., 2013a). There were also highly significant differences in IL-18, IGFBP-2, contactin 1 and MMP3 levels. IL-18 is a proinflammatory cytokine (Osborn et al., 2008), and well researched with respect to cardiovascular death and plaque formation (Libby, 2006). It is mainly produced by macrophages (Pirhonen et al., 1999), but recent work has implicated adipose tissue as a contributing factor (Wood et al., 2005). Hyperglycaemia has also been positively correlated with circulating levels of IL-18, in normal and diabetes patients with impaired glucose tolerance (Esposito et al., 2002). It is currently a validated measure of endothelial integrity and atherosclerosis (Straface et al., 2010), but has potential to be used as a characteristic marker of obesity. Insulin growth factor (IGF) proteins regulate migration and tube formation of vascular endothelial cells (Nakao-Hayashi et al., 1992). IGF-1 has been shown to affect atherosclerosis (Thum et al., 2007) and insulin sensitivity (Nam et al., 1997). IGFs are regulated by IGF binding proteins (IGFBP) (Monzavi and Cohen, 2002). IGFBP-2 was reduced in overweight patients in the present study, this supports other work that has shown the sensitivity of this molecule to insulin (Nam et al., 1997) and that it is directly correlated with insulin resistance (Frystyk et al., 1999).

Enrichment analysis implicated hypoxia-inducible factor (HIF) 1α signalling with the proteins in the obese patient cluster. HIF is regulated by cellular 0_2 (Wang et al., 2005b), however it is now known that insulin and IGF can induce expression (Feldser et al., 1999). HIF-1α increases IGFBP-2 expression (Tazuke et al., 1998), but in diabetes and obesity, insulin signalling is severely impaired (Spolcova et al., 2014); this would impact IGFBP-2 (Shin et al., 2017) and downstream IGF mediated angiogenesis (Nakao-Hayashi et al., 1992). The fact that MMP3 is also reduced further characterises the cohort as high vascular risk. MMP3 has been shown to have a negative regulatory role in adipocyte differentiation and low levels increase fat mass (Wu et al., 2017b). This would exacerbate cardiovascular risk (Burke et al., 2008). Any direct relationship or interaction between IL-18, IGFBP2 and MMP3 has yet to be defined.

There were 30 dysregulated proteins in patients with a HbA_{1c} >53<80 mmol/mol and in patients with a HbA_{1c} >80 mmol/mol (9.5%), but only 7 proteins were common between the two groups. Kidney injury molecule-1 (KIM1) was the most significant. It is thought to move into circulation as elevated glucose damages the kidney (Vos et al., 2012a). KIM1 is a sensitive and specific biomarker for chronic kidney injury (Sabbisetti et al., 2014), and levels are thought to be representative of altered actin cytoskeletons in renal microvascular endothelial cells. This cellular pathology ultimately reduces cell adhesion (Sutton et al., 2002). To date there is no research correlating KIM1 with HbA_{1c}. Enrichment analysis highlighted a pathway associated with endothelial integrity and cellular adhesion. Alterations in integrin family cell surface interactions were identified as being a major characteristic of poor glycaemia. Pre-clinical studies have shown that integrins function as adhesion receptors for extracellular ligands, transmitting biochemical signals into the cell (Michie et al., 1998). More than 150 proteins interact with integrin binding sites (Liu et al., 2000). Key insulin signalling proteins PI3K, AKT and MAPK are controlled by integrins (Delcommenne et al., 1998). These proteins are essential for maintaining insulin sensitivity and glucose homeostasis (Elghazi et al., 2006). Interestingly extremely poor glycaemic control (HbA_{1c}- 80 mmol/mol, 9.5%) was characteristic of an alternate pathway, the uPA and uPAR signalling cascade. This cascade is activated in times of high glucose (Flores-López et al., 2016) and one of the main components

of the uPA/uPAR system is MMP9 (Kong et al., 2007). This enzyme increases endothelial cell damage and vascular permeability, and is a leading cause of vascular pathology (Kowluru and Mishra, 2017). It is notable that MMP9 was non-significantly increased in patients with a HbA_{1c} >53 mmol/mol. Studies have indicated that in those without diabetes this protein is at very low levels and a high level is indicative of pathogenesis (Ayuk et al., 2016).

There were only 3 common proteomic targets; SCF, GAL-4 and TNFRSF13, universally changed in patients with alterations in HDL, LDL and total cholesterol. Alterations in HDL (>1mmol/l) had the greatest effect on plasma proteins; a total of 81 were significantly changed, compared to 32 in LDL (>2 mmol/l) and 30 in elevated total cholesterol (>4 mmol/l) patients. This is interesting as elevated HDL is thought to be protective, opposite in nature to LDL and cholesterol (Lim, 2017). Generally plasma HDL concentration correlated best with SCF, PON3, LPL and LDL receptor protein levels, when compared to proteins detected in elevated LDL and total cholesterol patients. Molecular pathway activity was different dependent on what aspects of a patients blood lipids were elevated. This is not surprising as HDL, LDL and total cholesterol have different pathophysiological effects (Batiste and Schaefer, 2002). Interestingly, β1 integrin cell surface interactions, and HIF-1α transcription factor alterations were distinctive of altered HDL and these pathways were also characteristic of poor glycaemia (>53 mmol/mol). There is a close link between blood lipoprotein abnormalities and diabetes (Shishino et al., 2007), and is probably why there is a degree of overlap. HDL concentration can differentially control integrin function and cell motility (Pan et al., 2012). Research has shown HDL regulates β1 integrin recycling, cell adhesion and migration, having a significant impact on vascular integrity (Camont et al., 2011).

Diabetes regime intensification is characteristic of poor pharmacological response, high HbA_{1c} and long diabetes duration according to NICE (NICE, 2015). Data from our previous work showed this is not always the case and intensification and drug prescription occurs on an individual basis. Peripheral proteomic changes are poorly described during diabetes regime intensification. This work identified 3 proteins that were negativity correlated with the number of prescribed diabetes medications; Sortilin (SORT), Caspase-3 (CASP3), and CD84. SORT has recently been

identified as a novel molecular link between insulin resistance (Li et al., 2015a), Apolipoprotein B production and atherosclerosis (Strong et al., 2012). SORT inhibits Apolipoprotein B, lowering plasma cholesterol (Dubé et al., 2011), but during states of insulin resistance is degraded (Li et al., 2015a). It is thought SORT activity is dependent on PI3K activity (Li et al., 2017), and due to its sensitivity to insulin resistance (Li et al., 2015a) could be a good marker to inform diabetes prescription. CASP3 has been shown to be a marker of β-cell apoptosis (Liadis et al., 2005), high levels are indicative of a low β-cell population. It is thought this marker could inform on whether medications that target the β-cell would be effective (Holz et al., 1993). CD84 is part of the signalling lymphocyte activation molecule (SLAM) family (Romero et al., 2004) and is involved in the adaptive immune response (Cannons et al., 2010). There have been indications that SLAM proteins could be involved in the autoimmune destruction of βcells (Dufour et al., 2008), and that this could be occurring in T2D as well as T1D (Velloso et al., 2013). This area of research is poorly described and only recently being explored but could provide an explanation as to why CD84 is predictive of diabetes medication intensification.

Proteomic profiles were generated for all classifications of anti-diabetes drugs (Biguanides, Dipeptidyl peptidase-4 inhibitors (DDP-IVi), Sodium-glucose Cotransporter-2 inhibitors (SGLT2i), Sulphonylureas (SU), Thiazolidinediones (TZDs), Glucagon-like peptide-1 receptor agonists (GLP-1Ra) and insulin. This type of profiling could aid in screening for patients that are responsive or tolerant to a particular medication. An interesting finding was that there were not any proteins (368 total) common to all classes. Although, from a mechanistic perspective this may not be surprising, all have different physiological effects. Metformin decreases hepatic glucose production (Liang and Giacomini, 2017), DDP-IVi increases the half-life of GLP-1 (Bourdel-Marchasson et al., 2011), SGLT2i reduce glucose reabsorption in the kidney (Steen and Goldenberg, 2017), SU (Kuhn, 1988) and GLP-1 increase insulin secretion from the β-cell (Bourdel-Marchasson et al., 2011) and TZDs increase insulin sensitivity by impacting lipid metabolism (Sengupta et al., 2012). Insulin signaling signals tissues to take glucose from the blood (Wilcox, 2005). GLP-1Ra and insulin prescribed patients both had dysregulated Carboxypeptidase A1 (CPA1) and Carboxypeptidase B1 (CPB1). It is known that CPA1 positive cells have the capacity to

generate numerous types of pancreatic cells, including β-cells (Pagliuca and Melton, 2013), and reductions indicate compromised β-cell populations. Both medication groups can directly affect the β-cell but characteristically have opposite expression levels (Buteau, 2011, Linden and Brooker, 1978). CPA1 and CPB1 were increased in GLP-1Ra patients and decreased in insulin dependent patients. CPA1 and CPB1 could be promising targets to quantify β-cell function, and warrant further research in this regard.

We reported in Chapter 4 that the DiaStrat cohort has poorly managed glycaemia, and ~40% were prescribed insulin. In this study we identified that insulin treated patients have elevated plasma GH and FABP2. Other work has shown that GH neutralises the effect of insulin on glucose and lipid metabolism (Clemmons, 2004), decreasing oxidation and supressing glucose uptake into muscle (Møller et al., 1991). FABP2 is widely associated with increased fat oxidation and severe insulin resistance (Xu et al., 2016). This proteomic profile would indicate that insulin prescribed patients may not benefit from insulin appropriately due to high levels of inhibitory proteins. Other pharmacological approaches may be more effective.

Establishing measures of insulin resistance is of high clinical significance for effective prescribing (Church and Haines, 2016). To date, fasting blood glucose tests or homeostatic model assessment (HOMA) have been used (McAuley et al., 2001), but are time consuming and labour intensive. In this study we measured plasma C-peptide and classified patients according to other published work (Radaelli et al., 2010). A diabetic patient with a plasma C-peptide level >9 ng/ml was said to be insulin resistant. The levels of 4 proteins were found to directly correlate with C-peptide; Angiotensin-converting enzyme (ACE) was most strongly associated. This enzyme is a potent vasodilator and causes increased vascular permeability, and oedema (Fang et al., 2014). IL-18 and LEP were also positively correlated with increasing C-peptide, as well as obesity, supporting the established links between obesity and insulin resistance (Patel and Abate, 2013).

There were 32 proteins specifically linked to severe insulin resistance, indicated by elevated C-peptide levels (>9 ng/ml C-peptide). MGMT and ZBTB16 were most significant. MGMT is essential for genome stability and dysregulation is a well-established risk in many cancers (Sharma et al., 2009). Clinical studies have

implicated MGMT dysregulation in leukocytes as a contributing factor to various immune related diseases (Akçay et al., 2003). It is known that if the immune system cannot respond to inflammation, tissues become unresponsive to insulin and fat cells release more fatty acids into the blood which can compromise the vasculature leading to CVD (Lumeng, 2013). ZBTB16 is involved in lipid metabolism and increased levels have been correlated with decreased body fat storage, reductions overall bodyweight and glucose oxidation (Plaisier et al., 2012).

There is a link between insulin resistance, hyperglycaemia and comorbidity (Castro et al., 2014). Current diabetes measures such as HbA_{1c} are associated with a number of micro and macrovascular complications as well as increased mortality (Kranenburg et al., 2015), but HbA_{1c} levels do not necessarily correlate with the number of comorbidities a patient may suffer from, rather stratifies them as high or low risk. A single plasma proteomic marker was identified that is strongly negatively correlated with the number of comorbidities in T2D patients. Chymotrypsin C (CTRC) decreased with increasing comorbidity; a protein directly linked to chronic pancreatitis and the destruction of insulin producing cells (Rosendahl et al., 2008). CTRC regulates trypsin, which when cleaved in the pancreas ultimately causes pancreatic self-digestion (Afghani et al., 2015). CTRC declines with increasing physiological stress. This potentiates the need for insulin therapy due to the onset of chronic pancreatitis

N-terminal prohormone of brain natriuretic peptide (NT-proBMP) and NCR1 were uniquely increased in macrovascular (CVD) and microvascular (nutritional diseases, disorders of the eye, genitourinary disorders) disorders. Globacom-1 (GLO1) and hOSCAR were specifically increased in other diabetes related comorbidities such as digestive and musculoskeletal disorders. Interestingly none of these proteins were increased in clinically diagnosed depression patients and there were no proteins common to all comorbid conditions. Depression and cognitive decline are twice as common in diabetes patients (Holt et al., 2014), and are now considered diabetes complications (Bădescu et al., 2016). Few studies have explored how plasma proteins are changed in depression and diabetes patients, but recent work has identified key lipid biomarkers; cholesterol, omega 3 and omega 6 fatty acids was being potential markers of major depressive disorder (Parekh et al., 2017).

There were 11 proteins alterations shared between depression and macrovascular/ microvascular conditions and only 2 proteins linking depression to other diabetes related conditions, indicating that macrovascular complications are more highly associated with depression. Kidney Injury Molecule-1 (KIM1) and PD-L1 were most strongly linked. KIM1 is associated with CKD and vascular endothelial dysfunction (Panduru et al., 2015) which is caused by increased blood glucose and LDL (Atkins, 2005). Many studies have shown KIM1 to be a reliable marker for low eGFR reads, and highly predictive of nephropathy (Panduru et al., 2015). Microvascular complications are correlated with longer duration diabetes and old age which are risk factors for depression (Iglay et al., 2017). The low grade proinflammatory state associated with vascular disease could also perpetuate a depressive state (Bădescu et al., 2016). KIM1 may therefore be predictive of depression risk.

Programmed-death ligand 1 (PD-L1) is an interesting novel marker for cardiometabolic health and depression. PD-L1 is primarily thought to be a regulatory protein involved in the adaptive immune response; activation reduces proliferation of cytotoxic and helper T-cells (Shi et al., 2013). Studies have shown that alterations in PD-L1 and T-cell activation can negatively affect β-cell populations, causing insulin deficiency and insulin dependence (Wang et al., 2008), leading to hyperglycemia and vascular endothelial damage (Wongpiyabovorn et al., 2008). PD-L1 is poorly described with respect to diabetes and depression and further work is required to determine how and why PD-L1 is increased in depression and CVD patients.

The UK Prospective Diabetes Study (UKPDS) and Diabetes Control and Complications Trial (DCCT) stressed the importance of suitable and timely introduction of insulin (American Association of Diabetes, 2002). Introducing insulin too early can present the patient to unnecessary risk associated with treatment, but too late and the patient will be exposed to comorbidity (Gale, 2008). GLP-1Ras are the only other injectable anti-diabetes medication and they primarily signal via c-AMP to mediate insulin secretion (Holz, 2004), but have also been shown to have significant effects on insulin sensitivity and weight loss (Vilsbøll et al., 2008). They are an attractive anti diabetes treatment option and may be more suitable than insulin in some instances. Unfortunately, they are expensive and are thought to have approximately a 50% response rate (Iepsen et al., 2015b). This study aimed to

characterise poorly managed blood glucose and bodyweight in GLP-1Ra prescribed patients, indicative of poor response. There was only one protein; ITGA11, specifically decreased in GLP-1Ra prescribed patients, with well managed blood glucose (<53 mmol/mol). Pathway enrichment analysis identified alterations in integrin cell surface interactions as being a key characteristic of all the altered proteins in GLP-1Ra prescribed patients. This corresponds with earlier data integrating the whole cohort which also identified integrin alterations as being of important during high glycaemia. Interestingly the proteomic markers involved are different in GLP-1Ra prescribed patients, ITGA11, VEGFD, ITGB2, PD-42 were the most significant. These proteins are involved in vascular integrity rather than mediating signals involved in insulin signalling. ITGA11 is involved in cardiac fibrosis progression and upregulation has been linked to heart failure (Talior-Volodarsky et al., 2012). VEGFD is associated with angiogenesis and endothelial cell growth (Girling and Rogers, 2009). This profile indicates GLP-1Ra treated patients with poor glycaemic control have a more compromised vascular system than those with good glycaemic control. Although GLP-1Ra treated patients represent are a more severe phenotype than the remainder of the cohort, global measurement of these markers could provide useful information on vascular endothelial cell function and may be indicative of likelihood to derive benefit from GLP-1 analogues

Obese (>30 Kg/m^2), GLP-1Ra treated patients could be distinguished from GLP-1Ra negative patients by Ep-CAM. Pathway enrichment analysis using the most significantly changed proteins in obese GLP-1Ra (+) patients indicated these patients characteristically had altered HIF-1α transcription factor signalling. This pathway was also significantly altered in obese diabetes patients in general (whole cohort). The most significantly affected protein in GLP-1Ra prescribed patients was Ep-CAM, which was reduced in the cohort as a whole, although to a lesser extent. This would indicate that BMI has a significant effect on Ep-CAM whose primary function is cell adherence and promoting cell-cell interactions (Schnell et al., 2013). *In vitro* Ep-CAM has been found to be a key mediator of cell proliferation, differentiation, and cell migration (Litvinov et al., 1996), these characteristics are key for vascular tissue development (Hedin et al., 2004). Ep-CAM has a close relationship with HIF-1α in cancer (Yamada et al., 2014), so it is possible they interact in diabetes, although

currently the molecular mechanisms are unclear. Pathway enrichment analysis indicates a relationship and it is known HIF-1α is involved in angiogenesis via IGFBP-2 (Feldser et al., 1999).

Limitations

This analysis has several limitations, the most significant being the modest sample size and small number of healthy controls included in analyses. Another major limitation was that all proteomic measurements were obtained from one time point and therefore no conclusions can be drawn with respect to individual drug response and comorbidity onset and progression. Resampling the patients and reassessing the same protein panels would provide useful longitudinal information on how key proteins respond to specific weight changes, pharmacological interventions and disease progression. Vulnerabilities in accurate reporting of comorbidity due to collation of data from a number of healthcare providers by various professionals within the ECR cannot be ignored. DiaStrat participants were recruited from secondary care and likely represent a severe diabetes phenotype. Few participants had a low or normal BMI or HbA_{1c} therefore changes associated with treatment and comorbidity were minimal.

Conclusion

In the present study we have identified numerous proteins characteristic of T2D, HbA_{1c} and BMI GLP-1Ra 'response'. We also identified proteins associated with alterations in blood lipids (HDL, LDL, total cholesterol), diabetes treatment regime, and comorbidity progression. Interestingly there was commonalty between proteomic markers and their associated pathways. Vascular endothelial damage and alterations were a common feature between variables. This type of pathology is well documented in diabetes (Avogaro et al., 2011) and ultimately results in endothelial cell swelling, necrosis and progressive tissue damage (Sena et al., 2013). Follow up validation studies will determine the utility of the proposed panels of markers to help characterise and better inform clinical practice on efficient and effective management of T2D.

Chapter VI

SNPs associated with T2D and response to GLP-1 analogue therapy in the DiaStrat cohort

Abstract

Single nucleotide polymorphisms (SNPs) have become the genetic markers of choice and many loci are associated with T2D onset and progression. Studies have shifted to identifying markers of drug response. The aim of this study was to define variants on 20 genes (GLP-1R, GIPR, INSR, IRS-1, IGFBP2, AKT, MAPK1, MTOR, LEP-R, STAT3, SH2B1, POMC, IL-6, IL-10, IL-18, IL-1RN, HGF, HAVCR1, SORT1, CASP3) related to insulin and leptin signalling in patients with T2D vs. controls, and in T2D GLP-1Ra responders vs. non-responders.

Genotyping was conducted using the Affymetrix UK Biobank array (CGS, UK). A total of 2489 SNPs were identified across all genes; 105 were indicative of T2D, and 25 of response to GLP-1Ra therapy. Rs140868873 (IRS1) was specific to T2D; 100% of controls had the AB genotype while 99.6% of T2D patients had the BB genotype. Rs117029769 (IGFBP2) was also specific to T2D (control 89.5% AB; T2D 97.2% BB, P<0.0000). In Rs72881029 (LEP-R) the AB genotype was associated with 100% of controls, while 97.6% T2D patients had the BB genotype (P<0.0000). In Rs11085808 (HGF) 100% of controls had the AB genotype while 100% of T2D patients had the BB genotype (P<0.0000).

T2D GLP-1Ra responders had different SNP genotypes to non-responders in a number of genes. In Rs71480142 (GLP-1R), 95.5% of non-responders had the AB genotype, while 87.7% of responders had the BB genotype (P<0.0000). In Rs115516351 (mTOR) the AA genotype was present in 100% of responders while the BB genotype was more prevalent in non-responders (63.6%, P<0.0000). In Rs72881029 (LEP-R) the AB genotype was associated with 100% of responders and 40.9% in non-responders (P<0.0000). SNPs were associated with inflammation and immunity in IL-6, IL-10, IL1RN and IL-18 were also identified but were less predictive than those associated with insulin and leptin signalling.

Many SNPs identified in this work are supported on the T2D knowledge portal, and some are novel. The highly specificity of SNPs implicated in response to GLP-1 analogue therapy suggests that GLP-1Ra response may be predicted by SNP genotyping. The findings reported herein will be validated in secondary cohorts.

6.1 Introduction

In clinical practice, guidance is provided by NICE to manage T2D in a stepwise protocol driven manner (NICE, 2015). Recent data and new advice from the American Diabetes Association has prompted a move away from this approach to more patient specific management plans (Chamberlain et al., 2017a). These are directly influenced by individual signs, symptoms, drug characteristics and possible adverse events (ADA, 2016).

Ensuring phenotypic homogeneity is important for effective treatment plans (Inzucchi et al., 2015), but studies have shown drug response is highly variable irrespective of accurate phenotype classification. Research has highlighted genetics as being a major causal factor of response variation (Arar et al., 2008). Response to metformin with respect to glycaemia has recently been shown to have a heritability figure of ~34%; therefore drug response is, in part, predicated by genetics (Zhou et al., 2014a).

The effectiveness of a drug is dependent on its ability to reach its site of action in a high enough concentration to produce an effect (Zhou et al., 2016). Pharmacogenomics has predominantly focused on genes implicated in drug transport and metabolism to this regard (Daniels et al., 2016). Single Nucleotide Polymorphisms (SNPs) in genes involved in transport and metabolism affect drug action and a patients propensity to experience side effects (Holstein and Beil, 2009). In T2D metformin and SUs have been widely studied. Metformin is a first line therapy in T2D, but it is often insufficient as a monotherapy at controlling the condition (Mahrooz et al., 2015). Recently genetic variation in Organic Cation Transporter 1 (OCT1) gene has been shown to have a significant effect on metformin tolerance and response (Shu et al., 2007). OCT1 is involved in metformin metabolism in the liver (Wu et al., 2017a). It is now known that ~8% of white Europeans carry variants that negatively impact OCT1 action, which ultimately results in patients being at twice the risk of developing a severe intolerance (Dujic et al., 2016). SU are often a second line therapy in T2D (NICE, 2015), and drug action is usually inactivated by the liver enzyme cytochrome P450 2C9 (Dujic et al., 2017). Six percent of the population have a polymorphic version of cytochrome P450 2C9 and cannot

inactivate the SU; such patients are ~4x more likely attain an HbA_{1C} of <53 mmol/mol (7%) (Dujic et al., 2017), and are at a higher risk of hypoglycaemia (Ragia et al., 2009).

The development of low cost genome wide arrays has moved research away from individual candidate gene studies towards multiple gene variant analysis (Scott et al., 2017). This high throughput approach, has a higher utility when the exact mechanism of a drug is uncertain (Volkman et al., 2017). Genetic variants or SNPs are becoming the genetic markers of choice (Akpinar et al., 2017). SNPs are densely and relativity evenly distributed throughout the human genome, most are non-functional present in non-coding regions of the genome (Zhang and Lupski, 2015). Characterisation of pathogenic SNPs is a high priority in precision medicine (Florez, 2017).

Genome-Wide Association Studies (GWAS) are commonly used to assess traits of disease (Chung et al., 2015) but have had limited success with respect to drug response (Pollastro et al., 2015). The most significant success of GWAS studies for drug outcome was the identification of genetic variants on the Solute Carrier Organic Anion Transporter Family Member 1B1 (SLCO1B1) gene (Postmus et al., 2014). This gene encodes for a statin transporter protein, and alterations have been shown to result in a 16x increased risk of statin induced myopathy (Link et al., 2008). Metformin is the only diabetes drug implicated by GWAS data for having a locus that affects response (Zhou et al., 2011). The UK Prospective Diabetes Study (UKPDS) identified a locus on chromosome 11 that altered drug response which was later confirmed in two independent cohorts and replicated in a European and Chinese cohort (van Leeuwen et al., 2012). The locus is identified by RS11212617, and multiple studies have implicated Ataxia Telangiectasia Mutated (ATM) as being the encoded candidate gene (Zhou et al., 2014b). ATM translates a DNA damage protein that is altered in multiple cancers but is also a cause of ataxia telangiectasia (Ambrose and Gatti, 2013). In clinical studies patients with this condition have increased blood glucose and insulin resistance, supporting the notion that ATM has a significant effect on insulin metabolism (Connelly et al., 2016b).

Glucagon-Like Peptide 1 analogues (GLP-1Ra) are a 4^{th} line anti-diabetes treatment (NICE, 2015), and are also licenced as a weight loss therapy in the USA

(Danne et al., 2017). Clinical trials have indicated that these drugs are particularly prone to response variability. It is thought ~50% of patients respond, achieving significant reductions in HbA_{1c} (1%+); some individuals have no effect or display gastrointestinal side effects (Iepsen et al., 2015b). Some of this variability may be contributed to lifestyle and non-compliance, but it is likely that a genetic component is involved (Nuffer and Trujillo, 2015). There is no current pharmacogenomic data in European or any other populations for response to GLP-1Ras. A clinical study in Asia looked specifically at the GLP-1R and failed to correlate any polymorphisms to GLP-1Ra response (Lin et al., 2015). RS10305492, within the GLP-1 receptor, has been shown to protect against high glucose and cardiovascular disease (Wessel et al., 2015). We hypothesise that it is likely that genes related to GLP-1's insulinotropic and appetite regulatory effect (associated with phenotypic alterations in response to therapy) may be important in T2D generally and in the likelihood of response to GLP-1 analogue therapy. To this end variants identified on the Glucose-Dependent Insulinotropic Polypeptide Receptor (GIPR) (Qi et al., 2012), Pro-opiomelanocortin (POMC) (Ternouth et al., 2011) and Insulin Receptor Substrate 1 (IRS1) (Zheng et al., 2013) have been associated with diabetes onset, appetite regulation and insulin resistance, but not yet in response to therapy.

In this study we aimed to characterise variants across 20 insulin and appetite signalling genes including GLP-1R, GIPR, INSR, IRS-1, IGFBP2, AKT, MAPK1, MTOR, LEP-R, STAT3, SH2B1, POMC, IL-6, IL-10, IL-18, IL-1RN, HGF, HAVCR1, SORT1 and CASP3. SNPs across all genes in patients with T2D were compared to controls to assess their importance in T2D generally. Additionally the same SNPs were assessed in patients that have responded to GLP-1R analogue treatment compared to those who have not achieved glycaemic control in response to GLP-1 analogue therapy. Identification of SNPs involved in response to GLP-1 analogue therapy may allow prediction of drug response at baseline and could have a significant impact on prescribing practice.

6.2 Methods

6.2.1 Participant recruitment

Type 2 diabetes participants were recruited from the Altnagelvin Hospital diabetes clinic, NI. Controls were identified and sampled at the University of the Third Age (U3a), Derry NI, as described in *Chapter 2, Section 2.7*. Participants were considered to be GLP-1Ra responders if they met NICE criteria for response (reduction of 1% HbA1c and/or 3% reduction in bodyweight) or had a HbA_{1c} <54 mmol/mol. Non-responders were individuals receiving GLP-1 analogue therapy who failed to meet NICE criteria or had a HbA_{1c} >65 mmol/mol.

6.2.2 Blood sampling

Blood samples were obtained using 21 G Vacuette® safety needle (Greiner Bio-One, Stonehouse, UK; Cat no. 450091), as described in *Chapter 2, Section 2.8.1.2.* Approximately 26ml of blood was extracted into 2 x 9 ml EDTA (K3E K3EDTA) coated Vacuette® tubes (Greiner Bio-One, Stonehouse, UK; Cat no. 455036) and 1x 8 ml Serum (Z Serum Sep Clot Activator) tube (Greiner Bio-One, Stonehouse, UK; Cat no. 455071). Blood samples were processed immediately.

6.2.3 Blood processing

Blood sample polypropylene tubes were pre-labelled using Item Tracker© software (ItemTracker Software Ltd, Suffolk, UK), prior to processing for serum, plasma, protein, RNA and whole blood. Full protocol described in *Chapter 2, Section 2.8.1.3*. Samples were maintained at -80^{o}C until required.

6.2.4 DNA extraction

DNA was extracted from EDTA whole blood samples using the Gentra Puregene blood kit (Qiagen, Manchester, UK; Cat no. 158445) as described in *Chapter 2, Section 2.9.2*.

6.2.5 DNA quantification

The Qubit®Fluorometer (ThermoFisher Scientific, UK; Cat no. Q33216) and Qubit™ dsDNA HS Assay kit (ThermoFisher Scientific, UK; Cat no. Q32854) were used for DNA quantification, as described in *Chapter 2, Section 2.9.3.*

6.2.6 SNP genotyping

SNP genotyping was conducted using the Affymetrix UK Biobank array by Cambridge Genomic Services (CGS, UK). Samples were prepared to CGS instructions as described in *Chapter 2, Section 2.13.1.*

6.2.7 Variant calling

In bound SNPs were identified using Axiom's Genotype Console software (Affymetrix, UK). Samples with a SNP call rate of less than 97.2% or a QC value of < 0.82 were considered to have failed QC and were excluded from subsequent analyses. A full list of target genes and comparisons described in *Chapter 2, Section 2.13.3.*

6.2.8 Statistical analyses

Differences in genotypes in SNPs between T2D and controls and GLP-1Ra 'responders and non-responders' were calculated using a Z-test for proportions. Significance was accepted at $P<0.05$. Full description in *Chapter 2, Section 2.15*.

6.3 Results

6.3.1 Identifying prevalent variants within the DiaStrat cohort

This study consisted of two analyses; (A1) characterised the prevalence of SNPs in 20 genes (*Chapter 2, Section 2.13.3*) associated with insulin and leptin signalling, inflammation and immunity in 254 T2D patients compared to 19 controls. The second analysis (A2) characterised the same SNPs in patients that responded GLP-1R analogues (HbA_{1c} <53 mmol/mol) compared to those who did not respond or failed to achieve acceptable glycaemic control (HbA_{1c} >65 mmol/mol). Table 6.1 shows that 105 of 2489 SNPs analysed were associated with significantly different prevalence between T2D and controls (A1) while 25 were significantly different between GLP-1 analogue responders and non-responders (A2).

Gene	Total number of SNPs per gene	Number of significant SNPs Control vs. T2D (A1)	Number of significant SNPs responders vs. non-responders (A2)
GLP-1R	61	*1*	*1*
GIPR	65	*2*	*2*
INSR	49	*1*	*0*
IRS-1	38	*2*	*1*
IGFBP2	57	*2*	*0*
AKT	952	*33*	*10*
MAPK1	68	*7*	*0*
MTOR	59	*2*	*1*
LEP-R	93	*3*	*1*
STAT3	39	*4*	*2*
SH2B1	1	*0*	*0*
POMC	91	*0*	*0*
IL6	60	*1*	*1*
IL10	47	*1*	*0*
IL18	104	*1*	*1*
IL1RN	73	*4*	*0*
HGF	69	*2*	*0*
HAVCR1	4	*0*	*0*
SORT1	35	*0*	*0*
CASP3	524	*39*	*5*
TOTAL	**2489**	***105***	***25***

Table 6.1 – **Summary of genes of interest and SNPs associated with T2D and response to GLP-1 analogue therapy**. A1 = T2D (n=254) vs. controls (n=19). A2 = GLP-1 responders (n=21) vs. non-responders (n=22).

6.3.2.1 SNPs significantly associated with T2D

Overall, ~98% of the analysed SNPs (Table 6.1) did not differ in prevalence between those with T2D and controls, although there were 105 SNPs associated with insulin, leptin, inflammation and immunity, which were significantly associated with T2D. The characteristics of all significant SNPs are illustrated below in Tables 6.2 – 8. All tables detail gene name abbreviation, Reference SNP cluster ID (RS number), Allele A and B, SNP call rate, Minor Allele Frequency (MAF) score and Hardy-Weinberg P value.

6.3.2.1.1 Insulin signalling related SNPs in T2D

Tables 6.2-4 illustrate 50 insulin related SNPs with significantly different prevalence in T2D patients versus controls. SNPS associated with GLP-1R, GIPR, INSR, IRS-1, IGFBP-2, MAP-K and MTOR are listed in Table 6.2. Notable SNPs include: rs13374714 in IRS1; controls exclusively had the AB genotype (100%), while in T2D the BB genotype was present in 99.6% of individuals (P<0.0000). Rs117029769 of IGFBP2 (control 89.5% AB; T2D 97.2% BB. P<0.0000). Rs117029769 is illustrated graphically in Figure 6.1. AKT was the most polymorphic gene screened, a total of 952 SNPs were identified (Table 6.1). Rs72726656 (AKT) was the most significant, 100% of controls had the AB genotype and 94.0% of the diabetes cohort had the BB genotype (p<0.0000). Interestingly the most notable SNPs in this study have not been previously linked to insulin signalling on the type 2 diabetes portal (T2D-GENES Consortium, 2017) (Table 6.9).

6.3.2.1.2 Leptin signalling related SNPs in T2D

Table 6.5 lists 7 SNPs related to leptin signalling genes. Of the 3 identified on the LEP-R, rs72881029 was most significant (P<0.0000). The rs72881029 SNP AB genotype was associated with 100% of controls, while 97.6% of the diabetes group had the BB

genotype, this is graphically illustrated in Figure 6.2. Polymorphisms present on the POMC gene were not as distinctive as those on LEP-R, however, rs112365258 was the most significant, with AB genotype more common in the control population (15.8% vs. 2.4% in diabetes (P<0.001)) and the BB genotype more common in T2D (97.6% vs 84.2% (P<0.001)). The importance of this SNP is supported on the type 2 diabetes portal (T2D-GENES Consortium, 2017) were GWAS studies have linked it to increased BMI (Table 6.9). No significant SNPs were identified In the STAT3 or SH2B1.

6.3.2.1.3 Inflammatory gene related SNPs in T2D

There were a total of 8 SNPs significantly associated with T2D across 4 inflammatory related genes (IL-6, IL-10, IL-1RN, IL-18). Rs79653684 (IL1RN) was the most significant, the AB genotype accounted for 15.8% of controls and only 1.2% of the diabetes cohort (P<0.0000, Table 6.6, Fig. 6.3). Conversely the BB genotype accounted for 98.8% of diabetes patients and 84.2% of controls (P<0.001). The Rs62363106 SNP present on the IL6 gene was the only polymorphism significantly associated with pathogenesis on the type 2 diabetes portal (T2D-GENES Consortium, 2017) (insulin response, Table 6.9), the distribution of this SNP is illustrated in Figure 6.3.

6.3.2.1.4 Immunity gene related SNPs in T2D

There were 41 SNPs on immune related genes (HGF, CASP3), with different prevalence rates in T2D patients compared to controls. In Rs11085808 of HGF (Fig. 6.4) 100% of controls had the AB genotype while 100% of T2D patients had the BB genotype (P<0.0000, Table 6.7). CASP3 was highly polymorphic, 39 SNPs differed from control. The most notable were rs114350724 (control, AB 100%; diabetes, BB 100%; P<0.0000), rs115209861 (control AB 100%; diabetes BB 98.8%; P<0.0000), rs117301440 (control AB 94.7%; diabetes AA 99.6; P<0.0000) and rs1718840 (control AB 100%; diabetes BB 95.6%; P<0.0000). Eighteen of the 39 SNPs identified in this study were linked to pathogenesis on the diabetes portal (T2D-GENES Consortium, 2017) (Table 6.9), but only rs114350724 has been linked with diabetes onset (Table 6.9). HAVCR1 and SORT1 had no SNPs which differentiated T2D from control.

Gene	db SNP RS ID	A allele	B allele	SNP call rate (%)		MAF		H.W. P value		% AA			% AB			% BB		
				Control	Diabetes	Control	Diabetes	Control	Diabetes	Control	Diabetes	p-value	Control	Diabetes	p-value	Control	Diabetes	p-value
GLP-1R	rs17415505	T	C	100	99.6	0.16	0.06	0.41	0.07	0.00	1.30	*0.6311*	31.60	11.30	*0.0099*	68.40	87.40	*0.0192*
GIPR	rs10415769	T	G	100	99.6	0.32	0.26	0.00	0.08	31.58	8.76	*0.0016*	0.00	33.86	*0.0022*	68.42	56.97	*0.3298*
	rs57462612	T	C	100	100	0.24	0.20	0.01	0.30	68.42	63.75	*0.4097*	15.79	33.47	*0.1118*	15.79	2.79	*0.0038*
INSR	rs140868873	A	G	100	100	0.03	0.00	0.91	1.00	0.00	0.00	\	5.26	0.00	*0.0002*	94.74	100	*0.0002*
IRS1	rs13374714	A	T	100	99.6	0.50	0.00	0.00	1.00	0.00	0.00	\	100	0.00	*<0.0000*	0.00	99.60	*<0.0000*
	rs200483572	A	G	100	99.6	0.03	0.00	0.91	1.00	0.00	0.00	\	5.25	0.00	*0.0003*	94.74	99.60	*0.1689*
IGFBP2	rs12786837	T	C	100	95.2	0.11	0.02	0.61	0.72	78.95	95.22	*0.0037*	21.05	4.38	*0.0022*	0.00	0.00	\
	rs117029769	T	C	100	99.6	0.45	0.01	0.00	0.85	10.53	0.00	*<0.0000*	89.47	2.39	*<0.0000*	0.00	97.21	*<0.0000*
MAPK1	rs115587686	T	G	100	88.1	0.05	0.19	0.81	0.31	0.00	2.39	*0.4955*	10.53	29.48	*0.0764*	89.47	56.18	*0.0044*
	rs2170185	A	G	100	100	0.47	0.34	0.11	0.68	31.58	43.43	*0.3138*	31.58	45.82	*0.2287*	36.84	10.76	*0.0009*
	rs28380311	T	C	84.2	100	0.06	0.00	0.79	1.00	0.00	0.00	\	10.53	0.00	\	73.68	100	*<0.0000*
	rs72743421	T	C	100	99.6	0.13	0.04	0.51	0.55	73.68	92.43	*0.0055*	26.32	7.17	*0.0039*	0.00	0.00	\
	rs74809741	T	C	100	88.1	0.13	0.03	0.18	0.00	5.26	2.39	*0.4471*	15.79	0.40	*<0.0000*	78.95	85.26	*0.4596*
	rs79193319	A	G	100	98.8	0.26	0.06	0.71	0.04	5.26	1.20	*0.1566*	42.11	10.36	*<0.0000*	52.63	87.25	*<0.0000*
	rs9276825	A	G	100	100	0.45	0.41	0.27	0.15	36.84	14.34	*0.0097*	36.84	52.59	*0.1853*	26.32	33.07	*0.5448*
MTOR	rs79267611	T	C	100	100	0.08	0.01	0.71	0.82	0.00	0.00	\	15.79	2.79	*0.0038*	84.21	97.21	*0.0038*
	rs35612501	A	G	100	98.0	0.13	0.04	0.51	0.53	0.00	0.00	\	26.32	7.57	*0.0056*	73.68	90.44	*0.0228*

Table 6.2 – **SNPs associated with insulin signalling genes in T2D vs. controls**. Included is db SNP RS ID, A allele, B allele, SNP call rate, Minor Allele Frequency (MAF), Hardy Weinberg (H.W) P value, % AA, %AB, % BB in control and T2D participants in SNPS associated with GLP-1R, GIPR, INSR, IRS-1, IGFBP2, MAKP1, MTOR. Control n=19, Diabetes n=254. Significance was determined using a Z-test for proportions. Significant differences in the prevalence of genotypes are highlighted in red. Significance was accepted at P<0.05.

Gene	dbSNP RS ID	A allele	B allele	SNP call rate (%)		MAF		H.W. P value		% AA			% AB			% BB		
				Control	Diabetes	Control	Diabetes	Control	Diabetes	Control	Diabetes	p-value	Control	Diabetes	p-value	Control	Diabetes	p-value
AKT	rs6497630	C	G	100	100	0.32	0.19	0.04	0.41	36.84	64.94	*0.0144*	63.16	32.27	*0.0062*	0.00	2.79	*0.4607*
	rs72726656	A	G	100	100	0.50	0.03	0.00	0.63	0.00	0.00	\	100	5.98	*<0.0000*	0.00	94.02	*<0.0000*
	rs73156841	T	C	100	99.6	0.11	0.05	0.05	0.43	84.21	90.04	*0.4214*	10.53	9.56	*0.1375*	5.26	0.00	*0.0003*
	rs73304795	A	G	100	100	0.42	0.37	0.01	0.20	31.58	41.04	*0.4177*	21.05	43.03	*0.0607*	47.37	15.94	*0.0006*
	rs74381725	A	G	100	99.6	0.11	0.02	0.05	0.70	5.26	0.00	*0.0002*	10.53	4.78	*0.2759*	84.21	94.82	*0.0587*
	rs75709259	A	T	94.7	100	0.11	0.01	0.60	0.82	73.68	97.21	*<0.0000*	21.05	2.79	*0.0001*	0.00	0.00	\
	rs76028668	T	C	100	99.6	0.11	0.02	0.61	0.77	0.00	0.00	\	21.05	3.59	*0.0006*	78.95	96.02	*0.0012*
	rs76975836	A	T	100	99.2	0.16	0.13	0.36	0.02	5.26	0.00	*0.0003*	21.05	25.10	*0.6937*	73.68	74.10	*0.9679*
	rs77182850	T	C	94.7	99.6	0.17	0.04	0.40	0.55	0.00	0.00	\	31.58	7.17	*0.0003*	63.16	92.43	*<0.0000*
	rs77369429	A	G	100	100	0.05	0.00	0.81	0.97	89.47	99.60	*<0.0000*	10.53	0.40	*<0.0000*	0.00	0.00	\
	rs77709798	A	G	100	100	0.08	0.02	0.01	0.75	5.26	0.00	*0.0003*	5.26	3.98	*0.7856*	89.47	96.02	*0.1818*
	rs78307740	A	G	100	100	0.16	0.04	0.36	0.27	5.26	0.40	*0.0168*	21.05	6.77	*0.0249*	73.68	92.83	*0.0039*
	rs7907973	A	G	100	98.4	0.42	0.26	0.12	0.53	42.11	54.18	*0.3087*	31.58	36.65	*0.6573*	26.32	7.57	*0.0056*
	rs79327580	T	C	100	98.0	0.08	0.05	0.71	0.00	0.00	3.98	*0.3752*	15.79	1.99	*0.0006*	84.21	92.03	*0.2387*
	rs7994925	A	T	100	99.2	0.39	0.37	0.00	0.28	0.00	15.14	*0.0672*	78.95	43.03	*0.0024*	21.05	41.04	*0.0859*
	rs80124654	T	C	100	98.4	0.08	0.01	0.71	0.82	84.21	95.62	*0.0304*	15.79	2.79	*0.0037*	0.00	0.00	\
	rs8192849	A	C	31.6	99.6	0.42	0.03	0.08	0.60	0.00	0.00	\	26.32	6.37	*0.0017*	5.26	93.23	*<0.0000*
	rs9308962	T	C	100	100	0.08	0.02	0.71	0.80	0.00	0.00	\	15.79	3.19	*0.0073*	84.21	96.81	*0.0073*

Table 6.3 – **SNPs associated with insulin signalling genes in T2D vs. controls (continued)**. Included is db SNP RS ID, A allele, B allele, SNP call rate, Minor Allele Frequency (MAF), Hardy Weinberg (H.W) P value, % AA, %AB, % BB in control and T2D participants in SNPS associated with AKT. Control n=19, Diabetes n=254. Significance was determined using a Z-test for proportions. Significant differences in the prevalence of genotypes are highlighted in red. Significance was accepted at P<0.05.

Gene	dbSNP RS ID	A allele	B allele	SNP call rate (%)		MAF		H.W. P value		% AA			% AB			% BB		
				Control	Diabetes	Control	Diabetes	Control	Diabetes	Control	Diabetes	p-value	Control	Diabetes	p-value	Control	Diabetes	p-value
AKT	rs11038533	T	C	100	99.6	0.08	0.05	0.01	0.37	5.26	0.00	*0.0002*	5.26	10.76	*0.4488*	89.47	88.84	*0.9329*
	rs114321579	A	G	100	99.6	0.11	0.02	0.61	0.70	78.95	94.82	*0.0059*	21.05	4.78	*0.0037*	0.00	0.00	\
	rs115891681	T	C	73.7	96.4	0.46	0.04	0.00	0.29	5.26	0.40	*0.0168*	68.42	6.77	*<0.0000*	0.00	89.24	*<0.0000*
	rs116108356	T	C	100	100	0.11	0.01	<0.00	0.85	10.53	0.00	*<0.0000*	0.00	2.39	*0.4955*	89.47	97.61	*0.0435*
	rs116146530	T	C	100	100	0.13	0.03	0.51	0.09	0.00	0.40	*0.7828*	26.32	5.18	*0.0003*	73.68	94.42	*0.0006*
	rs116193743	T	C	100	99.6	0.11	0.03	0.61	0.67	0.00	0.00	\	21.05	5.18	*0.0060*	78.95	94.42	*0.0090*
	rs116771750	T	C	100	99.2	0.05	0.05	0.00	0.42	94.74	89.64	*0.7139*	0.00	9.56	*0.1579*	5.26	0.00	*0.0003*
	rs117945130	A	G	100	99.6	0.08	0.03	0.01	0.67	89.47	94.42	*0.3781*	5.26	5.18	*0.9873*	5.26	0.00	*0.0003*
	rs118098404	T	C	100	99.6	0.13	0.03	0.18	0.62	78.95	93.63	*0.0184*	15.79	5.98	*0.0980*	5.26	0.00	*0.0003*
	rs118183140	T	C	100	100	0.11	0.03	0.61	0.67	0.00	0.00	\	21.05	5.18	*0.0060*	78.95	94.82	*0.0060*
	rs12073329	A	G	100	98.4	0.05	0.10	0.81	0.00	0.00	9.56	*0.1579*	10.53	0.40	*<0.0000*	89.47	88.45	*0.8922*
	rs2468774	C	G	100	100	0.47	0.26	0.50	0.78	26.32	54.58	*0.0173*	42.11	39.04	*0.7921*	31.58	6.37	*0.0001*
	rs34243925	A	G	100	100	0.11	0.05	0.05	0.45	5.26	0.00	*0.0003*	10.53	9.16	*0.8432*	84.21	90.84	*0.3449*
	rs34644046	T	G	100	100	0.13	0.05	0.18	0.45	5.26	0.00	*0.0003*	15.79	9.16	*0.3449*	78.95	90.84	*0.0956*
	rs358832	T	C	100	97.2	0.18	0.14	0.59	0.01	68.42	70.92	*0.8176*	26.32	26.29	*0.9984*	5.26	0.00	*0.0002*

Table 6.4 – **SNPs associated with insulin signalling genes in T2D vs. controls (Final)**. Included is db SNP RS ID, A allele, B allele, SNP call rate, Minor Allele Frequency (MAF), Hardy Weinberg (H.W) P value, % AA, %AB, % BB in control and T2D participants in SNPS associated with AKT. Control n=19, Diabetes n=254. Significance was determined using a Z-test for proportions. Significant differences in the prevalence of genotypes are highlighted in red. Significance was accepted at P<0.05.

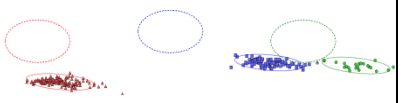
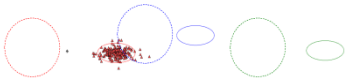
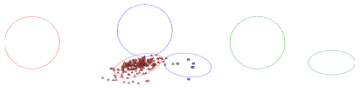
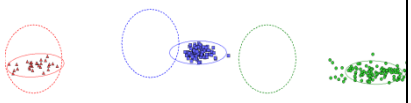
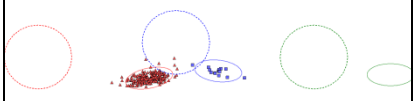
Insulin signalling	**T2D**	**Control**
GIPR rs10415769		
IRS1 rs13374714		
IGFBP2 rs12786837		
IGFBP2 rs117029769		
MAPK1 rs2170185		
AKT rs72726656		

Figure 6.1 – **Cluster analysis for most significant SNPs in insulin signalling related genes.** GIPR, rs10415769; IRS1, rs13374714; IGFBP2 rs12786837; IGFBP2, rs10415769; MAKP1 rs2170185; AKT; rs72726656. Control n=19, Diabetes n=254. All cluster diagrams are plotted on an X axis log scale, Y axis strength scale.

Gene	dbSNP RS ID	A allele	B allele	SNP call rate (%)		MAF		H.W. P value		% AA			% AB			% BB		
				Control	Diabetes	Control	Diabetes	Control	Diabetes	Control	Diabetes	p-value	Control	Diabetes	p-value	Control	Diabetes	p-value
LEP-R	rs14004	A	C	100	100	0.39	0.33	0.00	0.66	0.00	11.55	*0.1168*	78.95	43.03	*0.0024*	21.05	45.42	*0.0389*
	rs9268556	T	C	100	100	0.39	0.31	0.00	0.53	21.05	47.81	*0.0240*	78.95	41.43	*0.0015*	0.00	10.76	*0.1318*
	rs72881029	T	C	100	100	0.50	0.01	0.00	0.00	0.00	0.40	*0.7828*	100	1.99	*<0.0000*	0.00	97.61	*<0.0000*
POMC	rs3934473	A	G	100	100	0.05	0.02	0.00	0.70	5.26	0.00	*0.0002*	0.00	4.78	*0.3296*	94.74	95.22	*0.9245*
	rs112365258	A	G	100	100	0.08	0.01	0.71	0.85	0.00	0.00	\	15.79	2.39	*0.0017*	84.21	97.61	*0.0017*
	rs116605122	T	C	100	100	0.08	0.02	0.71	0.00	0.00	0.40	*0.7828*	15.79	3.19	*0.0073*	84.21	96.41	*0.0127*
	rs10772721	A	G	100	99.6	0.11	0.34	0.61	0.59	0.00	10.76	*0.1318*	21.05	46.22	*0.0332*	78.95	42.63	*0.0021*
SH2B1	*No significant SNPs identified*																	
STAT3	*No significant SNPs identified*																	

Table 6.5 – **SNPs associated with Leptin signalling genes in T2D vs. controls**. Included is db SNP RS ID, A allele, B allele, SNP call rate, Minor Allele Frequency (MAF), Hardy Weinberg (H.W) P value, % AA, %AB, % BB in control and T2D participants in SNPS associated with LEP-R, POMC, SH2B1, STAT3. Control n=19, Diabetes n=254. Significance was determined using a Z-test for proportions. Significant differences in the prevalence of genotypes are highlighted in red. Significance was accepted at $P<0.05$.

Gene	dbSNP RS ID	A allele	B allele	SNP call rate (%)		MAF		H.W. P value		% AA			% AB			% BB		
				Control	Diabetes	Control	Diabetes	Control	Diabetes	Control	Diabetes	*p-value*	Control	Diabetes	*p-value*	Control	Diabetes	*p-value*
IL-6	rs62363106	T	C	100	100	0.05	0.01	0.81	0.92	89.47	98.80	*0.0036*	10.53	1.20	*0.0036*	0.00	0.00	\
IL-10	rs117999816	T	C	100	100	0.05	0.01	0.81	0.92	0.00	0.00	\	10.53	1.20	*0.0036*	89.47	98.80	*0.0036*
IL-1RN	rs61875109	A	C	100	99.6	0.42	0.22	0.55	0.80	21.05	5.18	*0.0059*	42.11	33.86	*0.4659*	36.84	60.56	*0.0427*
	rs146196345	T	G	100	99.6	0.08	0.02	0.01	0.70	5.26	0.00	*0.0003*	5.26	4.78	*0.9245*	89.47	94.82	*0.3263*
	rs79653684	A	G	100	100	0.08	0.01	0.71	0.92	0.00	0.00	\	15.79	1.20	*<0.0000*	84.21	98.80	*<0.0000*
	rs4823900	T	C	89.47	99.6	0.03	0.01	0.90	0.92	0.00	0.00	\	5.26	1.20	*0.1566*	84.21	98.41	*0.0002*
IL-18	rs71326432	T	C	100	81.67	0.03	0.20	0.91	0.00	94.74	57.37	*0.0013*	5.26	15.94	*0.2113*	0.00	8.37	*0.1892*
	rs333597	T	C	100	100	0.21	0.08	0.25	0.05	0.00	1.59	*0.5793*	42.11	13.15	*0.0007*	57.90	85.26	*0.0020*

Table 6.6 – **SNPs associated with inflammatory genes in T2D vs. controls**. Included is db SNP RS ID, A allele, B allele, SNP call rate, Minor Allele Frequency (MAF), Hardy Weinberg (H.W) P value, % AA, %AB, % BB in control and T2D participants in SNPS associated with IL-6, IL-10, IL-1RN, IL-18. Control n=19, Diabetes n=254. Significance was determined using a Z-test for proportions. Significant differences in the prevalence of genotypes are highlighted in red. Significance was accepted at $P<0.05$.

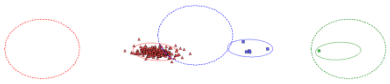
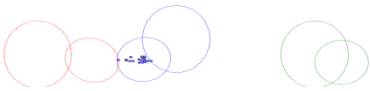
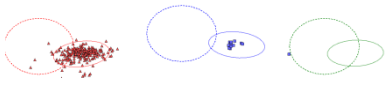
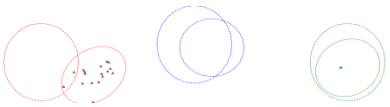
Leptin signalling	**T2D**	**Control**
LEP-R rs72881029		
POMC rs3934473		

Figure 6.2 – **Cluster analysis for defined SNPs of leptin signalling related genes.** LEPR rs72881029; POMC rs3934473. All cluster diagrams are plotted on an X axis log scale, Y axis strength scale.

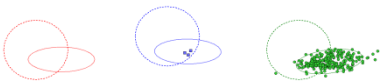
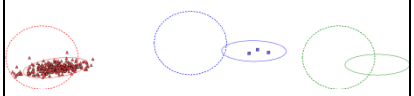
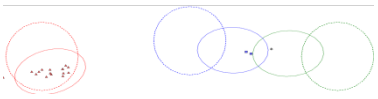
Inflammatory Genes	**T2D**	**Control**
IL-6 rs62363106		
IL-1RN rs79653684		

Figure 6.3 – **Cluster analysis for defined SNPs of inflammatory signalling related genes.** IL6 rs62363106; IL-1RN rs79653684. All cluster diagrams are plotted on an X axis log scale, Y axis strength scale.

Gene	dbSNP RS ID	A allele	B allele	SNP call rate (%)		MAF		H.W. P value		% AA			% AB			% BB		
				Control	Diabetes	Control	Diabetes	Control	Diabetes	Control	Diabetes	*p-value*	Control	Diabetes	*p-value*	Control	Diabetes	*p-value*
HGF	rs62149753	A	C	100	100	0.11	0.06	0.05	0.30	5.26	0.00	*0.0003*	10.53	12.35	*0.8148*	84.21	87.65	*0.6629*
	rs11085808	T	C	100	100	0.50	0.00	0.00	1.00	0.00	0.00	\	100	0.00	*<0.0000*	0.00	100	*0.0000*
CASP3	rs114350724	T	C	100	100	0.50	0.00	0.00	1.00	0.00	0.00	\	100	0.00	*<0.0000*	0.00	100	*<0.0000*
	rs115209861	T	C	100	99.6	0.50	0.00	0.00	0.95	0.00	0.00	\	100	0.80	*<0.0000*	0.00	98.80	*<0.0000*
	rs116910946	A	G	73.68	99.6	0.00	0.00	1.00	1.00	0.00	0.00	\	0.00	0.00	\	73.68	99.60	*<0.0000*
	rs117172277	A	G	63.16	99.6	0.46	0.08	0.54	0.19	10.53	84.46	*<0.0000*	36.84	15.14	*0.0143*	15.79	0.00	*<0.0000*
	rs117264186	T	C	100	100	0.18	0.05	0.04	0.45	10.53	0.00	*<0.0000*	15.79	9.16	*0.3450*	73.68	90.84	*0.0180*
	rs117301440	A	G	100	99.6	0.47	0.00	0.00	1.00	5.26	99.60	*<0.0000*	94.74	0.00	*<0.0000*	0.00	0.00	\
	rs117416733	T	C	63.16	100	0.13	0.04	0.62	0.47	0.00	0.00	\	15.79	8.76	*0.3082*	47.37	91.24	*<0.0000*
	rs117450330	T	C	100	99.6	0.08	0.02	0.71	0.80	84.21	96.41	*0.0127*	15.79	3.19	*0.0073*	0.00	0.00	\
	rs117651071	T	C	100	100	0.11	0.02	0.00	0.72	10.53	0.00	*<0.0000*	0.00	4.38	*0.3515*	89.47	95.62	*0.2275*
	rs117733754	T	C	94.74	97.61	0.47	0.03	0.00	0.67	5.26	0.00	*0.0003*	89.47	5.18	*<0.0000*	0.00	92.43	*<0.0000*
	rs117871273	A	G	100	100	0.11	0.02	0.61	0.70	78.95	95.22	*0.0037*	21.05	4.78	*0.0037*	0.00	0.00	\
	rs1227518	A	G	100	99.6	0.32	0.18	0.00	0.09	31.58	4.78	*<0.0000*	0.00	26.29	*0.0101*	68.42	68.53	*0.9924*
	rs12938916	A	G	100	100	0.16	0.08	0.00	0.67	15.79	0.80	*<0.0000*	0.00	13.94	*0.0810*	84.21	85.26	*0.9013*
	rs13121941	T	C	89.47	100	0.29	0.30	0.00	0.23	26.32	10.76	*0.0429*	0.00	39.04	*0.0006*	63.16	50.20	*0.2758*
	rs140868873	A	G	100	100	0.03	0.00	0.91	1.00	0.00	0.00	\	5.26	0.00	*0.0003*	94.74	100	*0.0003*
	rs148687847	C	G	100	100	0.45	0.12	0.00	0.34	0.00	76.89	*<0.0000*	89.47	22.31	*<0.0000*	10.53	0.80	*0.0007*
	rs1695	A	G	100	99.6	0.42	0.37	0.00	0.83	52.63	39.84	*0.2738*	10.53	45.82	*0.0028*	36.84	13.94	*0.0079*
	rs17056659	A	G	100	99.6	0.08	0.05	0.01	0.43	5.26	0.00	*0.0003*	5.26	9.56	*0.5330*	89.47	90.04	*0.9368*
	rs1718840	A	G	100	100	0.50	0.03	0.00	0.00	0.00	0.80	*0.6961*	100	3.59	*<0.0000*	0.00	95.62	*<0.0000*
	rs3733344	T	G	100	99.6	0.42	0.43	0.03	0.21	5.26	33.86	*0.0099*	73.68	45.02	*0.0158*	21.05	20.72	*0.9723*

Table 6.7 – **SNPs associated with immunity related genes in T2D vs. controls**. Included is db SNP RS ID, A allele, B allele, SNP call rate, Minor Allele Frequency (MAF), Hardy Weinberg (H.W) P value, % AA, %AB, % BB in control and T2D participants in SNPS associated with HGF and CASP3. Control n=19, Diabetes n=254. Significance was determined using a Z-test for proportions, significant differences in the prevalence of genotypes are highlighted in red. Significance was accepted at P<0.05.

Gene	dbSNP RS ID	A allele	B allele	SNP call rate (%)		MAF		H.W. P value		% AA			% AB			% BB		
				Control	Diabetes	Control	Diabetes	Control	Diabetes	Control	Diabetes	*p-value*	Control	Diabetes	*p-value*	Control	Diabetes	*p-value*
CASP3	rs4382459	T	C	100	100	0.21	0.13	0.11	0.50	10.53	1.20	*0.0036*	21.05	23.51	*0.8074*	68.42	75.30	*0.5053*
	rs55690953	T	C	100	81.67	0.05	0.23	0.81	0.33	0.00	5.18	*0.3093*	10.53	26.69	*0.1192*	89.47	49.80	*0.0008*
	rs56059137	A	G	100	99.6	0.00	0.00	1.00	1.00	0.00	0.00	\	0.00	0.00	\	1000	9.60	*<0.0000*
	rs56259105	T	C	100	99.6	0.26	0.15	0.42	0.21	57.89	71.31	*0.2166*	31.58	27.09	*0.6723*	10.53	1.20	*0.0036*
	rs56324835	T	C	100	99.6	0.11	0.07	0.05	0.25	5.26	0.00	*0.0003*	10.53	13.55	*0.7089*	84.21	86.06	*0.8235*
	rs5771096	T	C	100	99.6	0.05	0.21	0.81	0.00	0.00	20.72	*0.0272*	10.53	0.40	*<0.0000*	89.47	78.49	*0.2546*
	rs62411887	T	C	94.74	100	0.17	0.07	0.01	0.88	10.53	0.40	*0.0000*	10.53	12.75	*0.7782*	73.68	86.85	*0.1113*
	rs649628	T	C	100	99.6	0.08	0.06	0.01	0.28	5.26	0.00	*0.0003*	5.26	12.75	*0.3367*	89.47	86.85	*0.7429*
	rs6831849	A	C	100	100	0.05	0.01	0.81	0.92	89.47	98.80	*0.0036*	10.53	1.20	*0.0036*	0.00	0.00	\
	rs72826199	T	C	94.74	99.6	0.06	0.01	0.00	0.90	5.26	0.00	*0.0003*	0.00	1.59	*0.5793*	89.47	98.01	*0.0239*
	rs72829254	T	C	47.37	99.6	0.44	0.03	0.76	0.17	10.53	0.40	*<0.0000*	21.05	5.98	*0.0132*	15.79	93.23	*<0.0000*
	rs75943855	A	C	63.16	100	0.33	0.02	0.08	0.75	0.00	0.00	\	42.11	3.98	*0.0000*	21.05	96.02	*<0.0000*
	rs76975836	A	T	100	99.2	0.16	0.13	0.36	0.02	5.26	0.00	*0.0003*	21.05	25.10	*0.6937*	73.68	74.10	*0.9679*
	rs77037706	A	G	84.21	99.6	0.03	0.01	0.90	0.00	0.00	0.40	*0.7828*	5.26	1.99	*0.3507*	78.95	97.21	*0.0001*
	rs77588920	T	C	100	94.02	0.05	0.00	0.81	0.95	0.00	0.00	\	10.53	0.80	*0.0007*	89.47	93.23	*0.5372*
	rs7818461	C	G	100	99.2	0.11	0.03	0.61	0.67	78.95	94.02	*0.0132*	21.05	5.18	*0.0060*	0.00	0.00	\
	rs7907973	A	G	100	98.41	0.42	0.26	0.12	0.53	42.11	54.18	*0.3088*	31.58	36.65	*0.6573*	26.32	7.57	*0.0056*
	rs79139872	T	C	100	100	0.11	0.01	0.05	0.85	5.26	0.00	*0.0003*	10.53	2.39	*0.0435*	84.21	97.61	*0.0017*
	rs79250505	A	G	100	100	0.18	0.06	0.32	0.04	0.00	1.20	*0.6318*	36.84	10.36	*0.0007*	63.16	88.45	*0.0018*
HAVCR1	*No significant SNPs identified*																	
SORT1	*No significant SNPs identified*																	

Table 6.8 – **SNPs associated with immunity related genes in T2D vs. controls (Final)**. Included is db SNP RS ID, A allele, B allele, SNP call rate, Minor Allele Frequency (MAF), Hardy Weinberg (H.W) P value, % AA, %AB, % BB in control and T2D participants in SNPS associated with CASP3, HAVCR1 and SORT1. Control n=19, Diabetes n=254. Significance was determined using a Z-test for proportions. Significant differences in the prevalence of genotypes are highlighted in red. Significance was accepted at P<0.05.

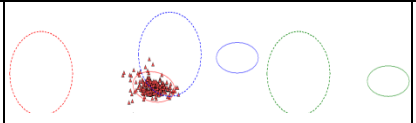
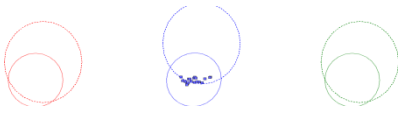
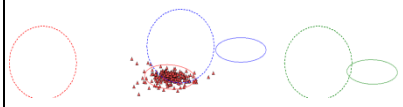
Immunity Genes	**T2D**	**Control**
HGF rs11085808		
CASP3 rs114350742		

Figure 6.4 – **Cluster analysis for defined SNPs of immune related genes.** HGF rs11085808; CASP3; rs114350742. All cluster diagrams are plotted on an X axis log scale, Y axis strength scale.

6.3.2.2 Previous association of identified SNPs in the Type 2 Diabetes Knowledge Portal

Gene	dbSNP RS ID	P-Value	Association
Insulin signalling genes			
GIPR	rs10415769	*<0.0000*	*Lipid metabolism*
IGFBP2	rs12786837	*<0.05*	*Diabetes onset*
MAPK1	rs115587686	*<0.05*	*Lipid / Diabetes onset*
	rs2170185	*<0.05*	*Creatine metabolism*
	rs9276825	*<0.05*	*Lipid metabolism*
AKT	rs11038533	*<0.05*	*weight gain*
	rs12073329	*<0.001*	*increases adiposity*
	rs7994925	*<0.001*	*Proinsulin, triglycerides*
	rs2468774	*<0.05*	*Diabetes onset*
	rs116108356	*<0.05*	*Diabetes onset*
	rs72726656	*<0.05*	*Diabetes onset*
	rs80124654	*<0.05*	*Diabetes onset*
	rs74381725	*<0.05*	*Insulin sensitivity*
	rs8192849	*<0.001*	*Hba1c, insulin sensitivity*
Leptin signalling genes			
LEP-R	rs14004	*<0.0000*	*Adiposity, T2D, BMI*
	rs9268556	*<0.0000*	*Adiposity, T2D, BMI*
POMC	rs3934473	*<0.05*	*BMI*
	rs112365258	*<0.05*	*BMI*
Inflammation related genes			
IL6	rs62363106	*<0.05*	*Insulin response*
Immune related genes			
CASP3	rs114350724	*<0.05*	*Diabetes onset*
	rs117416733	*<0.001*	*Insulin sensitivity*
	rs117733754	*<0.001*	*Insulin sensitivity*
	rs117871273	*<0.05*	*Diabetes onset*
	rs1227518	*<0.001*	*Height, weight, Hba1c*
	rs12938916	*<0.01*	*Adiposity*
	rs148687847	*<0.001*	*T2D, BMI*
	rs1695	*<0.001*	*Lipid metabolism*
	rs17056659	*<0.05*	*Adiposity*
	rs3733344	*<0.0001*	*Insulin response*
	rs4382459	*<0.05*	*T2D Schizophrenia*
	rs55690953	*<0.05*	*Heart disease*
	rs56059137	*<0.01*	*BMI, lipids*
	rs56259105	*<0.05*	*Heart disease*
	rs62411887	*<0.05*	*Diabetes onset*
	rs6831849	*<0.01*	*BMI*
	rs77037706	*<0.001*	*Heart disease*
	rs7818461	*<0.001*	*Diabetes onset*

Table 6.9 – **SNPs identified on type 2 diabetes portal and associations related to T2D.** Table includes all SNPs from current analysis that are reported on the type 2 diabetes portal (T2D-GENES Consortium, 2017). Association is highest ranking trait from GWAS studies. P-value represents probability that the observed frequency could occur by chance. Table includes SNPS from insulin, leptin, inflammation and immune related genes.

6.3.3.1 SNPs associated with response to GLP-1 analogue therapy

There were 25 SNPs that had a significantly different prevalence in patients that responded to GLP-1 analogues and had a HbA_{1c} of <54 mmol/mol compared to 'non-responders' who had a HbA_{1c} of >65 mmol/mol. The characteristics of all significant SNPs are displayed in Tables 6.10 – 14, with cluster diagrams of 8 most significantly altered SNPs visualised graphically in Figures 6.5-8. Tables detail gene name abbreviation, Reference SNP cluster ID (RS number), Allele A and B, SNP call rate, Minor Allele Frequency (MAF) score and Hardy-Weinberg P value.

6.3.3.1.1 Insulin signalling related SNPs associated with response to GLP-1 analogue therapy: a GLP-1 polymorphism is highly significantly associated with response to therapy

Table 6.10 illustrates 15 insulin related SNPs with significantly different prevalence in GLP-1Ra responders and non-responders. SNPS associated with GLP-1R, GIPR, INSR, IRS-1, IGFBP-2, MAP-K, MTOR and AKT are listed in Table 6.10. The GLP-1R had one SNP associated with response. In Rs71480142 the AB genotype was prevalent in non-responders (>65 mmol/mol, 95.5%, P<0.0000) while the BB allele was prevalent in responders (<53 mmol/mol, 87.7%, P<0.0000). This cluster diagram for this SNP is illustrated in Figure 6.5. Rs115516351 (MTOR) was also highly distinctive between responders and non-responders. The AA allele combination was 100% associated with responders (P<0.0000), while the BB allele was linked with non-responders (63.6%, P<0.0000, Fig. 6.5). It is worth noting that the call rate for Rs115516351 in the >65 group was 77.3%.

AKT had two noteworthy SNPs. Rs7952176 showed differential prevalence levels dependent on GLP-1Ra response. Responders typically had an AB genotype (61.9%. vs. 27.3%, P<0.05), while non-responders usually had the BB genotype (72.7% vs, 33.3% P<0.01, Table 6.10). In Rs8192849 low AA and AB genotype prevalence was documented, and 90.91% of non-responders had the BB genotype while only 19.05% of responders were BB (P<0.0000). Call rate in non-responders, however, was 33.3% which may account for the difference observed in this SNP. Rs8192849 has been strongly correlated with HbA_{1c} and insulin sensitivity on the type 2 diabetes portal (T2D-GENES Consortium, 2017) (Table 6.14), and cluster diagram of genotypes in responders and non-responders is illustrated in Figure 6.5. INSR, IGFBP2 and MAPK1 did not express any SNPs that differed between responders and non-responders.

6.3.3.1.2 Leptin signalling related SNPs associated with response to GLP-1 analogue therapy

Table 6.11 shows 3 SNPs associated with leptin signalling genes. In Rs72881029 of the LEP-R the AB genotype was 100% prevalent in responders and 40.9% in non-responders. In Rs12410054 of POMC prevalence levels are significantly different, no responders had the BB genotype while 31.8% of non-responders were BB (P<0.01). Rs12410054 is identified on the type 2 diabetes portal (T2D-GENES Consortium, 2017) as having a significant effect on adiposity (Table 6.14). Rs72881029 and Rs12410054 genotype cluster diagrams are illustrated in Figure 6.6. SH2B1 and STAT3 did not express any SNPs that differed significantly between groups.

6.3.3.1.3 Inflammatory gene related SNPs associated with response to GLP-1 analogue therapy

There were 2 SNPs identified on inflammatory related genes that differed between responders and non-responders (Table 6.12). Rs184477 of IL-6 was more prevalent as the AA genotype in responders vs. non-responders (90.5% vs. 54.6%, P<0.01), and as a AB genotype in non-responders (45.5% v 9.5%, P<0.01). The prevalence of each genotype is illustrated as cluster diagram in Figure 6.7.

6.3.3.1.4 Immunity gene related SNPs associated with response to GLP-1 analogue therapy

Rs2228591 on the CASP3 gene was the only SNP identified to be significantly associated with GLP-1 analogue response. The AB genotype was significantly associated with responders (27.6% v 0%, P<0.01), while the AA genotype was associated with 100% of non-responders compared to 71.4% of responders (P<0.01). These genotype clusters can be seen in Figure 6.8. Rs2228591 has been linked to bodyweight on the type 2 diabetes portal (T2D-GENES Consortium, 2017) (Table 6.14).

Gene	dbSNP RS ID	A allele	B allele	SNP call rate (%)		MAF		H.W. P value		% AA			% AB			% BB		
				<53.9	>65	<53.9	>65	<53.9	>65	<53.9	>65	p-value	<53.9	>65	p-value	<53.9	>65	p-value
GLP-1R	rs71480142	A	G	100	95.5	0.07	0.50	0.72	0.00	0	0	\	14.29	95.45	*<0.0000*	85.71	0	*<0.0000*
GIPR	rs4941804	A	G	100	100	0.36	0.43	0.03	0.07	23.81	9.09	*0.1913*	23.81	68.18	*0.0035*	52.38	22.73	*0.0443*
	rs6864090	A	G	100	100	0.50	0.20	0.83	0.92	23.81	63.64	*0.0086*	52.38	31.82	*0.1719*	23.81	4.55	*0.0684*
IRS1	rs77857696	A	G	71.4	100	0.07	0.00	0.78	1.00	61.90	100	*0.0013*	9.52	0.00	*0.1382*	0.00	0.00	\
MTOR	rs115516351	T	G	100	77.3	0.00	0.41	1.00	0.00	100	13.64	*<0.0000*	0.00	63.64	*<0.0000*	0.00	0.00	\
INSR	*No significant SNPs identified*																	
IGFBP2	*No significant SNPs identified*																	
MAPK1	*No significant SNPs identified*																	
AKT		-	T	71.4	59.1	0.50	0.04	0.00	0.89	0.00	54.55	*<0.0000*	71.43	4.55	*<0.0000*	0.00	0.00	\
	rs6552231	T	C	81.0	100	0.12	0.00	0.58	1.00	61.90	100	*0.0013*	19.05	0.00	*0.0316*	0.00	0.00	\
	rs11261052	C	G	100	100	0.00	0.18	1.00	0.30	0.00	0.00	\	0.00	36.36	*0.0022*	100	63.64	*0.0022*
	rs8192849	A	C	33.3	100	0.29	0.05	0.43	0.82	4.76	0.00	*0.3004*	9.52	9.09	*0.9610*	19.05	90.91	*<0.0000*
	rs4789687	A	G	100	95.5	0.29	0.48	0.45	0.00	4.76	4.55	*0.9731*	47.62	81.82	*0.0187*	47.62	9.09	*0.0049*
	rs250101	A	G	100	95.5	0.21	0.48	0.96	0.51	4.76	18.18	*0.1700*	33.33	54.55	*0.1615*	61.90	22.73	*0.0092*
	rs7952176	A	G	100	100	0.36	0.14	0.11	0.46	4.76	0.00	*0.3004*	61.90	27.27	*0.0223*	33.33	72.73	*0.0096*
	rs425774	A	G	100	100	0.45	0.16	0.79	0.48	19.05	72.73	*0.0004*	52.38	22.73	*0.0443*	28.57	4.55	*0.0329*
	rs6864090	A	G	100	100	0.50	0.20	0.83	0.92	23.81	63.64	*0.0086*	52.38	31.82	*0.1719*	23.81	4.55	*0.0684*
	rs1901440	A	C	100	100	0.29	0.45	0.17	0.18	57.14	13.64	*0.0028*	28.57	63.64	*0.0212*	14.29	22.73	*0.4771*

Table 6.10 – **SNPs associated with insulin signalling genes in GLP-1 analogue responders and non-responders.** Included is db SNP RS ID, A allele, B allele, SNP call rate, Minor Allele Frequency (MAF), Hardy Weinberg (H.W) P value, % AA, %AB, % BB in GLP-1R, GIPR, INSR, IRS-1, IGFBP2, MAKP1, MTOR. Responders (HbA1c <53.9) n=21. Non-responders (HbA1c >65) n=22. Significance was determined using a Z-test for proportions. Significant differences in the prevalence of genotypes are highlighted in red. Significance was accepted at P<0.05.

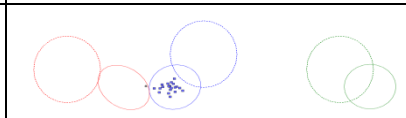
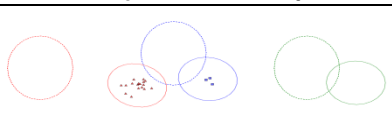
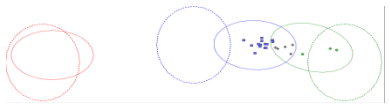
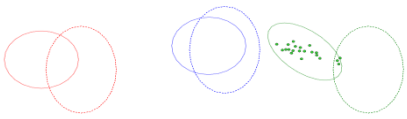
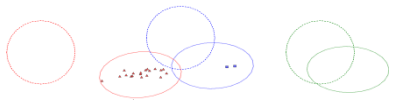
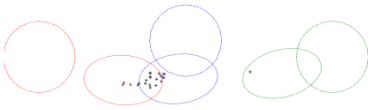
Insulin signalling	**Non responders HbA_{1c} >65 mmol/mol**	**Responders HbA_{1c} <53.9 mmol/mol**
GLP1-R rs71480142		
MTOR rs115516351		
AKT rs8192849		
AKT rs7952176		

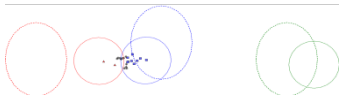
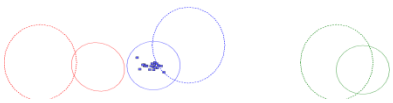
Figure 6.5 – **Cluster analysis for most significant insulin signalling related SNPs in GLP-1 analogue responders and non-responders.** GLP1-R rs71480142; MTOR rs115516351; AKT rs8192849; AKT; rs795`2176. Responders (HbA1c <53.9) n=22. Non-responders (HbA1c >65) n=21. All cluster diagrams are plotted on an X axis log scale, Y axis strength scale.

Gene	dbSNP RS ID	A allele	B allele	SNP call rate (%)		MAF		H.W. P value		% AA			% AB			% BB		
				<53.9	>65	<53.9	>65	<53.9	>65	<53.9	>65	*p-value*	<53.9	>65	*p-value*	<53.9	>65	*p-value*
LEP-R	rs72881029	T	C	100	50	0.50	0.41	0.00	0.02	0.00	0.00	\	100	40.91	*<0.0000*	0.00	9.09	*0.1571*
POMC	rs1281146	A	G	85.71	100	0.08	0.00	0.70	1.00	0.00	0.00	\	14.29	0.00	*0.0660*	71.43	1000	*0.0069*
POMC	rs12410054	A	G	100	100	0.05	0.16	0.00	0.37	4.76	0.00	*0.3004*	0.00	31.82	*0.0047*	95.24	68.18	*0.0227*
SH2B1	*No significant SNPs identified*																	
STAT3	*No significant SNPs identified*																	

Table 6.11 – **SNPs associated with leptin signalling genes in GLP-1 analogue responders and non-responders.** Included is db SNP RS ID, A allele, B allele, SNP call rate, Minor Allele Frequency (MAF), Hardy Weinberg (H.W) P value, % AA, %AB, % BB in LEP-R, POMC, SH2B1, STAT3. Responders (HbA1c <53.9) n=21. Non-responders (HbA1c >65) n=22. Significance was determined using a Z-test for proportions. Significant differences in the prevalence of genotypes are highlighted in red. Significance was accepted at P<0.05.

Gene	dbSNP RS ID	A allele	B allele	SNP call rate (%)		MAF		H.W. P value		% AA			% AB			% BB		
				<53.9	>65	<53.9	>65	<53.9	>65	<53.9	>65	*p-value*	<53.9	>65	*p-value*	<53.9	>65	*p-value*
IL6	rs184477	A	G	100	100	0.05	0.23	0.82	0.17	90.48	54.55	*0.0086*	9.52	45.45	*0.0086*	0.00	0.00	\
IL18	rs74782463	A	G	100	100	0.17	0.00	0.51	1.00	71.43	100	*0.0069*	23.81	0.00	*0.0149*	4.76	0.00	*0.3004*
IL10	No significant SNPs identified																	
IL-1RN	No significant SNPs identified																	

Table 6.12 – **SNPs associated with inflammatory genes in GLP-1 analogue responders and non-responders.** Included is db SNP RS ID, A allele, B allele, SNP call rate, Minor Allele Frequency (MAF), Hardy Weinberg (H.W) P value, % AA, %AB, % BB in IL6, IL18, IL10, IL-1RN. Responders (HbA1c <53.9) n=21. Non-responders (HbA1c >65) n=22. Significance was determined using a Z-test for proportions. Significant differences in the

Leptin signalling	**Non responders HbA_{1c} >65 mmol/mol**	**Responders HbA_{1c} <53.9 mmol/mol**
LEP-R rs72881029		
POMC rs12410054		

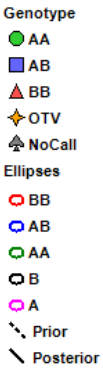


Figure 6.6 – **Cluster analysis for most significant leptin signalling related SNPS in GLP-1 analogue responders and non-responders.** LEPR rs72881029; POMC rs12410054. All cluster diagrams are plotted on an X axis log scale, Y axis strength scale.

Inflammatory gene	**Non responders HbA_{1c} >65 mmol/mol**	**Responders HbA_{1c} <53.9 mmol/mol**
IL6 rs184477		

Figure 6.7 – **Clustering of genotypes of rs184477 of IL-6 between GLP-1 analogue responders and non-responders.** Cluster diagrams are plotted on an X axis log scale, Y axis strength scale.

Gene	dbSNP RS ID	A allele	B allele	SNP call rate (%)		MAF		H.W. P value		% AA			% AB			% BB		
				<53.9	>65	<53.9	>65	<53.9	>65	<53.9	>65	*p-value*	<53.9	>65	*p-value*	<53.9	>65	*p-value*
CASP3	rs2228591	C	G	100	100	0.14	0.00	0.45	1.00	71.43	1000	*0.0069*	28.57	0.00	*0.0069*	0.00	0.00	\
	rs250101	A	G	100	95.45	0.21	0.48	0.96	0.51	4.76	18.18	*0.1700*	33.33	54.55	*0.1615*	61.90	22.73	*0.0092*
	rs7746807	A	C	100	100	0.36	0.41	0.11	0.24	33.33	22.73	*0.4383*	61.90	36.36	*0.0940*	4.76	40.91	*0.0050*
	rs56259105	T	C	100	100	0.10	0.27	0.04	0.08	85.71	45.45	*0.0056*	9.52	54.55	*0.0016*	4.76	0.00	*0.3004*
	rs1997035	T	C	100	100	0.40	0.43	0.00	0.93	0.00	31.82	*0.0047*	80.95	50.00	*0.0333*	19.05	18.18	*0.9419*
HGF	*No Significant SNPs identified*																	
HAVCR1	*No Significant SNPs identified*																	
SORT1	*No Significant SNPs identified*																	

Table 6.13 – **SNPs associated with immunity related genes in GLP-1 analogue responders and non-responders.** Included is db SNP RS ID, A allele, B allele, SNP call rate, Minor Allele Frequency (MAF), Hardy Weinberg (H.W) P value, % AA, %AB, % BB in SNPs within CASP3, HGF, HAVCR1, SORT1. Responders (HbA_{1c} <53.9) n=21. Non-responders (HbA_{1c} >65) n=22. Significance was determined using a Z-test for proportions. Significant differences in the prevalence of genotypes are highlighted in red. Significance was accepted at P<0.05.

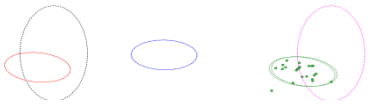
Immune related gene	**Non responders HbA_{1c} >65 mmol/mol**	**Responders HbA_{1c} <53.9 mmol/mol**
CASP3 rs2228591		

Figure 6.8 – **Cluster analysis for CASP3 rs2228591 in GLP-1 analogue responders and non-responders.** Cluster diagrams are plotted on an X axis log scale, Y axis strength scale.

6.3.3.2 Previous association of identified SNPs in the Type 2 Diabetes Knowledge Portal

Gene	dbSNP RS ID	P-Value	Association
Insulin Signalling genes			
AKT	rs250101	*<0.05*	*Diabetes onset*
	rs8192849	*<0.001*	*HbA_{1c}, insulin sensitivity*
	rs11261052	*<0.05*	*Insulin sensitivity*
	rs6552231	*<0.01*	*Lipid metabolism*
Leptin Signalling genes			
POMC	rs12410054	*<0.001*	*Adiposity*
Immunity related genes			
CASP3	rs2228591	*<0.001*	*Weight, BMI*
	rs250101	*<0.001*	*Diabetes onset*
	rs7746807	*<0.0001*	*Height, lipids*
	rs56259105	*<0.001*	*Heart disease*
	rs1997035	*<0.001*	*Adiposity*

Table 6.14 – **Associations linked with insulin related SNPs in GLP-1Ra responders and non-responders.** Table includes all SNPs from current analysis that have a defined function on the type 2 diabetes portal. Association is highest ranking trait from GWAS studies. P-value represents probability that the observed frequency could occur by chance. SNPS from insulin, leptin, and immune related genes were identified.

6.4 Discussion

In the present study, informed by previous work (*Chapter 3, 5*), and the literature (Valentinis and Baserga, 2001, Zhao et al., 2012) we analysed 20 genes associated with T2D and GLP-1R agonist action. A total of 2489 SNPs were screened across the 20 genes. One hundred and five SNPs were significantly different in those with T2D compared to controls, while we identified 25 SNPs associated with response to GLP-1 analogue therapy. This study demonstrated that genetic alterations in insulin and leptin genes are characteristic of T2D and response to GLP-1 analogue therapy and suggests that drug response may be predicted in this drug class.

There were eight insulin signalling related genes analysed; results indicated IRS1, IGFBP2 and AKT were most polymorphic and exhibited the highest degree of differentiation between T2D and controls. IRS1 is known to play a key role in the insulin stimulated PI3K pathway (Dong et al., 2006), and alterations in this gene have been linked to major impairments in insulin action (Li et al., 2016). A north American study reported SNPs present on the IRS1 gene may cause inflammation and oxidative DNA damage, negatively affecting the ability of IRS1 to bind to the insulin receptor (Feng et al., 2013b). The SNPs identified in this study are not present in the literature and will be validated in secondary cohorts within the UK and Europe.

There were two SNPs on IGFBP2 indicative of diabetes. IGFBP2 is known to interact with IRS1 (Lavin et al., 2016). Low protein levels can affect IGF and IRS1 bioavailability (Grimberg et al., 2006), which is necessary to potentiate the PI3K pathway and its downstream target AKT (Guo, 2014). IGFBP2 also has a close relationship with appetite regulatory hormone, leptin (Hedbacker et al., 2010). Clinical studies have shown that IGFBP2 increases weight loss, ameliorating T2D (Holden et al., 2009). Recent preclinical studies have shown that leptin increases IGFBP2 to regulate plasma insulin and glucose concentrations (Hedbacker et al., 2010). Two polymorphisms in IGFBP2, rs9341105 and rs7603372, have been linked to long term weight loss and reduced plasma glucose levels in Caucasians, and were validated in large cohorts (n= 991) in the UK (Narayanan, 2013).

IRS and IGF protein signalling converges on AKT to mediate various insulinotrophic actions (Shpakov, 2014). AKT was the most polymorphic gene

screened in this study, with many SNPs that are indicative of T2D (n=33). Other studies have shown AKT to be highly polymorphic (Matsubara et al., 2001), but few SNPs have been linked to T2D pathogenesis (Sun et al., 2011). A recent, small study in Asia showed that the presence of two SNPs on AKT, rs2494746 and rs2494738, impacted the PI3K/AKT/mTOR pathway, likely contributing to the progression of T2D (Yin et al., 2017). These were not identified in the current work. The importance of AKT signalling for healthy glucose metabolism is well established (Elghazi et al., 2006). It is likely that genetic variation on the AKT pathway, affects diabetes progression. Screening these genes may be useful in patient risk stratification.

In recent years leptin has become a promising target for treating obesity (Cui et al., 2017), diabetes (Iepsen et al., 2015a), and brain insulin resistance (Greco et al., 2009). The present study identified three SNPs on the LEP-R and four SNPs on its downstream target POMC (De Jonghe et al., 2012) that are characteristic of T2D in this cohort that have not previously been reported. Other studies have demonstrated that SNPs on the LEP-R can affect BMI and lipid metabolism (Park et al., 2006a), while SNPs on the POMC gene have been shown to alter obesity traits (rs1009388, rs1866146) (Sutton et al., 2005). The exact physiological effect of the SNPs identified in this study is unknown. It is likely that they are genetic contributors of obesity and diabetes, and are promising candidates for further validation experiments.

Inflammation is a characteristic of both diabetes and obesity (Wellen and Hotamisligil, 2005) and is a main cause of insulin resistance (Cefalu, 2009). Pro-inflammatory cytokines negatively influence leptin and insulin signalling. There were four pro-inflammatory mediators IL-6 (1 SNP), IL10 (1 SNP), IL-1RN (4 SNPs) and IL18, (2 SNPs) analysed and 8 SNPs identified as having a significantly different prevalence in T2D. IL-6 is an established marker of insulin resistance in diabetes (Allen and Febbraio, 2010), but large meta-analysis (n=5383) have failed to link reported IL-6 polymorphisms (rs2069827, rs1800797, rs1800795) to diabetes risk or circulating IL-6 levels (Qi et al., 2006). The SNP identified in this study is novel, and there have not been any mechanistic studies to elucidate its effect. Recent studies have focused on correlating SNP-SNP interactions across multiple genes to account for disease progression with respect to inflammation (Murk and DeWan, 2016) as many

cytokines have tissue specific expression profiles (Kim et al., 2017). In T2D results have indicated that interplay between IL-6, TNFα and IL-10 exists; a combination of SNPs across these genes greatly increases diabetes risk and associated pathogenesis (Saxena et al., 2013). It is possible that the cytokines and SNPs identified in this study interact to affect disease progression, but further analysis will be required to support this suggestion.

Caspase 3 (CASP3) was the most polymorphic immune related gene screened in this study, 39 SNPs were found to be different in T2D vs. controls indicative of a significant role in T2D pathogenesis. CASP3 is known to be involved in apoptotic cell death and β-cell loss in T2D (Mathis et al., 2001), and increased IL-6 has been linked to elevated CASP3 (Jin et al., 2016). There are few studies linking CASP3 SNPs to diabetes, but a large body of work implicates this gene in cancer progression (Chen et al., 2008) and other autoimmune disease (Onouchi, 2017). This data suggests it should be considered in future SNP analyses in T2D.

The 20 genes analysed in the present study are highly polymorphic and a number of SNPs were significantly associated with T2D. As GLP-1 analogues affect insulin and leptin signalling, inflammation and the immune response we hypothesised that polymorphisms in genes associated with these processes may be associated with response and non-response to GLP-1 analogue therapy. Polymorphisms were found on both the GIP and GLP-1 receptors. Studies have shown that activation of each receptor can have glucose and lipid regulating effects (Kazafeos, 2011), and recent work has indicated how dual functionality results in a greater incretin effect than any single activation (Skow et al., 2016). Whether the genetic variation in the GIPR interacts with the GLP-1R is unknown. The one SNP on the GLP-1R (rs71480142) was highly significantly associated with response to therapy. The BB genotype (GG) was prevalent exclusively in responders. This novel finding will be replicated in secondary cohorts, but is suggestive that it may be possible to predict response to therapy utilising SNP genotyping.

Rs115516351 on the MTOR gene was different in responders and non-responders. GLP-1 can activate the IRS/ AKT / MTOR pathway to increase β-cell growth and viability (Park et al., 2006b). It is thought SNPs on the MTOR gene negatively affect this process, impacting the long term effects of GLP-1 on islet

function (Van de Velde et al., 2011). Currently Rs115516351 is not linked with diabetes pathogenesis, in the literature or on the type 2 diabetes knowledge portal (T2D-GENES Consortium, 2017). The only evidence of polymorphisms on MTOR with clinical impact were reported in a recent small study (n=134), which specified two SNPs (rs7212142, and rs9674559) as being strongly correlated with nephropathy (Zhu et al., 2015). Although not conclusive, this study demonstrated that MTOR alterations may affect diabetes outcome.

Rs72881029 (LEP-R) was the only SNP in this study that was noteworthy in both T2D versus controls, and GLP-1Ra responders versus non-responders. This highlights the importance of leptin signalling in both T2D and GLP-1 analogue drug response. All control patients had the AB genotype and 97.6% of T2D patients had the BB genotype. GLP-1Ra responders were also 100% positive for the AB genotype, whereas 40% of non-responders were AB. Chapter 3 and studies in the literature have reported that leptin signalling may have insulin sensitising effects (Duan et al., 2004). It is possible that this SNP impacts T2D and GLP-1Ra response. More work is warranted in this area.

CASP3 was highly polymorphic and rs2228591 was one of the most interesting SNPs on this gene. The AA genotype (CC) was 100% prevalent in non-responders, while responders were split between the AA and the AB genotypes, 71.4% and 28.6% respectively. Although this gene is usually associated with cell death and β-cell dysfunction (Liadis et al., 2005), rs2228591 has been linked with increased BMI and weight on the type 2 diabetes knowledge portal (T2D-GENES Consortium, 2017).

This studies main limitation is sample size, particularly for controls. Recruitment is ongoing and validation will be completed in larger sample sets. Due to well documented differences in ethnicity and geographical genotypes (T2D-GENES Consortium, 2017), validation in other cohorts would be valuable. Further multivariate analysis would also provide key insights in terms to gene-gene and SNP-SNP interactions.

In conclusion we have analysed 2489 SNPs across 20 genes associated with insulin and leptin signalling, and defined that many SNPs were associated with T2D generally and with response to GLP-1 analogues in a Northern Irish type 2 diabetes

population. Many of the SNPs identified are supported by findings on the type 2 diabetes portal (T2D-GENES Consortium, 2017) and within the literature. Some are also novel and suggestive that treatment response to GLP-1 analogues may be predicted, if findings are replicated in secondary cohorts.

Chapter VII

Depression, Anxiety and Cognitive Function in GLP-1 Analogue Responders and Non-responders

Abstract

Type II diabetes (T2D) is a significant socioeconomic burden, with many long term complications that are now known to include depression and cognitive dysfunction. Clinically GLP-1Ras are an effective glycaemic and weight reducing therapy, and preclinically have been shown to improve memory and learning. This study aimed to assess the effect of GLP-1Ras on cognition, depression and anxiety in T2D patients. SMMSE and qMCI assessments, and Beck depression and anxiety inventories were used, and common biochemical measures including BMI, HbA_{1c} and FBC taken for correlation analysis. Non diabetes controls (n=19, 47% male), diabetes controls (GLP-1Ra naïve; n=13, 62% male), T2D GLP-1Ra responders (n=22, 59% male) and non-responders (n=15, 80% male) were included.

GLP-1Ra non-responders had a higher HbA_{1c} than responders ($P<0.01$). Non-responders, specifically, were on more anti-diabetes medications ($P<0.05$), and had a lower neutrophil count ($P<0.05$) than diabetes controls. They also had the highest percentage of cardiovascular (23%, $P<0.01$) related comorbidity. Non-responders were the only group to score higher ($P<0.05$) than controls in depression and anxiety tests. Cognitive dysfunction was more prevalent in non-responders; memory and perception scores were lower in non-responders compared to responders in the SMMSE ($P<0.0001$). In the qMCI, non-responders scored lower in delayed recall ($P<0.05$) and logical memory ($P<0.05$) domains compared to non-diabetes controls. Significant correlation between BMI and depression scores was observed ($R=0.22$, $P=0.02$) and qMCI, but not SMMSE, was significantly correlated with HbA_{1c} ($R=-0.53$, $P<0.0001$). Neutrophil number was highly significantly positively correlated with both depression ($R=0.27$, $P<0.0001$), and anxiety ($R=0.36$, $P<0.0001$) but not with cognitive function. These preliminary results indicate response to GLP-1Ra therapy in diabetes may reduce comorbidity and have a positive effect on mood and cognition.

7.1 Introduction

The advancement of medicine means T2D patients are living longer (Kirkman et al., 2012b). This presents clinicians with difficult decisions with respect to diabetes management as multimorbidity becomes more prevalent (Ligthelm et al., 2012). Micro and macrovascular complications are well known (Forbes and Fotheringham, 2017), but the effect T2D has on cognitive function is poorly described (Yang et al., 2015). Cognitive function in diabetes is of high clinical relevance because of the impact it has on self-care, regime adherence and quality of life (Vinik et al., 2017). Cognitive decline is a general term that incorporates 5 domains; learning, memory, attention, mental flexibility and executive function (O'Caoimh et al., 2012).

Patients with T2D have been shown to exhibit a 3 times increased risk of deficits in learning, memory and executive function (Ott et al., 1999), and onset is often accompanied by mood disorders such as depression (Deschenes et al., 2017). Pathologically, T2D post mortem brains show vascular lesions and atrophy indicative of AD (Manschot et al., 2007). Longitudinal studies following asymptomatic individuals have shown that brain volume decreases with increased age and HbA_{1c} (Kocahan and Doğan, 2017). Cognitive dysfunction in diabetes is thought to be affected by glycaemia, inflammation, and vascular complications (Prickett et al., 2015). Cumulatively these disorders likely reduce blood flow to the brain decreasing the threshold at which cognition is impacted by other neurological illnesses (Feinkohl et al., 2015).

Clinically, cognitive dysfunction in diabetes manifests via alterations in behaviour (Venditti, 2016). Effective diabetes management requires good planning and coordination to effectively monitor blood glucose, regime frequency and diet (NICE, 2017). As cognition declines the risk of glycaemic dysregulation increases (Kirkman et al., 2012a). In patients with simple regimes these signs may go unnoticed, but in high risk patients on complex insulin dependent regimes unintentional non-compliance can have a significant effect on patient outcome (Weinstock et al., 2016). It has been proposed that regular screening for executive dysfunction in elderly, T2D patients before prescribing complex insulin regimes would be clinically beneficial (Sinclair et al., 2013).

Studies have assessed the link between chronic disease, mood and cognitive decline (Iglay et al., 2017), but few correlations have been made between antidepressants and cognition; vortioxetine has been shown to increase cognitive performance (McIntyre et al., 2016).

There is a convergence of cognitive dysfunction in patients with mood disorders and T2D, suggestive of intersecting neurobiological pathways (Geijselaers et al., 2015). It is possible that currently available treatments targeting T2D may mitigate cognitive insufficiencies (McIntyre et al., 2013). Currently it is not clear whether tighter glycaemic control can reduce the risk or prevent further cognitive decline in T2D patients. Glucagon-Like Peptide 1 Receptor analogues (GLP-1Ra) have been shown to be effective at treating metabolic syndrome, by acting not only on blood glucose but on appetite, and lipolysis (Iepsen et al., 2015b). There are many preclinical studies in various models that have shown GLP-1Ras to have protective, proliferative (Kelly et al., 2015) and anti-inflammatory effects in brain (Hou et al., 2012), all of which reduce classic AD pathologies (Luchsinger, 2010). Other behavioural studies have shown GLP-1Ras to improve learning and memory (McClean and Holscher, 2014). This significant body of evidence suggests GLP-1Ras restore learning and memory in animals, and has prompted clinical trials for this class of drugs in AD; a phase 2b trial, Evaluating Liraglutide in Alzheimer's disease (ELAD, NCT 01843075), is ongoing but excludes participants with T2D.

Preclinical results with GLP-1 analogues are promising (Kelly et al., 2015), but a disconnect exists between preclinical and clinical findings (Ruggeri et al., 2014), and data in human cohorts on the effect of GLP-1Ras on depression, anxiety and cognition in those with T2D are lacking. A small, domain based, pilot study (n=19) found that the GLP-1Ra, liraglutide had beneficial effects on cognitive function in non-diabetic participants with major depressive disorder (MDD) (Mansur et al., 2017). Such findings are the earliest clinical indications that GLP-1Ra may be beneficial in patients with mood disorders. Further studies are required to define the therapeutic potential of liraglutide in psychopathological domains.

Here we report interim findings assessing the effect of the GLP-1Ra, liraglutide, on aspects of cognitive function, depression and anxiety in a Northern Irish T2D population in GLP-1 analogue responders and non-responders, diabetes

controls and controls without T2D. HbA_{1c}, drug prescriptions, self-reported comorbidity, and full blood count were also assessed. This study will develop a better understanding of liraglutide's cognitive effects in T2D, and will provide evidence for the utility of GLP-1 analogues for the prevention of mood disturbances and cognitive decline in T2D.

7.2 Methods

7.2.1 Participant Recruitment

Participants with T2D were identified by clinicians at the Altnagelvin Hospital diabetes clinic. Controls were identified and sampled at the University of the Third Age (U3a), Derry, NI. Patient groups, inclusion, exclusion and ethical considerations are described in *Chapter 2, Section 2.7.2.*

7.2.2 Sample Collection

7.2.2.1 Blood sampling

All blood samples were obtained using 21G Vacuette® safety needles (Greiner Bio-One, Stonehouse, UK; Cat no. 450091), as described in *Chapter 2, Section 2.8.2.1* Approximately 50 ml of blood was extracted into 2x 9 ml EDTA (K3E K3EDTA) coated Vacuette® tubes (Greiner Bio-One, Stonehouse, UK; Cat no. 455036), 2x 4 ml EDTA (K3E K3EDTA) coated Vacuette® tubes (Greiner Bio-One, Stonehouse, UK; Cat no. 455021) and 1x 8 ml Serum (Z Serum Sep Clot Activator) tube (Greiner Bio-One, Stonehouse, UK; Cat no. 455071). Blood samples were processed immediately after collection.

7.2.2.2 Blood processing

Polypropylene tubes were labelled using Item Tracker© software (ItemTracker Software Ltd, Suffolk, UK), prior to processing for serum, plasma, protein, RNA and whole blood, as described in *Chapter 2, Section 2.8.2.2*. Samples were stored at -80° C in HTA compliant freezers.

7.2.3 Anxiety, Depression and cognitive screening

7.2.3.1 Beck Anxiety Inventory (BAI)

Participants completed a self-score inventory with 21 symptoms, according to how much each one had affected them the previous week. Full description in *Chapter 2,*

Section 2.14.1, and the full inventory can be found in *Appendices; supplementary screen 1*.

7.2.3.2 Beck Depression inventory (BDI)

Participants self-scored 21 questions ranking severity from 0-3. Higher scores are indicative of greater symptom severity. Full description in *Chapter 2, Section 2.14.2*, and the full inventory can be found in *Appendices; Supplementary Screen 2*.

7.2.3.3 Standardized Mini-Mental State Examination (SMMSE)

The SMMSE examination was conducted with Dr Molloy's proposed script (Molloy and Standish, 1997). The test consisted of 12 questions with a maximum score of 30. The full protocol is described in *Chapter 2, Section 2.14.3*, and full test can be found in *Appendices; Supplementary Screen 3*.

7.2.3.4 Quick mild cognitive impairment screen (qMCI)

The questionnaire consists of 6 sections designed to address 6 domains of cognition. Orientation, registration, clock drawing, verbal memory (VM), verbal fluency (VF) and logical memory (LM), and is described fully in *Chapter 2, Section 2.14.4* and the full test can be found in *Appendices; Supplementary Screen 4*.

7.2.4 Clinical database construction

Relevant clinical information for all participants was obtained from Western Health and Social Care Trusts, Hicom Diamond.NET diabetes management system (Hitcom, Surrey, UK) and Orion Health technologies, Northern Ireland Electronic Care Record (NIECR) (Orion health, Hammersmith, UK) as described in *Chapter 2, Section 2.11.2.*

7.2.5 Statistical analysis

All statistical analysis was conducted using Graphpad Prism software (Graphpad Software Inc., La Jolla, CA, USA; v6.0h), as described in *Chapter 2; Section 2.15.*

7.3 Results

Cohort characteristics for the 'GLP-1 response study' are shown in Table 7.1 for healthy controls, diabetes controls, GLP-1 analogue responders and GLP-1 analogue non-responders. All data presented represent interim analyses as patient recruitment and analysis is ongoing. All groups, with the exception of controls (47%), were predominantly male, diabetes 62%, diabetes GLP-1 analogue responders 59%, diabetes GLP-1 analogue non-responders 80%. GLP-1Ra non-responders were the only group that differed in age between groups, they were significantly younger than non-diabetes controls (59yrs, P<0.05). Patients prescribed a GLP-1Ra were significantly heavier compared to GLP-1 naïve diabetes controls (P<0.001-P<0.0001) and non-diabetes controls (P<0.05-P<0.01). HbA_{1c} was higher in GLP-1 analogue non-responders compared to diabetes control (73 vs. 57.5 mmol/mol, p<0.01). The age participants left school was no different between groups.

GLP-1 Response Study	Non-diabetes Controls			Diabetes Controls			GLP-1 analogue responders			GLP-1 analogue non-responders		
	Total (n)	mean (SD)	%	Total (n)	mean (SD)	%	Total (n)	mean (SD)	%	Total (n)	mean (SD)	%
Number of participants	19			13			22			15		
Male	9		47.4	8		61.5	13		59.1	12		80.0
Female	10			5			9			3		
Age (yrs)	19	68.4 (9)	100.0	13	66.1 (12)	100.0	22	62.8 (10)	100.0	15	59.1 (9)*	100.0
BMI (Kg/m2)	18	26.8 (3)	94.7	13	28.0 (9)	100.0	21	36.2 (8)****/ΔΔ	95.5	14	35.6 (7)***/Δ	93.3
Overweight – >25	10	27.7 (2)	52.6	13	28.0 (9)	100.0	20	36.8 (7)**/ ΔΔ	90.9	14	35.6 (7)* /Δ	93.3
HbA1c (mmol/mol)	18	36.2 (2)	94.7	11	57.5 (12)****	84.6	21	56.7 (9)****	95.5	15	70.7 (20)**** /Δ/λλ	100.0
above target - >48.9	0	36.2 (2)	0.0	9	61.4 (9)****	69.2	17	59.8 (7)****	77.3	14	73.0 (9)**** Δ/λλ	93.3
Smoking Status	19		100.0	13		100.0	21		95.5	15		100.0
Never	9		47.4	5		38.5	10		47.6	5		33.3
Ex	10		52.6	7		53.8	8		38.1	6		40.0
Current	0		0.0	1		7.7	3		14.3	4		26.7
Age left school (yrs)	17	18.2 (3)	89.5	13	16.4 (2)	100.0	20	16.2 (2)	90.9	15	16.1 (1)	100.0

Table 7.1 – **Demographics and clinical features of non-diabetes controls, diabetes controls, and T2D GLP-1 responders and non-responders.** Data is presented as control, diabetes control, GLP-1 analogue responders and GLP-1 analogue non-responders. Each column describes the total number of participants, mean ± SD and % of total for each measure. All participants were aged 18-100 years. Targets used for HbA_{1c}, and BMI were obtained from NICE. Significance was determined by one-way ANOVA and Tukey's post hoc test. *P<0.05, **P<0.01 vs. Non-diabetes. ΔP<0.05, ΔΔ P<0.01 vs Diabetes controls, λλ P<0.01 vs Responders.

7.3.1 Diabetes patients have significantly increased polypharmacy compared to controls

Non-diabetes controls were prescribed on average 1.5 medications while all groups with T2D were prescribed significantly more non-diabetes medications (P<0.0001, Fig. 7.1A). GLP-1Ra non-responders were prescribed significantly more diabetes medications than the diabetes control group (P<0.05, Fig. 7.1B).

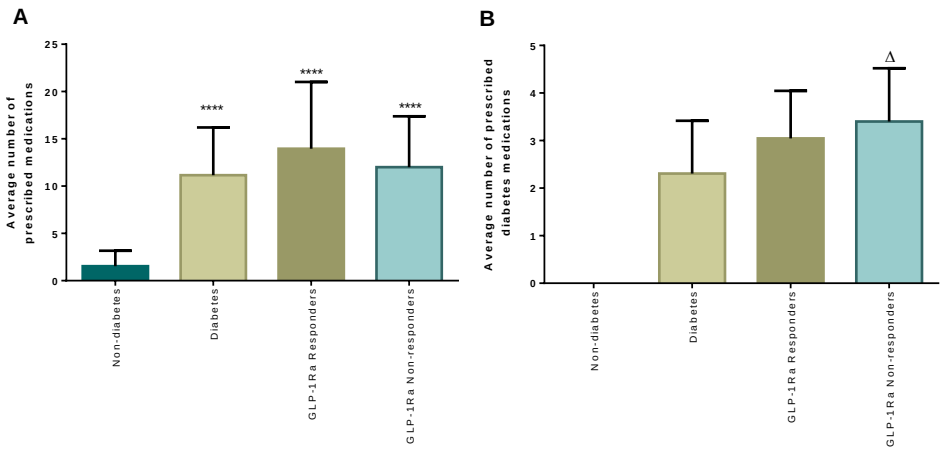


Figure 7.1 – **Diabetes patients have significantly increased polypharmacy compared to controls** (A) Average number of drugs prescribed per group. (B) Average number of anti-diabetes drugs prescribed per group. Each graph shows non-diabetes controls (n=19), diabetes controls (n=13), GLP-1Ra responders (n=22) and GLP-1Ra non-responders (n=15). Significance was determined using one-way ANOVA and Tukey's post hoc test. ****P<0.0001 vs. non-diabetes controls. ΔP<0.05 vs. diabetes controls.

7.3.2 Full blood count (FBC)

There was a weak but significant positive (R=0.31, P=0.01) correlation between HbA_{1C} and WBC count (Fig. 7.2).

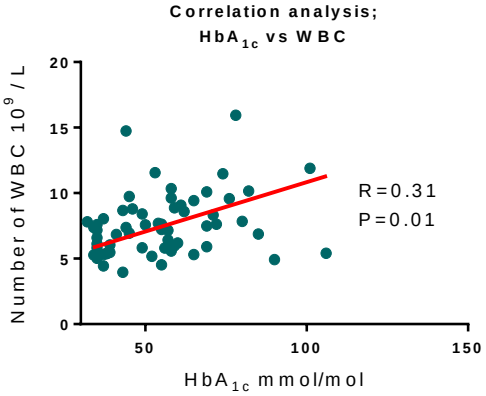


Figure 7.2 – **Correlation analysis between HbA_{1c} and white blood cell (WBC) count.** Data presented from total cohort (N=69) Data presented as a scatter plot with linear regression best line fitted. R value representative of relationship.

Total red blood cell (average 4.7 x 10^{12}/l, Fig. 7.3A, RBC) and platelet (average 241 x 10^{9}/l, Fig. 7.3B, PLT) counts were no different between groups. The white blood cell (WBC) count differed significantly (Fig. 7.3A). Diabetes controls (GLP-1Ra naïve) had a significantly higher WBC count (9 x 10^{9}/l, P<0.0001) than non-diabetes controls (average 6 x 10^{9}/l), as did GLP-1Ra responders (8 x 10^{9}/l, P<0.01) and non-responders (7.5 x 10^{9}/l, P<0.05). WBC differentiation revealed lymphocytes and monocytes did not differ between groups and variance could be attributed to neutrophil number (Fig. 7.3C). Non-diabetes controls had the lowest neutrophil number (3.6 x 10^{9}/l), and diabetes control patients had most (5.7 x 10^{9}/l, P<0.0001). GLP-1Ra responders and non-responders also had increased neutrophils compared to controls, 5.0 x 10^{9}/l (P<0.001) and 4.6 x 10^{9}/l (P<0.05) respectively (Fig. 7.3C). GLP-1 analogue non-responders had reduced neutrophil counts compared to diabetes controls (P<0.05, Fig. 7.3C).

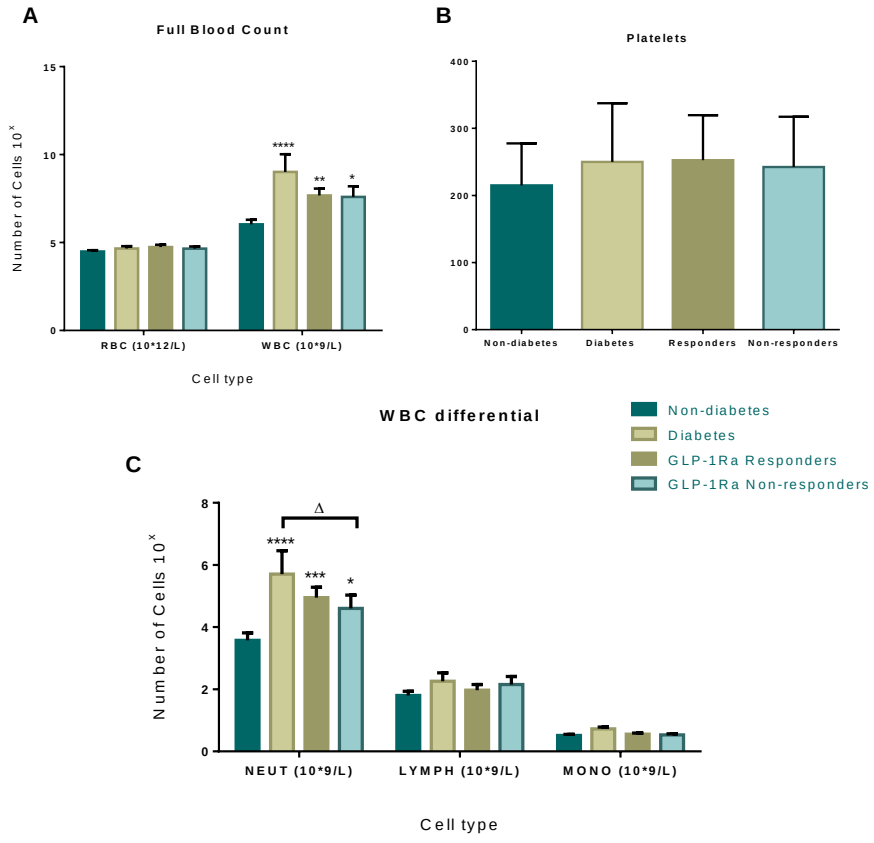


Figure 7.3 – **White blood cell counts are significantly increased in diabetes** (A) RBC and WBC count. (B) platelet count. (C) WBC differentiation count, including neutrophils, lymphocytes and monocytes. Each graph shows non-diabetes controls (n=19), diabetes controls (n=13), GLP-1Ra responders (n=22) and GLP-1Ra non-responders (n=15). Significance was determined using one-way ANOVA and Tukey's post hoc test. *P<0.05, **P<0.01, ****P<0.0001 vs. non-diabetes controls. ΔP<0.05 vs. diabetes controls.

7.3.3 Comorbidity

The non-diabetes control group had on average 1.3 chronic conditions, and the diabetes GLP-1Ra responders (average 2.8) and non-responders (average 2.9) had significantly more (P<0.05, Fig. 7.4). It is interesting to note that GLP-1Ra naïve diabetes controls had on average of 4 chronic conditions, this was significantly more than controls (P<0.0001), but at this interim stage in the study, no difference could be determined from the GLP-1Ra positive groups.

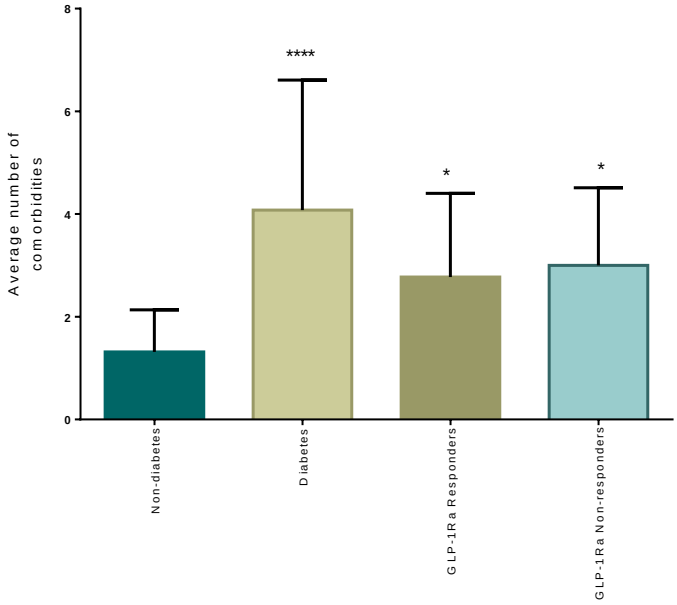


Figure 7.4 – **Number of chronic conditions reported by participants of the GLP-1 study cohort (including diabetes).** Graph shows non-diabetes controls (n=19), diabetes controls (n=13), GLP-1Ra responders (n=22) and GLP-1Ra non-responders (n=15). Significance was determined using one-way ANOVA and Tukey's post hoc test. *P<0.05, ****P<0.0001 vs non-diabetes.

Circulatory disorders contributed 4% of reported comorbidity in non-diabetes controls compared to 12% (P<0.05) in diabetes controls, 20% (P<0.05) in GLP-1Ra responders and 23% (P<0.01) in non-responders. Musculoskeletal disorders were more common in non-diabetes (24%) and diabetes controls (GLP-1 naïve, 20%) compared to GLP-1Ra responders (12%, P<0.05), and non-responders (0%, P<0.01). In contrast endocrine and nutritional disorders were less common in non-diabetes (8%) and diabetes (7%), when compared to GLP-1Ra positive patients (Responders;

18%, non-responders 17%). Neoplasms made up a higher percentage of reported comorbidity in non-diabetes controls (12%), when related to diabetes controls (5%), responders (7%) or non-responders (3%) (Fig. 7.5).

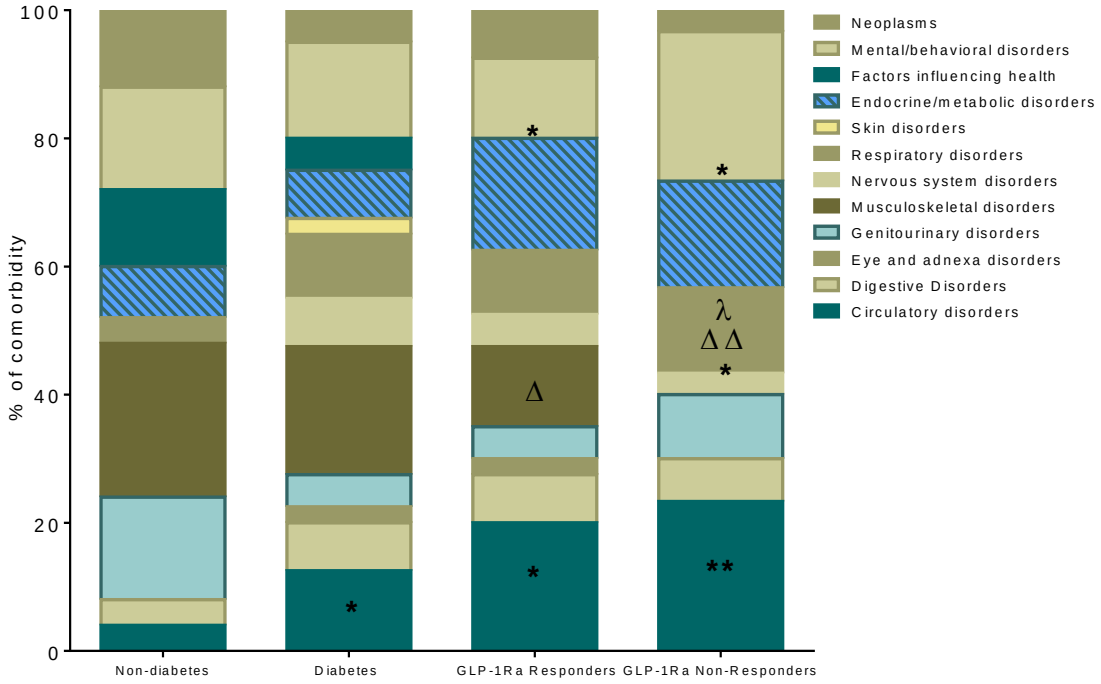


Figure 7.5 – **Percentage of comorbidity type reported by participants of the GLP-1 study.** All comorbidities were coded according ICD-10 classification system. Graph shows non-diabetes controls (n=19), diabetes controls (n=13), GLP-1Ra responders (n=22), GLP-1Ra non-responders (n=15). Significance was determined using a 2 proportion Z-test. *P<0.05, **P<0.01 vs. non-diabetes controls. ΔP<0.05, ΔΔP<0.01 vs. Diabetes controls. λ P<0.05 vs GLP-1 Responders.

7.3.4 Depression and anxiety in GLP-1 analogue therapy

As illustrated in Figure 7.6 GLP-1 analogue non-responders displayed significantly increased scores (P<0.05) in the Beck depression Inventory (Fig. 7.6A) and Beck Anxiety Inventory (P<0.05, Fig. 7.6B) compared to non-diabetes controls. No significant differences were observed in other groups.

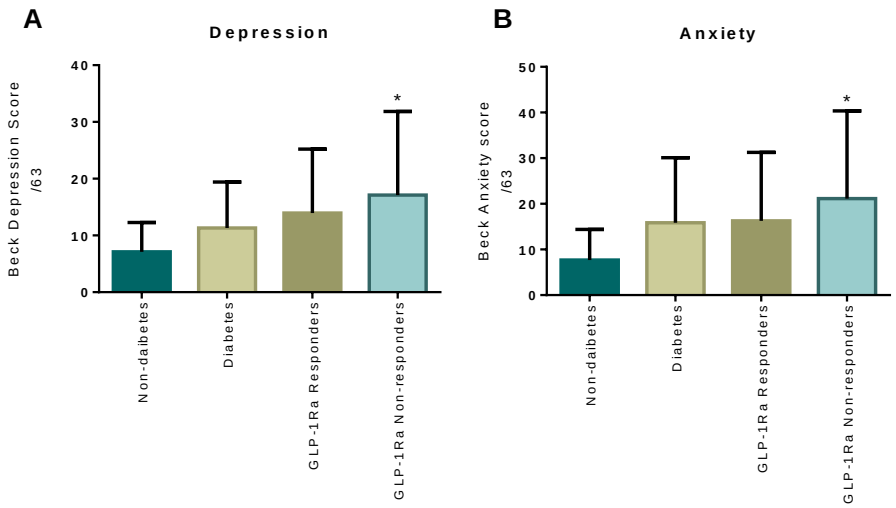


Figure 7.6 – **Beck Depression and Anxiety scores in GLP-1 analogue responders and non-responders.** Beck depression inventory (A), and anxiety inventory (B) scores in non-diabetes controls (N=19), diabetes controls (n=13), GLP-1 analogue responders (n=22) and GLP-1 analogue non-responders (n=15). Maximum score in both inventories was 63. Significance was determined using one-way ANOVA and Tukey's post hoc test. *P<0.05 vs. non-diabetes control.

7.3.5 Cognitive function

7.3.5.1 The Standardized Mini-Mental State Examination (SMMSE)

Total scores in SMMSE were high, non-diabetes control participants scored 29.3 ± 1.2, diabetes control patients scored 29.2 ± 1.2, T2D GLP-1Ra responders 28.5 ± 2.5 and non-responders 27.7 ± 1.9. There was no difference between the groups' total scores. Aphasia (Q6) was affected in diabetes controls and GLP-1 analogue non-responders (P< 0.05), both groups scored ~20% more on these questions compared to controls and GLP-1Ra responders. GLP-1 analogue non-responders exhibited a ~30% reduction in scores obtained in questions associated with memory and perception compared to controls, diabetes controls, and GLP-1 analogue responders (Q11, P<0.01-P<0.0001). There were no differences in scores in questions associated with orientation, registration, attention, perception, and short-term memory (Fig. 7.7).

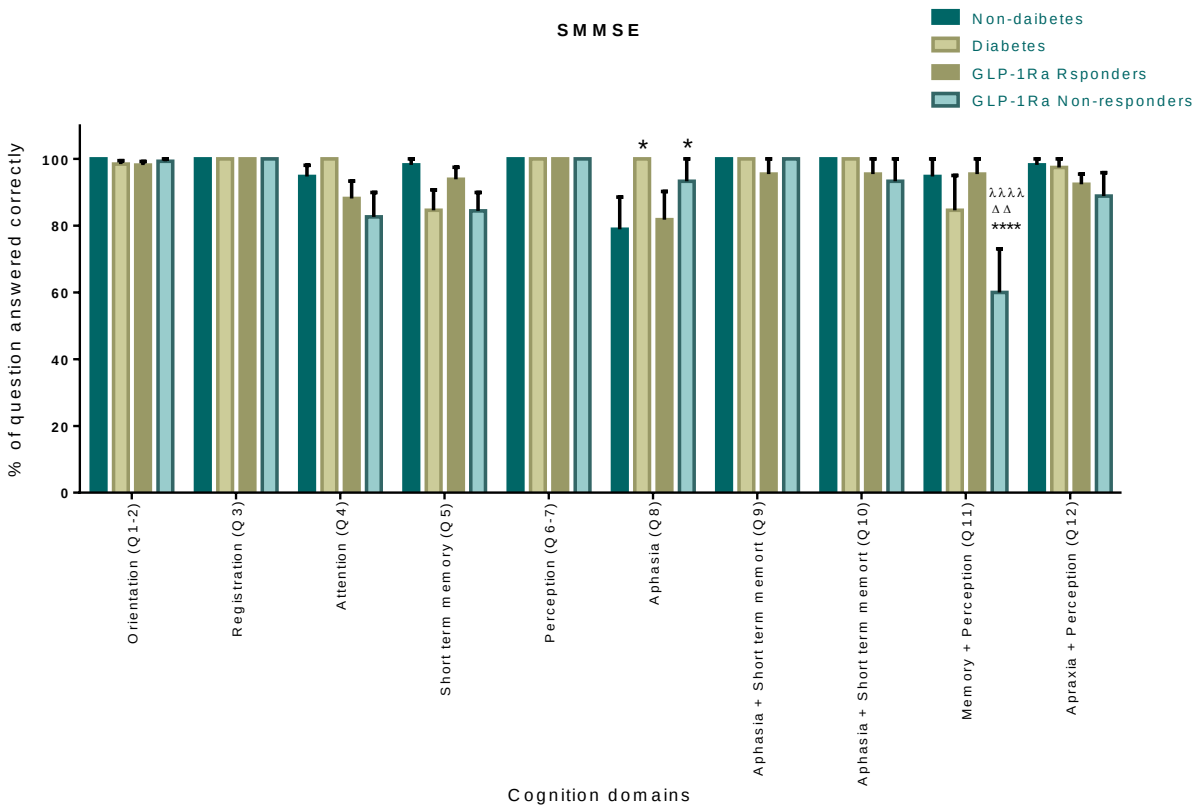


Figure 7.7 – **Difference in SMMSE scores across different cognitive domains.** Illustrated are scores associated with different cognitive domains within the SMMSE. Non-diabetes control (n=19), diabetes control (n=13), GLP-1 analogue responders (n=22), GLP-1 analogue non-responders (n=15). The total score for the MMSE is 30, while each domain represents a different number of marks. Data presented as a percentage of the question answered correctly (Q1=5, Q2=5, Q3=3, Q4=5, Q5=3, Q6=1, Q7=1, Q8=1, Q9=1, Q10=1, Q11=1, Q12=3). Significance determined using two-way ANOVA and Holm-Šídák's post hoc test. ****P<0.0001 vs non-diabetes. ΔΔ P<0.01 vs Diabetes. λλλλ P<0.0001 vs Responders.

7.3.5.2 The Quick Mild Cognitive Impairment screen (qMCI)

Overall scores in qMCI were not significantly different between groups, however non-diabetes controls scored 73.2 ± 6.9, diabetes controls scored 69.0 ± 4.6, diabetes GLP-1Ra responders 69.7 ± 10.5 and non-responders 67.5 ± 11.3. GLP-1 analogue non-responders displayed reduced delayed recall (Q4, ~17%, P<0.05) and logical memory (Q6, ~12%, P<0.05) compared to non-diabetes controls. Diabetes controls also displayed reduced logical memory compared to controls (Q6, P<0.05), while GLP-1 analogue responders performed similarly to controls. Orientation, registration visuospatial and verbal fluency were no different between groups (Q1, 2, 3, 5, Fig. 7.8).

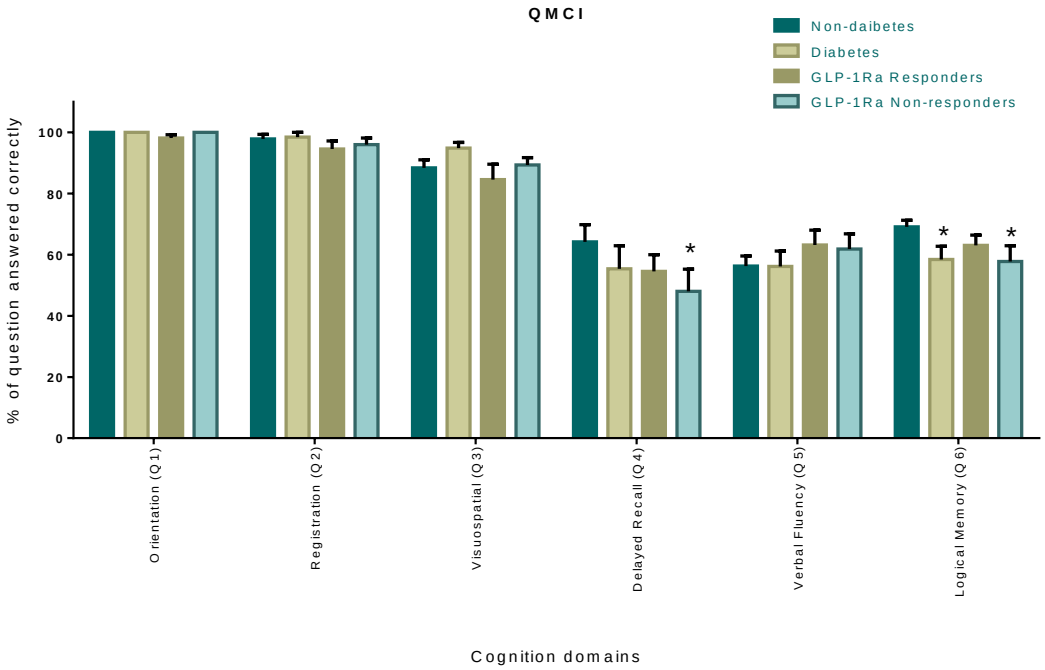


Figure 7.8 – **Difference in qMCI scores separated by cognitive domains.** Illustrated are scores associated with different cognitive domains within the qMCI. Non-diabetes controls (n=19), diabetes controls (n=13), GLP-1 analogue responders (n=22), GLP-1 analogue non-responders (n=15). Test scored out of 100, each domain represents a different number of marks. Data presented as a percentage of the question answered correctly (Q1=10, Q2=5, Q3=15, Q4=20, Q5=20, Q6=30). Significance determined using two-way ANOVA and Holm-Šídák's post hoc test. *P<0.05 vs non-diabetes.

7.3.6 Relationship between BMI and HbA_{1c} and depression, anxiety SMMSE and qMCI scores

Correlation analysis determined that BMI significantly correlated with Beck depression inventory score (R=0.22, P<0.02, Fig. 7.9A) but not anxiety (R=0.16, P=0.18, Fig. 7.9C), SMMSE (R=-0.23, P=0.054, Fig. 7.9E) or qMCI (R=0.06, P=0.62, Fig 7.9G). HbA_{1c} did not significantly correlate with Beck depression (R=0.21, P<0.08, Fig. 7.9B) or anxiety (R=0.22, P<0.07, Fig. 7.9D) inventory scores, nor with SMMSE performance (R=-0.08, P=0.48, Fig. 7.9F). The qMCI, however, showed a highly significant negative correlation with HbA_{1c} (R= - 0.53, P<0.0001, Fig. 7.9H).

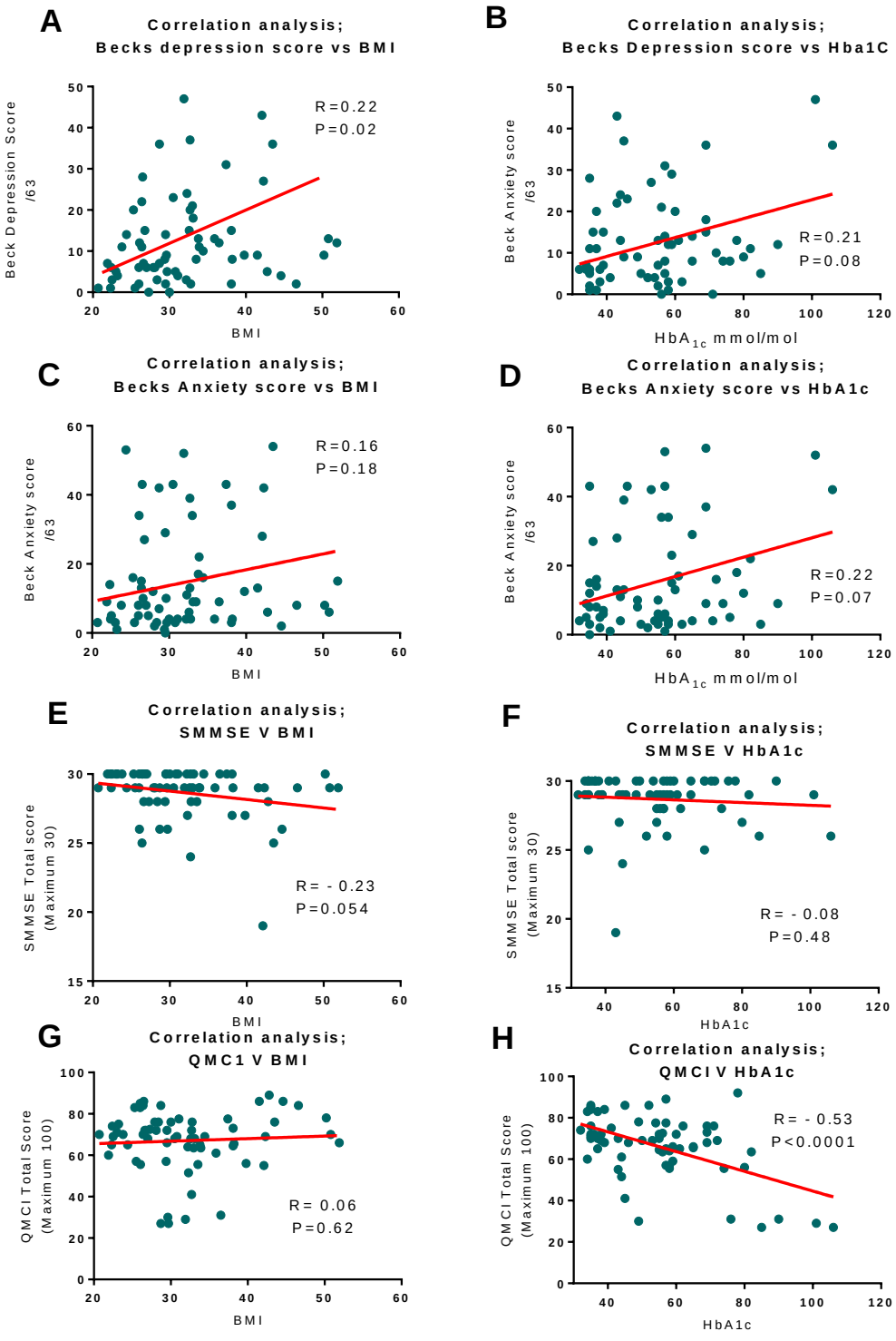


Figure 7.9 – **Correlation analysis between Beck Depression and Anxiety inventories, SMMSE, qMCI and HbA_{1c} and BMI.** (A) Beck depression scores vs. BMI. (B) Beck depression scores vs. HbA_{1c}. (C) Beck anxiety scores vs. BMI. (D) Beck anxiety scores vs. $HbA_{1c.}$ (E) SMMSE scores vs. BMI. (F) SMMSE scores vs. HbA_{1c}. (G) qMCI scores vs. BMI. (H) qMCI scores vs. HbA_{1c}. All data from non-diabetes controls (n=19), diabetes controls (n=13), GLP analogue responders (n=22) and GLP-1 analogue non-responders (n=15) were plotted. Data presented as scatter plots with linear regression best line fitted. R value representative of relationship.

7.3.7 Mood and Neutrophil correlations

Due to the high degree of neutrophil variation between groups, cell number was correlated with Beck depression and anxiety scores and SMMSE and qMCI total scores. Both depression (R=0.27, Fig. 7.10A) and anxiety (R=0.36, Fig. 7.10B) scores were strongly positively correlated with neutrophil number (P<0.0001), however neither SMMSE (Fig. 7.10C) nor qMCI (Fig. 7.10D) showed significant correlation with neutrophil number.

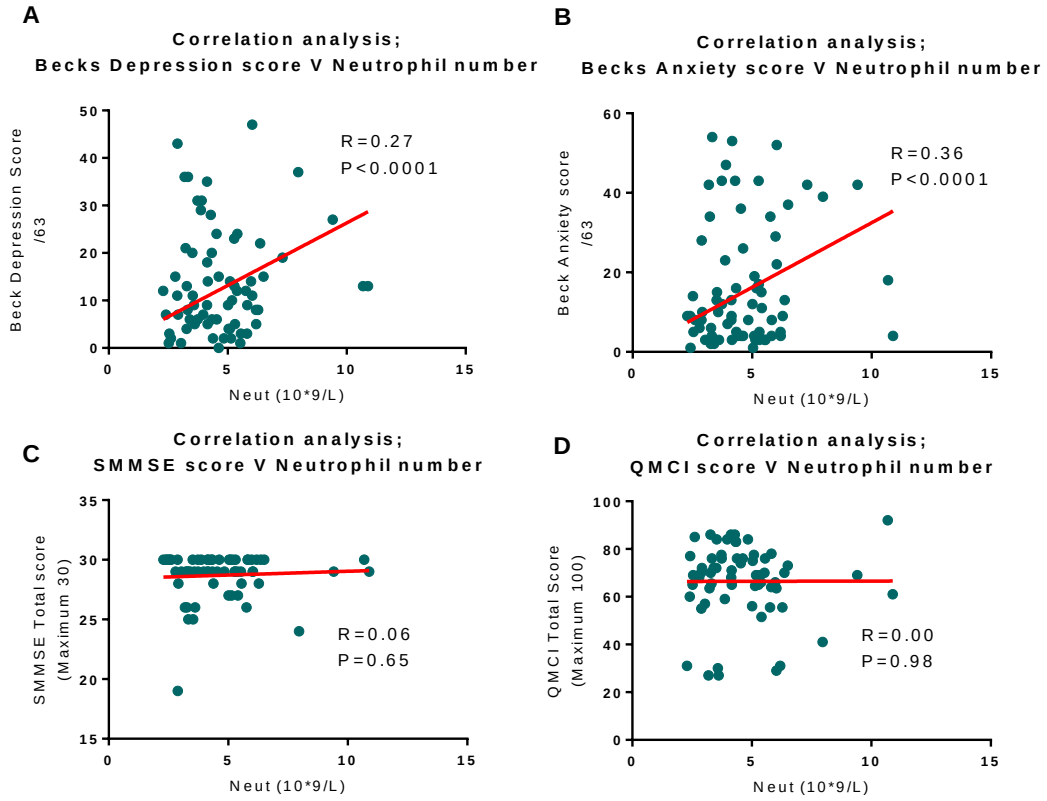


Figure 7.10 – **Neutrophil count is positively correlated with Beck Depression and Anxiety scores.** (A) Depression score vs. Neutrophil count. (B) Anxiety score vs. neutrophil count. (C) SMMSE scores vs. Neutrophil count. (D) qMCI scores vs. Neutrophil count. All data from non-diabetes controls (n=19), diabetes controls (n=13), GLP analogue responders (n=22) and GLP-1 analogue non-responders (n=15) were plotted. Data presented as scatter plots with linear regression best line fitted. R value representative of relationship.

7.4 Discussion

This clinical study assessed depression, anxiety and cognitive function in T2D patients treated with GLP-1R analogues. Patients were grouped depending on whether they were responding or not responding with respect to their HbA_{1c} (-1%) and/or weight (-3%), as stipulated by NICE (NICE, 2015). Prescribed medications, HbA_{1c}, full blood count, comorbidity records, and cognition measures (SMMSE, qMCI), depression and anxiety inventory scores were compared in GLP-1Ra responders and non-responders, non-diabetes controls and diabetes controls who were GLP-1Ra naïve.

All diabetes groups were predominantly, unintentionally male (diabetes controls 62%; GLP-1Ra responders 59%; GLP-1Ra non-responders 80%), and males made up ~50% of the non-diabetes control group. This is not unusual, studies have shown diabetes to be more prevalent in males (van Zon et al., 2017). The only group to differ in age was the diabetes GLP-1Ra non-responders who were younger than the rest of the cohort. Both groups of GLP-1Ra treated patients were also significantly heavier than non-diabetes controls and diabetes controls. This may also be expected as GLP-1Ra are commonly prescribed to severe or late stage T2D patients, and are recommended for obese individuals (NICE, 2015). GLP-1 analogues have been shown to be an effective weight loss therapy (Nuffer and Trujillo, 2015). GLP-1Ra non-responders had a higher HbA_{1c} than the responder group, GLP-1 is known to affect glycaemia (Iepsen et al., 2015b). There were no differences between the groups in the average age participants left school. Interestingly, research has shown education does not affect dementia risk, or either assessments ability to differentiate between MCI and Alzheimer's (Sharrett, 2012, O'Caoimh et al., 2012).

Diabetes patients were prescribed ~12 medications irrespective of their diabetes treatment regime, while non-diabetes controls were prescribed 2. Our previous work (*Chapter 4*) and a number of other studies have indicated that this difference is due to the high degree of comorbidity present in diabetes (Jelinek et al., 2017). GLP-1Ra non-responders were on more anti-diabetes medications than the diabetes control GLP-1Ra naive group, while GLP-1Ra responders were not. Our previous results indicated GLP-1Ras are prescribed as part of dual or triple therapy,

likely due to early, aggressive weight and glucose management plans in line with American Diabetes Association advice (Chamberlain et al., 2017a).

A full blood count was determined for all participants. It is well established that haemoglobin, platelet and WBC levels are associated with a number of conditions (George-Gay and Parker, 2003). WBCs are often used as markers of cancer (low levels) (Ong et al., 2017) or infection (high levels) (Rempel et al., 2013), but studies have shown strong correlations with diabetes progression (Vozarova et al., 2002). In this study we positively correlated WBC levels to HbA_{1c}. This is not unusual as diabetes pathogenesis is associated with chronic low grade inflammation (Duncan et al., 2003), driven by insulin resistance (Saltiel and Olefsky, 2017). Other clinical studies have reported elevated WBCs in glucose metabolism disorders (Jiang et al., 2014). This study indicated WBCs were elevated in diabetes patients compared to non-diabetes controls, and to a greater extent in GLP-1Ra naïve diabetes patients. Although significance could not be determined between GLP-1Ra treated and naïve patients at this interim stage, data is suggestive that GLP-1Ras impact WBC number, possibly due to anti-inflammatory effects (Hou et al., 2012). WBC differentiation showed neutrophils were the only cell type that was altered in diabetes, following a similar trend to the total WBC number. Neutrophils are part of the innate immune system, and fight infection (Lumeng, 2013). High levels have been shown to be indicative of non-infectious inflammation (Soehnlein et al., 2017), stress, and various other chronic conditions (Scott et al., 2011a). In the data reported here the highest levels were seen in GLP-1Ra naïve diabetes patients who also have the highest number of comorbid chronic conditions.

GLP-1Ra non-responders had the highest percentage of circulatory and endocrine disorders, both of which, greatly affect quality of life (Ruo et al., 2003) and are linked to the highest rate of mortality in T2D (Micha et al., 2017). This is reflected in the Beck depression and anxiety inventory scores; non-responders were the only group to score significantly higher than the non-diabetes controls. Many studies have linked comorbidity, and quality of life to mood (Naicker et al., 2017). The high degree of cardiovascular and endocrine related conditions in non-responders appears to affect both depression and anxiety scores. These results support findings highlighting the importance of depression in the management of diabetes (Verma et

al., 2010), and highlights the importance of effectively managing glycaemia (Schmitt et al., 2017).

In cognitive assessments total scores were not different between groups, although individual cognitive domains varied. Non-responders scored lower in questions relating to memory and perception, when compared to all other groups. GLP-1Ra are well-established as having positive cognitive effects in preclinical studies (McClean and Holscher, 2014), but these effects are poorly reported in clinical cohorts. Similar findings were found when assessing cognitive domains using the qMCI. No difference could be determined between total scores; however diabetes controls (GLP-1Ra naïve) and GLP-1Ra non-responders, scored lower than non-diabetes controls in delayed recall and logical memory. The qMCI has been shown by many studies as being highly sensitive at differentiating mild cognitive impairment, with delayed recall and logical memory being the most influential components (O'Caoimh et al., 2012). GLP-1Ra responders do not differ from controls in these domains, and therefore GLP-1 analogues may be protective in terms of cognitive decline in T2D in a clinical setting. This has not been previously reported; we await findings from ongoing studies to verify this result.

The data infers GLP-1Ra response, comorbidity, mood and cognition are linked. We conducted correlation analysis of BMI and HbA_{1c} to Beck depression and anxiety scores and observed that only depression scores positively correlated with BMI. This was surprising as weight and glycaemia have both been linked to quality of life, diabetes complications (Steele et al., 2016) and mood (Nigatu et al., 2016); these findings are likely a result of small sample numbers recruited to date (~n=20). Correlation analysis of BMI and HbA_{1c} vs. SMMSE and qMCI demonstrated that qMCI was strongly negatively correlated with HbA_{1c}. This supports previously reported links between poor glycaemic control and cognitive decline (van den Berg et al., 2009). Neutrophil levels were also correlated with depression and anxiety inventories, SMMSE and qMCI. A highly significant positive correlation was recorded between neutrophil number depression and anxiety. Other studies have reported similar correlations in neutrophil to lymphocyte ratio in depression (Aydin Sunbul et al., 2016). Neutrophil levels, however, did not correlate with scores in SMMSE and qMCI, which is of interest. The present study indicates WBC counts and neutrophils

in particular are indicative of depression and anxiety in T2D, and warrants further investigation.

The main limitation of this study is the small group sizes, as this represents an interim analysis. This limited sensitivity to distinguish alterations in cognitive domains and small differences in depression and anxiety scores. All comorbidities and BMI records were self-reported by participants, which allows for a degree of unintentional inaccuracy. A true definition of medication response was difficult to determine retrospectively; response was determined using electronic health record data. Prospective cohorts will definitively assess the efficacy of GLP-1 analogues on cognitive outcomes.

This interim analysis provides an early indication that GLP-1Ra response has a positive effect on comorbidity, depression, anxiety and cognitive performance, specifically related to memory and perception. WBCs and neutrophil number may also be a useful measure of T2D, depression and anxiety severity.

Chapter VIII

Discussion

In this thesis we hypothesised that current clinical diabetes measures insufficiently stratify patients, ultimately resulting in poor anti-diabetes pharmacological response, comorbidity and cognitive decline. The identification of novel peripheral genetic and proteomic biomarkers indicative of T2D and response to GLP-1 analogue therapy may be used to target GLP-1 therapy towards known responders, and provide rationale for the use of GLP-1 analogues earlier in the T2D disease course.

8.1 Summary of findings and field advancements

In the third chapter transgenic animal models were used to define molecular mechanisms downstream of the GLP-1R that may be indicative of drug response in brain and whole blood. Initial results supported other work that indicated GLP-1 acts on the melanocortin system (Goldstone et al., 1997a), and has insulin sensitising effects (Idris et al., 2002). Results confirmed relevance of all 13 genes for future analysis in clinical populations. All genes of interest were affected by a 70% knock down of the GLP-1R in the GLP-1 RKO mouse model. Early Aβ deposits that have been shown in young APP/PS1 mice also affected the panel (Radde et al., 2006). Target gene expression in C57BL/6 mice was highly sensitive to treatment duration. After 7 days most genes were upregulated and after 21 days all genes were globally down regulated. A relationship was identified between blood and brain gene expression. Peripheral levels after 1-7 days GLP-1Ra treatment mirrored what was recorded centrally after 3 weeks.

In the fourth chapter we assessed currently available biochemical and anthropometric measures, and the clinical profiles of 500 T2D patients (DiaStrat) in the Western Trust. Data from electronic health records and primary insulin and C-peptide ELISA data was used. We observed that glycaemia and bodyweight were poorly managed. Average HbA_{1c} was 65 mmol/mol (8.1%), and 93% of patients were overweight. CVD was the most common comorbidity (25%), but hyperlipidaemia was well managed. It was evident from prescribing data that there was major deviation from NICE guidance (NICE, 2015). Cardiovascular disease (CVD) management appears to be prioritised over glycaemic control and bodyweight. We also demonstrated that C-peptide is a useful measure of diabetes and comorbidity

severity, and routine measurement should be incorporated into the clinic in addition to the regular monitoring of HbA_{1c}.

In chapters five and six proteomic and genetic biomarkers were identified using Proximity Extension Assays (PEA, OLink proteomics), that included 368 proteins, and analysis of SNPs within 20 insulin and leptin related genes. Multiple specific protein panels were identified for diabetes, obesity, hyperglycaemia, common comorbidities, and diabetes drug response. There were proteins linked to leptin and insulin signalling, however, data enrichment (Enrichr) identified pathways involved in vascular endothelial damage as being the common feature between the proteomic markers for all quantified measures. We identified 105 SNPs in insulin and leptin related genes, that could discriminate between T2D and healthy controls, most of which were novel, with some previously reported on the T2D portal (T2D-GENES Consortium, 2017). There were 25 SNPs identified specific to GLP-1Ra responders, with one highly specific SNP present on the GLP-1R.

In chapter seven we further assessed differences between GLP-1Ra responders and non-responders and the effect GLP-1Ras have on mood and cognitive decline. Corresponding with findings in the literature, our data indicated that GLP-1Ra non-responders were at a higher risk of comorbidities (Sleiman, 2012), and had significantly higher depression and anxiety scores. Total scores for both SMMSE and qMCI were no different between groups, but cognitive domains relating to memory and perception were negatively affected in GLP-1Ra non-responders.

8.2 Strengths and limitations

8.2.1 Preclinical

All experiments in chapter 3 were designed *in vivo*, to gain as much translational reliability as possible (Burkhardt and Zlotnik, 2013). Animal models are known to be a reproducible method for investigating disease and response mechanisms (Justice and Dhillon, 2016) that are less expensive and invasive than human trials (Vandamme, 2014).

We used three mouse models C57BL/6, GLP-1RKO and APP/PS1, which enabled us firstly to assess potential biomarkers in response to GLP-1Ra treatment in

WT, and secondly to define if these effects translated and could be used in a model of AD. These effects were not measured in a model of diabetes or obesity. A streptozotocin induced T2D model (Gilbert et al., 2011), or a HFD induced model of obesity (Wang and Liao, 2012) could provide valuable insights and help define links between obesity/T2D and AD and assess the possibility that biomarkers of GLP-1R agonist response may be common between conditions.

Central gene expression was detected consistently and accurately, while peripheral gene expression was possible for all genes, excepting GHSR. Peripheral data was more variable; this was most likely due to the small sample volume (50 µl) and sample number (n=5). There was also a discrepancy between cDNA load (25 ng in brain, 5 ng in blood); despite being normalised to internal control this may have affected variability observed. This work determined a relationship exists between the blood and the brain for our genes of interest. It is worth noting the blood and brain from the same mice were used for comparative experiments to reduce any inter animal variability, which has been documented in other work (Han et al., 2004).

8.2.2 Clinical

8.2.2.1 ECR Research

Diabetes participants were recruited from secondary care and all were registered to the Western Health and Social Care Trust. This experimental design allowed us to define the effect of severe diabetes on prescribing trends and comorbidity, allowing better stratification of this high-risk group. Future studies should focus on recruiting from primary care to enable comparisons between proteomic and genetic changes in pre-diabetes, newly diagnosed T2D and late T2D (Jiao et al., 2017). This may facilitate more efficient generation of preventative and early diabetes markers, and would permit prospective assessment of first line therapy response.

A strength of the DiaStrat study was access to multiple ECR systems, which covered all appointments of the participants in primary and secondary care. Computerised prescription records, drug interaction data, and longitudinal medical history information (Menachemi and Collum, 2011) allowed for effective clustering dependent on various biochemical and phenotypic characteristics. We first mined

the Diamond diabetes management solution by Hicom (HiCom, 2017). This is a web based application hosted by the Health Trust. From a research perspective, Diamond and other ECRs provided opportunities for participant identification (Casey et al., 2016), clinicians were able to effectively screen research exclusion and inclusion criteria, and contact potential participants. Access to recruited participants medical records allowed for novel epidemiological insights into disease history, drug application and the effects of comorbidity (De Moor et al., 2015).

The main limitation of the Diamond system at Altnagelvin is that it is solely focused on diabetes and is region specific. A lack of information relating to primary care or other health systems being used in the hospital resulted in a simplified, disease specific picture of healthcare (HiCom, 2017). In the DiaStrat study only T2D patients could be identified and recruited through Diamond. This limited our findings as there was no non-diabetes control ECR data to compare our data to.

Due to the limitations of the Diamond system we also utilised a second ECR, the Northern Ireland Electronic Care Record (NIECR) by Orion health (Orion Health, 2017). NIECR encompassed all primary and secondary care visits, and links all regional patient records across Northern Ireland (Orion Health, 2017). Diamond was incorporated into this system in 2016, ensuring no data loss. All data input was manually transferred from ECR systems to a separate spreadsheet for analysis. Manual data input increases the risk of human error (Menachemi and Collum, 2011), therefore quality control checks were carried out between diamond an NIECR data records to ensure the highest level of accuracy. The nature of electronic systems means multiple sources have access to patient accounts and are required to fill in patient interactions accurately. Different departments with different data input process can lead to a degree of error, or missing data (Menachemi and Collum, 2011). This factor was taken into account when analysing the data. Patients with missing data entries were excluded from specific cluster analysis.

8.2.2.2 Proteomic screening

In Chapter 5 we outsourced proteomic analysis to OLink proteomics, allowing for rapid high throughput targeted discovery of protein markers. This company is a specialist in the field of protein analysis, with advanced equipment and technical expertise not available at Ulster University (OLink, 2017). Utilising OLink services allowed for quick and reliable analysis of 374 T2D samples, and 20 non-diabetes control samples. Proximity Extension Assay (PEA) technology ensures precise and sensitive detection with a broad dynamic range (Assarsson et al., 2014). The array only requires 1 µl of sample (OLink, 2017).

Plasma proteome analysis was chosen, due to its minimal invasiveness (Dayon, 2013). Blood sampling is regularly conducted in the diabetes clinic, therefore provision of blood samples tends not to distress participants (NICE, 2017). Blood is a complex body fluid which contains a large diversity of proteins (Muthusamy et al., 2005), many are emitted by cells under various pathological or physiological processes (Anderson and Anderson, 2002). It is thought plasma is a promising source of peripheral biomarkers due to its circulation and contact with various tissues. It is likely a good indicator of overall health (Etzioni et al., 2003). Our results indicated that 92% of 368 screened proteins met QC and were detected. This is particularly high, much research has indicated that plasma can be a challenging tissue to screen (Omenn, 2006). It is acknowledged that a high degree of variability in the concentration and activation state of plasma proteins exists between patients (Nedelkov et al., 2005). There is a complex dynamic range of proteins in plasma, large plasma proteins; albumins (55%), globulins (38%) and fibrinogen (7%) make up the majority of total protein concentration (Tu et al., 2010), while low abundance hormones, cytokines and lipoproteins are usually difficult to detect (Anderson and Anderson, 2002). Fractionation techniques are usually carried out on samples to remove these proteins (Burnouf, 2007), but were not required for OLink analyses (OLink, 2017).

There are three main limitations associated with the data captured within this chapter. The first is that clustering of patients was dependent on ECR data available at the time of sampling. Some measures may have been within weeks of

sampling date which may have introduced a degree of error to a patients classification. Inaccurate grouping would confound observations reported. For validation studies ensuring accurate classification would be preferable; taking additional blood samples and carrying out relevant biochemical tests on the same day would address this issue. Secondly samples were taken at one time point, and protein-protein; protein-gene multivariate interaction analysis was not conducted. Sampling patients longitudinally would permit repeated measures analysis of how proteins change over time, and in response to interventions, improving diagnostic and prognostic accuracy. Assessing the relationship between proteins and genes would provide important and insightful information that could improve our understanding of disease (Jones and Thornton, 1996).

8.2.2.3 SNP genotyping of insulin and leptin associated genes

In chapter six 266 T2D whole blood DNA samples and 19 non-diabetes control samples were outsourced to Cambridge Genomic Services for genetic screening using the Affymetrix UK Biobank SNP array (UK BioBank, 2017). There are 820,967 SNP and indel markers on the array, that include ~95,500 markers of particular interest in various diseases, linked to phenotypic variation and 112,000 coding variants, with remaining content (629,000) covering a variety of genome wide markers found in Caucasian populations (UK BioBank, 2017).

Comparisons of SNP genotypes in 20 genes of interest were made between individuals with T2D and non-diabetes controls, with 105 SNPs indicative of diabetes. Comparisons were also made between T2D GLP-1Ra responders and non-responders, with 25 SNPs identified that significantly influenced response.

The main limitation associated with this experiment was sample size. Many studies have shown that to detect the influence of biological and technical variation, and rare biological variants large sample sizes are required (Nielsen et al., 2011). The non-diabetes control group consisted of 19 participants and was compared to 266 individuals with T2D, which enabled a satisfactory differentiation rate. The T2D GLP-1 responder and non-responder groups had ~20 participants; despite identifying some impressive SNP genotype differences, such low numbers may have affected

the detection of SNPs with low minor allele frequencies (Nielsen et al., 2011). Findings observed here will be validated in larger populations to determine their reproducibility, and further analysis is required on other SNPs associated with T2D and response to GLP-1 analogue therapy.

8.2.2.4 GLP-1 response study

In chapter 7 the effect of GLP-1Ra drug response on cognition, depression and anxiety was determined using SMMSE and qMCI assessments and Beck depression and anxiety inventories. The SMMSE and qMCI examinations are well accepted measures of cognition (O'Caoimh et al., 2012). The SMMSE is widely used in clinical practice to determine cognitive function (Molloy et al., 2005), but is known not to assess decision making abilities, limiting its usefulness in depressed, psychotic or frontal lobe disease patients (Freedman et al., 1991). The qMCI is more sensitive than the SMMSE for determining MCI particularly in older adults (O'Caoimh et al., 2012), attributed specifically to questions relating to episodic memory (O'Caoimh et al., 2013). Education level does not alter the ability of either examination to discriminate between dementia and MCI (O'Caoimh et al., 2012). The Beck depression and anxiety inventories are well established measures (Lovibond and Lovibond, 1995), used across various health services and in a number of disease areas (Beck et al., 1997, Richter et al., 1998). The main strength of this study was the recruitment of multiple control groups to normalise findings to GLP-1Ra response. Non-diabetes controls, diabetes controls (GLP-1Ra naïve) and diabetes GLP-1Ra non-responders were compared to diabetes GLP-1Ra responders. All cognitive, mood and blood samples were acquired at one appointment to ensure correlations were reflective of that time point. Official cognitive assessment scripts and protocols were followed for all participants to minimise appointment condition variability (Molloy and Standish, 1997, O'Caoimh et al., 2012).

The main limitation of this study was the small sample number, most groups were <n=20. This likely affected sensitivity and the ability to differentiate between the groups for cognitive, depression and anxiety screening instruments. Secondly, defining responders and non-responders is difficult. NICE define response as either a 1% reduction HbA_{1c} or a 3% reduction in body weight after 6 months exposure to

GLP-1 analogues (NICE, 2015). Using the ECR we found patients were kept on GLP-1Ra irrespective of response, if a single criteria was met, or if they had previously responded, and the drug is maintaining glycaemia or weight. Response was therefore subjective, and was defined on this study as 'currently responding' with respect to glycaemia, bodyweight or both.

8.3 Potential translational impact

Elevated or poorly controlled blood glucose, poor pharmacological response and resultant comorbidity are the main causes of poor quality of life and mortality in T2D (Cavalot et al., 2011). Diabetes biomarkers such as HbA_{1c} have not changed since 1975 (Tattersall et al., 1975). HbA_{1c} is still considered a good marker in terms of monitoring glycaemic control (NICE, 2015), but there is much room for improvement in diabetes management. Secondary complications and comorbidities are associated with significant personal and economic burdens. Strategies to predict or measure response to therapy more effectively, and better understand the development of comorbidity will improve outcomes for patients and reduce overall healthcare costs in the long term.

In chapter 4 we demonstrated that current biomarkers have limited predictive capability when correlated with disease progression, prescribing patterns and multimorbidity. Evidence was presented that suggests glycaemic control and bodyweight are ineffectively controlled compared to blood lipids and blood pressure. This type of epidemiological information may better inform clinical practice and policy; there is certainly an argument for the introduction of obesity clinics to manage the high levels of obesity in the same manner that CVD risk has been targeted. This work also highlighted the need for new and specific genetic and protein biomarker panels that are capable of predicting risk associated with poor glycaemic control and comorbidity and allowing for more accurate stratification of T2D patients

In diabetes, complications develop slowly over time (Iglay et al., 2016); we found evidence of this in Chapter 4 with most comorbidities linked to longer diabetes duration and older age. The use of ECR data in conjunction with biomarker

discovery techniques in chapters 5 and 6 permitted clustering analysis across numerous T2D and associated comorbidity variables. In this study we identified specific SNPs and proteins for T2D and GLP-1Ra response and investigated alterations and correlations with various biochemical measures. These novel findings have the potential to identify individuals at risk of T2D and assess the management of glycaemic control and predict onset of comorbidity in individuals with a T2D diagnosis. We report here, for the first time, a number of SNPs that are highly significantly associated with response to GLP-1Ra. SNP biomarkers, once reproducibility is confirmed in secondary cohorts, could allow for prediction of response to therapy. This would permit more efficient prescribing and reduced NHS costs by reducing the onset of comorbidities and secondary complications associated with non-response in those who will derive no clinical benefit.

Results from Chapter 7 indicate that response to GLP-1 analogues, from a diabetes perspective i.e. in those who achieve reductions in HbA_{1c} and bodyweight, may also be associated with reduced depression, anxiety and cognitive decline. This study is ongoing and more power is required to support the preliminary trends observed. Such a finding has high clinical and translational relevance due to the impact cognition has on drug regime adherence and disease progression in T2D (Iglay et al., 2017).

8.4 Future direction

From a preclinical perspective (Chapter 3) further profiling of peripheral leptin and insulin gene expression in response to liraglutide is required. A larger sample number would reduce the variability observed in peripheral gene expression data and permit more accurate definition of drug response. Genes of interest were expressed at much higher levels in brain than in blood, but we did not definitively determine whether transcriptional changes in brain affects blood mRNA or *vice versa*; it would be interesting to assess gene expression at the cellular level in blood using flow cytometry (Letzkus et al., 2014). It is possible that central and peripheral gene expression, in response to pharmacological treatment, is related to BBB function (Huntley et al., 2014); it would be interesting to assess this in future work. The effect of liraglutide in the APP/PS1 genotype should be expanded to include an

untreated APP/PS1 control and also liraglutide-treated and untreated diabetes model groups; this could provide valuable insights in the shared pathophysiology between T2D, AD (Luchsinger, 2010) and GLP-1Ra response.

Chapter 4 would be strengthened by comparing findings to an age and sex matched non-diabetes control population. This would allow for more informative baseline comparisons to be made on the effect of T2D on BP, BMI, blood lipids, comorbidity and prescribing. A large non-diabetes control population would also allow increased sensitivity when analysing the proteomic and genetic profiling data in chapters 5 and 6 respectively. This could enable identification of more proteomic changes and SNP alterations with low minor allele frequencies to be detected. (Steinthorsdottir et al., 2014). In chapter six there is a possibility of missing significant findings in our genes of interest by using a SNP array. In future validation experiments, with larger participant numbers, whole genome sequencing should be conducted to able exon and intron spanning analysis (Kalari et al., 2006). This will increase insights into variation in the genes of interest associated with T2D and GLP-1Ra response. All proteomic and genetic findings will be validated in larger multiregional cohorts to further assess accuracy and reliability.

The Chapter 7 GLP-1 response study is ongoing. Further recruitment should increase power significantly. Follow up work is also planned to prospectively follow individuals prescribed GLP-1 analogues to permit validation of biomarkers identified in Chapters 3, 5 and 6. RNA samples were collected for all participants of the study. The leptin and insulin markers screened *in* vivo (Chapter 3) will be assessed in human samples via qPCR to assess their clinical utility, and the models translational capacity.

8.5 Conclusion

Leptin and insulin related genes can be detected centrally and peripherally *in vivo*. Peripheral levels after 7 days were indicative of central changes after 3 weeks and may act as putative markers of GLP-1Ra response in diabetes and, potentially, Alzheimer's disease. ECR analysis of 500 T2D patients demonstrated current diabetes measures, HbA_{1c}, BMI and blood lipid profile, were ineffective at predicting and managing T2D and associated comorbidities. C-peptide may act as a valuable

addition in the clinic for determining disease progression, and potentially drug response. New proteomic and genetic biomarkers capable of differentiating T2D and non-diabetes controls were identified. We also identified specific and sensitive protein markers to comorbidity onset and proteomic and SNP markers associated with GLP-1Ra response. Preliminary findings on the effect of GLP-1 analogues on cognition, depression and anxiety supported the notion that identifying markers of GLP-1Ra response in diabetes is of high clinical significance. Non-responders to GLP-1Ras showed an increased risk of comorbidity and mood disorders, which may affect cognitive domains relating to memory and perception. The biomarker panels identified within this thesis, after further validation, may help stratify T2D patients that are at risk of poor anti-diabetes medication response and comorbidity, and better inform clinical practices on the best course of treatment, ultimately improving outcomes in T2D.

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Appendices

Supplementary Table 1 – All proteins measured by OLink analysis (Passed QC)

Number	Proteins	Number	Proteins	Number	Proteins
1	BMP 6	41	GLO1	81	CTSL1
2	ANG1	42	CD84	82	hOSCAR
3	ADM	43	PAPPA	83	TNFRSF13B
4	CD40 L	44	SERPINA12	84	TGM2
5	SLAMF7	45	REN	85	LEP
6	PGF	46	DECR1	86	CA5A
7	ADAM TS13	47	MERTK	87	HSP 27
8	BOC	48	KIM1	88	CD4
9	IL 4RA	49	THBS2	89	NEMO
10	SRC	50	TM	90	VEGFD
11	IL 1ra	51	VSIG2	91	PARP1
12	IL6	52	AMBP	92	HAOX1
13	TNFRSF10A	53	PRELP	93	TNFRSF14
14	STK4	54	HO1	94	LDL receptor
15	IDUA	55	XCL1	95	ITGB2
16	TNFRSF11A	56	IL16	96	IL17RA
17	PAR 1	57	SORT1	97	TNF R2
18	TRAIL R2	58	CEACAM8	98	MMP 9
19	PRSS27	59	PTX3	99	EPHB4
20	TIE2	60	PSGL1	100	IL2 RA
21	TF	61	CCL17	101	OPG
22	IL1RL2	62	CCL3	102	ALCAM
23	PDGF subunit B	63	MMP7	103	TFF3
24	IL27	64	IgGFc R IIb	104	SELP
25	IL17D	65	ITGB1BP2	105	CSTB
26	CXCL1	66	DCN	106	MCP 1
27	LOX 1	67	Dkk1	107	CD163
28	Gal 9	68	LPL	108	Gal 3
29	GIF	69	PRSS8	109	GRN
30	SCF	70	AGRP	110	MEPE
31	IL18	71	HB EGF	111	BLM hydrolase
32	FGF 21	72	GDF2	112	PLC
33	PIgR	73	FABP2	113	LTBR
34	RAGE	74	THPO	114	Notch3
35	SOD2	75	MARCO	115	TIMP4
36	CTRC	76	GT	116	CNTN1
37	FGF 23	77	BNP	117	CDH5
38	SPON2	78	MMP12	118	TLT2
39	GH	79	ACE2	119	FABP4
40	FS	80	PDL2	120	TFPI

Supplementary Table 1 – continued

Number	Proteins	Number	Proteins	Number	Proteins
121	PAI	161	uPA	201	PRDX1
122	CCL24	162	CPB1	202	PRDX3
123	TR	163	CHI3L1	203	FGF2
124	TNFRSF10C	164	ST2	204	PRDX5
125	GDF15	165	tPA	205	DPP10
126	SELE	166	SCGB3A2	206	TRIM5
127	AZU1	167	EGFR	207	DCTN1
128	DLK1	168	IGFBP 7	208	ITGA6
129	SPON1	169	CD93	209	CDSN
130	MPO	170	IL 18BP	210	GALNT3
131	CXCL16	171	COL1A1	211	FXYD5
132	IL6RA	172	PON3	212	TRAF2
133	RETN	173	CTSZ	213	TRIM21
134	IGFBP 1	174	MMP 3	214	LILRB4
135	CHIT1	175	RARRES2	215	NTF4
136	TRAP	176	ICAM 2	216	KRT19
137	CCL22	177	KLK6	217	ITM2A
138	PSP D	178	PDGF subunit A	218	HNMT
139	PI3	179	TNF R1	219	CCL11
140	Ep CAM	180	IGFBP 2	220	MILR1
141	APN	181	vWF	221	EGLN1
142	AXL	182	PECAM 1	222	NFATC3
143	IL 1RT1	183	NTproBNP	223	LY75
144	MMP 2	184	CCL16	224	EIF5A
145	FAS	185	PPP1R9B	225	EIF4G1
146	MB	186	GLB1	226	CD28
147	TNFSF13B	187	PSIP1	227	PTH1R
148	PRTN3	188	ZBTB16	228	BIRC2
149	PCSK9	189	IRAK4	229	HSD11B1
150	UPAR	190	TPSAB1	230	NF2
151	OPN	191	HCLS1	231	PLXNA4
152	CTSD	192	CNTNAP2	232	SH2B3
153	PGLYRP1	193	CLEC4G	233	FCRL3
154	CPA1	194	IRF9	234	CKAP4
155	JAM A	195	EDAR	235	JUN
156	Gal4	196	IL6	236	HEXIM1
157	IL 1RT2	197	DGKZ	237	CLEC4D
158	SHPS 1	198	CLEC4C	238	PRKCQ
159	CCL15	199	IRAK1	239	MGMT
160	CASP3	200	CLEC4A	240	TREM1

Supplementary table 1 – final

Number	Proteins	Number	Proteins	Number	Proteins	Number	Proteins
241	CXADR	281	GDNF	321	IL 10RB	361	TWEAK
242	IL10	282	CDCP1	322	IL 22 RA1	362	CCL20
243	SRPK2	283	CD244	323	IL 18R1	363	ST1A1
244	KLRD1	284	IL7	324	PD L1	364	STAMPB
245	BACH1	285	OPG	325	Beta NGF	365	IL5
246	PIK3AP1	286	LAPTGF beta 1	326	CXCL5	366	ADA
247	SPRY2	287	uPA	327	TRANCE	367	TNFB
248	STC1	288	IL6	328	HGF	368	CSF1
249	ARNT	289	IL 17C	329	IL 12B		
250	FAM3B	290	MCP 1	330	IL 24		
251	SH2D1A	291	IL 17A	331	IL13		
252	ICA1	292	CXCL11	332	ARTN		
253	DFFA	293	AXIN1	333	MMP 10		
254	DCBLD2	294	TRAIL	334	IL10		
255	FCRL6	295	IL 20RA	335	TNF		
256	NCR1	296	CXCL9	336	CCL23		
257	CXCL12	297	CST5	337	CD5		
258	AREG	298	IL 2RB	338	CCL3		
259	IFNLR1	299	IL 1 alpha	339	Flt3L		
260	DAPP1	300	OSM	340	CXCL6		
261	PADI2	301	IL2	341	CXCL10		
262	SIT1	302	CXCL1x	342	4E BP1		
263	MASP1	303	TSLP	343	IL 20		
264	LAMP3	304	CCL4	344	SIRT2		
265	CLEC7A	305	CD6	345	CCL28		
266	CLEC6A	306	SCF	346	DNER		
267	DDX58	307	IL18	347	EN RAGE		
268	IL12RB1	308	SLAMF1	348	CD40		
269	TANK	309	TGF alpha	349	IL33		
270	ITGA11	310	MCP 4	350	IFN gamma		
271	KPNA1	311	CCL11x	351	FGF 19		
272	LAG3	312	TNFSF14	352	IL4		
273	IL5	313	FGF 23	353	LIF		
274	CD83	314	IL 10RA	354	NRTN		
275	ITGB6	315	FGF 5	355	MCP 2		
276	BTN3A2	316	MMP 1	356	CASP8		
277	IL8	317	LIF R	357	CCL25		
278	VEGFA	318	FGF 21	358	CX3CL1		
279	BDNF	319	CCL19	359	TNFRSF9		
280	MCP 3	320	IL 15RA	360	NT3		

Supplementary Table 2 – OLINK Proteins that did not meet QC

Number	Protein	Number	Protein	Number	Protein
1	ARNT	12	IL-20	23	KPNA1
2	ARNT	13	IL-20RA	24	LIF
3	BDNF	14	IL-22RA1	25	NF2
4	BIRC2	15	IL-24	26	NRTN
5	BNP	16	IL2RB	27	PAD12
6	DGK2	17	IL3	28	Pi3
7	EIF5A	18	IL33	29	PRKCQ
8	FXYDS	19	IL4	30	T5LP
9	IFN-gamma	20	IL5	31	TNF
10	IL-1 alpha	21	IL5		
11	IL13	22	JUN		

Supplementary Table 3 – Proteins associated with other injectable anti diabetes medications

Number	Protein	Number	Protein
1	CTSZ	28	LAG3
2	TRAF2	29	IL-18BP
3	CA5A	30	IL-15RA
4	CD163	31	TFF3
5	LY75	32	CDCP1
6	CD5	33	IL13
7	ITGB6	34	IL-1RT1
8	IGFBP-7	35	CXCL10
9	Gal-4	36	TRANCE
10	HAOX1	37	CST5
11	IL-1ra	38	CCL28
12	Flt3L	39	SPON1
13	MMP7	40	TNFRSF13B
14	THBS2	41	NCR1
15	BTN3A2	42	NT-3
16	SCF	43	CXCL16
17	hOSCAR	44	TNFRSF14
18	AMBP	45	TNF-R2
19	IL18	46	MARCO
20	DDX58	47	CD83
21	OSM	48	FAM3B
22	LOX-1	49	IL-17C
23	CLEC4A	50	CCL22
24	CD84	51	CLEC7A
25	ITGA6	52	PAI
26	TNFSF14	53	CXCL9
27	PARP-1	54	SLAMF1
		55	CCL15

Supplementary Table 4 – Proteins associated with Microvascular and Macrovascular comorbidities

Number	Protein	Number	Protein	Number	Protein
21	TNFRSF14	54	IL12RB1	87	ICAM-2
22	IL-17C	55	Notch 3	88	SH2B3
23	LILRB4	56	GALNT3	89	STAMPB
24	CLEC4D	57	LAMP3	90	PECAM-1
25	CCL19	58	IL16	91	OPN
26	CDSN	59	DCBLD2	92	IGFBP-2
27	IL-10RB	60	CXADR	93	TRIM5
28	KLRD1	61	EGLN1	94	KLK6
29	EPHB4	62	ANG-1	95	NFATC3
30	PGF	63	HO-1	96	FAM3B
31	LAG3	64	HB-EGF	97	CD84
32	IL2-RA	65	AZU1	98	ST1A1
33	TNFRSF13B	66	TIE2	99	IL7
34	TIMP4	67	PARP-1	100	PLXNA4
35	IGFBP-7	68	CCL17	101	TF
36	MILR1	69	TR	102	HCLS1
37	TGF-alpha	70	MMP-10	103	ICA1
38	PRELP	71	CCL22	104	MEPE
39	ITGA6	72	Gal-3	105	CST5
40	PDGF subunit B	73	PSP-D	106	RARRES2
41	PON3	74	AREG	107	CX3CL1
42	TNFRSF10A	75	AXL	108	CXCL16
43	MMP7	76	Gal-4	109	PRTN3
44	MB	77	KRT19	110	MCP-1
45	CD4	78	GDF-15	111	CLEC4G
46	SHPS-1	79	FGF-21	112	COL1A1
47	CCL25	80	IgG Fc receptor II-b	113	MMP-3
48	SPON2	81	CXCL12		
49	SLAMF1	82	Ep-CAM		
50	MCP-3	83	DLK-1		
51	CD93	84	IL1RL2		
52	LPL	85	CD40-L		
53	AMBP	86	CXCL1		

Supplementary Screen 1 – Beck Anxiety Inventory

Beck Anxiety Inventory

Below is a list of common symptoms of anxiety. Please carefully read each item in the list. Indicate how much you have been bothered by that symptom during the past month, including today, by circling the number in the corresponding space in the column next to each symptom.

	Not At All	Mildly but it didn't bother me much.	Moderately - it wasn't pleasant at times	Severely – it bothered me a lot
Numbness or tingling	0	1	2	3
Feeling hot	0	1	2	3
Wobbliness in legs	0	1	2	3
Unable to relax	0	1	2	3
Fear of worst happening	0	1	2	3
Dizzy or lightheaded	0	1	2	3
Heart pounding/racing	0	1	2	3
Unsteady	0	1	2	3
Terrified or afraid	0	1	2	3
Nervous	0	1	2	3
Feeling of choking	0	1	2	3
Hands trembling	0	1	2	3
Shaky / unsteady	0	1	2	3
Fear of losing control	0	1	2	3
Difficulty in breathing	0	1	2	3
Fear of dying	0	1	2	3
Scared	0	1	2	3
Indigestion	0	1	2	3
Faint / lightheaded	0	1	2	3
Face flushed	0	1	2	3
Hot/cold sweats	0	1	2	3
Column Sum				

Scoring **-** Sum each column. Then sum the column totals to achieve a grand score. Write that score here _____ .

Interpretation

A grand sum between **0 – 21** indicates very low anxiety. That is usually a good thing. However, it is possible that you might be unrealistic in either your assessment which would be denial or that you have learned to "mask" the symptoms commonly associated with anxiety. Too little "anxiety" could indicate that you are detached from yourself, others, or your environment.

A grand sum between **22 – 35** indicates moderate anxiety. Your body is trying to tell you something. Look for patterns as to when and why you experience the symptoms described above. For example, if it occurs prior to public speaking and your job requires a lot of presentations you may want to find ways to calm yourself before speaking or let others do some of the presentations. You may have some conflict issues that need to be resolved. Clearly, it is not "panic" time but you want to find ways to manage the stress you feel.

A grand sum that **exceeds 36** is a potential cause for concern. Again, look for patterns or times when you tend to feel the symptoms you have circled. Persistent and high anxiety is not a sign of personal weakness or failure. It is, however, something that needs to be proactively treated or there could be significant impacts to you mentally and physically. You may want to consult a counselor if the feelings persist.

Supplementary Screen 2 – Beck Depression Inventory

Beck's Depression Inventory

This depression inventory can be self-scored. The scoring scale is at the end of the questionnaire.

1.
0 I do not feel sad.
1 I feel sad
2 I am sad all the time and I can't snap out of it.
3 I am so sad and unhappy that I can't stand it.

2.
0 I am not particularly discouraged about the future.
1 I feel discouraged about the future.
2 I feel I have nothing to look forward to.
3 I feel the future is hopeless and that things cannot improve.

3.
0 I do not feel like a failure.
1 I feel I have failed more than the average person.
2 As I look back on my life, all I can see is a lot of failures.
3 I feel I am a complete failure as a person.

4.
0 I get as much satisfaction out of things as I used to.
1 I don't enjoy things the way I used to.
2 I don't get real satisfaction out of anything anymore.
3 I am dissatisfied or bored with everything.

5.
0 I don't feel particularly guilty
1 I feel guilty a good part of the time.
2 I feel quite guilty most of the time.
3 I feel guilty all of the time.

6.
0 I don't feel I am being punished.
1 I feel I may be punished.
2 I expect to be punished.
3 I feel I am being punished.

7.
0 I don't feel disappointed in myself.
1 I am disappointed in myself.
2 I am disgusted with myself.
3 I hate myself.

8.
0 I don't feel I am any worse than anybody else.
1 I am critical of myself for my weaknesses or mistakes.
2 I blame myself all the time for my faults.
3 I blame myself for everything bad that happens.

9.
0 I don't have any thoughts of killing myself.
1 I have thoughts of killing myself, but I would not carry them out.
2 I would like to kill myself.
3 I would kill myself if I had the chance.

10.
0 I don't cry any more than usual.
1 I cry more now than I used to.
2 I cry all the time now.
3 I used to be able to cry, but now I can't cry even though I want to.

11.
0 I am no more irritated by things than I ever was.
1 I am slightly more irritated now than usual.
2 I am quite annoyed or irritated a good deal of the time.
3 I feel irritated all the time.

12.
0 I have not lost interest in other people.
1 I am less interested in other people than I used to be.
2 I have lost most of my interest in other people.
3 I have lost all of my interest in other people.

13.
0 I make decisions about as well as I ever could.
1 I put off making decisions more than I used to.
2 I have greater difficulty in making decisions more than I used to.
3 I can't make decisions at all anymore.

14.
0 I don't feel that I look any worse than I used to.
1 I am worried that I am looking old or unattractive.
2 I feel there are permanent changes in my appearance that make me look unattractive
3 I believe that I look ugly.

15.
0 I can work about as well as before.
1 It takes an extra effort to get started at doing something.
2 I have to push myself very hard to do anything.
3 I can't do any work at all.

16.
0 I can sleep as well as usual.
1 I don't sleep as well as I used to.
2 I wake up 1-2 hours earlier than usual and find it hard to get back to sleep.
3 I wake up several hours earlier than I used to and cannot get back to sleep.

17.
0 I don't get more tired than usual.
1 I get tired more easily than I used to.
2 I get tired from doing almost anything.
3 I am too tired to do anything.

18.
0 My appetite is no worse than usual.
1 My appetite is not as good as it used to be.
2 My appetite is much worse now.
3 I have no appetite at all anymore.

19.
0 I haven't lost much weight, if any, lately.
1 I have lost more than five pounds.
2 I have lost more than ten pounds.
3 I have lost more than fifteen pounds.

20.
0 I am no more worried about my health than usual.
1 I am worried about physical problems like aches, pains, upset stomach, or constipation.
2 I am very worried about physical problems and it's hard to think of much else.
3 I am so worried about my physical problems that I cannot think of anything else.

21.
0 I have not noticed any recent change in my interest in sex.
1 I am less interested in sex than I used to be.
2 I have almost no interest in sex.
3 I have lost interest in sex completely.

INTERPRETING THE BECK DEPRESSION INVENTORY

Now that you have completed the questionnaire, add up the score for each of the twenty-one questions by counting the number to the right of each question you marked. The highest possible total for the whole test would be sixty-three. This would mean you circled number three on all twenty-one questions. Since the lowest possible score for each question is zero, the lowest possible score for the test would be zero. This would mean you circles zero on each question.

You can evaluate your depression according to the Table below.

Total Score_____Levels of Depression

1-10_____These ups and downs are considered normal
11-16_____ Mild mood disturbance
17-20_____Borderline clinical depression
21-30_____Moderate depression
31-40_____Severe depression
over 40_____Extreme depression

Supplementary Screen 3 – Standardised Mini-Mental State Examination

Name of patient:		DOB:	/ /	Name of examiner:		Date of test:	/ /

Standardised Mini-Mental State Examination (SMMSE)

Please see accompanying guide for directions for administration

Say: I am going to ask you some questions and give you some problems to solve. Please try to answer as best you can.

1. **(Allow 10 seconds for each reply). Say:**

a) What year is this? (Accept exact answer only) ..... / 1

b) What season is this? (During the last week of the old season or first week of a new season, accept either) ..... / 1

c) What month is this? (On the first day of a new month or the last day of the previous month, accept either) ..... / 1

d) What is today's date? (Accept previous or next date) ..... / 1

e) What day of the week is this? (Accept exact answer only) ..... / 1

2. **(Allow 10 seconds for each reply). Say:**

a) What country are we in? (Accept exact answer only) ..... / 1

b) What county are we in? (Accept exact answer only) ..... / 1

c) What city/town are we in? (Accept exact answer only) ..... / 1

d) (At home) What is the street address of this house? (Accept street name and house number or equivalent in rural areas)

(In facility) What is the name of this building? (Accept exact name of institution only) ..... / 1

e) (At home) What room are we in? (Accept exact answer only)

(In facility) What floor of the building are we on? (Accept exact answer only) ..... / 1

3. **Say:** I am going to name three objects. When I am finished, I want you to repeat them. Remember what they are because I am going to ask you to name them again in a few minutes (Say slowly at approximately one-second intervals).

Ball Car Man

For repeated use: Bell, jar, fan; bill, tar, can; bull, bar, pan

Say: Please repeat the three items for me. (Score one point for each correct reply on the first attempt) ..... / 3

Allow 20 seconds for reply; if the person did not repeat all three, repeat until they are learned or up to a maximum of five times. (But only score first attempt).

4. **Spell the word WORLD.** ('You may help the person to spell the word correctly) **Say:** *Now spell it backwards please* (Allow 30 seconds; if the subject cannot spell World even with assistance, score 0) Refer to accompanying guide for scoring instructions (Score on reverse of this sheet) ..... / 5

5. **Say:** Now what were the three objects I asked you to remember? ..... / 3

(Score one point for each correct answer regardless of order; allow 10 seconds)

6. **Show wristwatch. Ask:** *What is this called?* ..... / 1

(Score one point for correct response; accept "wristwatch" or "watch"; do not accept "clock" or "time", etc.; allow 10 seconds)

7. **Show pencil. Ask:** *What is this called?* ..... / 1

(Score one point for correct response; accept "pencil" only; score 0 for pen; allow 10 seconds for reply)

8. **Say:** I would like you to repeat a phrase after me: *No if's, ands, or buts* ..... / 1

(Allow 10 seconds for response. Score one point for a correct repetition. Must be exact, e.g. no ifs or buts, score 0)

9. **Say:** *Read the words on this page and then do what it says* ..... / 1

Then, **hand** the person the sheet with CLOSE YOUR EYES (score on reverse of this sheet) on it. If the subject just reads and does not close eyes, you may repeat: Read the words on this page and then do what it says, (a maximum of three times. See point No. 3 in Directions for Administration section of accompanying guide). Allow 10 seconds, score one point only if the subject closes eyes. The subject does not have to read aloud.

10. **Hand** the person a pencil and paper. **Say:** *Write any complete sentence on that piece of paper.* (Allow 30 seconds. Score one point. The sentence must make sense. Ignore spelling errors) ..... / 1

11. **Place** design (see reverse of this sheet), pencil, eraser and paper in front of the person. **Say:** *Copy this design please.* Allow multiple tries. Wait until the person is finished and hands it back. Score one point for a correctly copied diagram. The person must have drawn a four-sided figure between two five-sided figures. Maximum time: One minute ..... / 1

12. **Ask** the person if he is right or left handed. **Take** a piece of paper, hold it up in front of the person and **say** the following:
Take this paper in your right/left hand (whichever is non-dominant), fold the paper in half once with both hands and put the paper down on the floor.

Takes paper in correct hand ..... / 1

Folds it in half ..... / 1

Puts it on the floor ..... / 1

TOTAL TEST SCORE: / 30

ADJUSTED SCORE : /

Molloy DW, Alemayehu E, Roberts R. Reliability of a standardized Mini-Mental State Examination compared with the traditional Mini-Mental state Examination. *American Journal of Psychiatry*, Vol. 14, 1991a, pp.102-105.

Supplementary Screen 4 – Quick Mild Cognitive Impairment (Qmci) screen

Administration and Scoring Guideline

1.1 Orientation

✎ ***Scoring*** 2 points for the correct answer, 1 point for wrong answers, and 0 points for no answer or a conceptually unrelated answer (see details below).

🕒 ***Timing*** Maximum of 10 sec for each answer.

Instructions and Scoring Guide

Year	If the person gives the correct year score 2 points, the incorrect year score 1 point, and 0 points if no year is given.
Country	Score 2 points for correct country, 1 point for incorrect country, and 0 if no country is named.
Month	Score 2 points for the correct month or for the previous or following month if within two days of the change of the month (for example, if the date is September 30th, score the full 2 points if person answers October. Similarly, if the date is October 2nd, score 2 points if person says September). Score 1 point if the month is incorrect and 0 if no month is named.
Date	Score 2 points for exact date or ± one day, 1 point for any other date, 0 if no date is named.
Day of week	2 points for correct day, 1 point for incorrect day, 0 if no day named.

To begin say...

"I'd like to ask you some questions and give you some problems to solve. Would that be OK?"

What country is this?	**_____**
What year is this?	**_____**
What month is this?	**_____**
What is today's date?	**_____**
What day of the week is this?	**_____**

Score _____ / 10

1.2 Word Registration

Scoring Score 1 point for each word recalled after the first reading. If subject recalls all five, repeat the five items once and then go on to clock drawing. If subject does not repeat all 5, repeat the 5 items and ask the subject to repeat them. Do this until the subject correctly recalls all 5 items or for a maximum of 3 trials. Do not score for trials 2 and 3. These trials are to help the person learn in preparation for the delayed recall task.

Timing Say the words very deliberately, one per second. Allow 10 sec for the recall.

To begin say…

"I am going to say 5 words. After I have said these 5 words, repeat them back to me. Are you ready?"

dog	**rain**	**butter**	**love**	**door**

Score _____ / 5

When finished, say… **"Remember these words because I'll ask you to recall them later."**

Alternate word groups include…

cat	**Dark**	**Pepper**	**fear**	**bed**
rat	**Heat**	**Bread**	**round**	**chair**

1.3 Clock Drawing

✎ ***Scoring.*** Place the circle of the transparent scoring template over the circle of the patient's completed clock. Rotate the template circle so that the "12" s align. Score 1 point each if the 1, 2, 4, 5, 7, 8, 10, and 11 are in the correct quadrants. Score 1 point each if the 12, 3, 6, and 9 touch their quadrant lines. Subtract one point for each number repeated or for numbers above 12. (Should the patient not have drawn a "12" align the template with the 3, 6, or 9.)

Score the placement of hands according to the tips and pivot. Give 1 point for each hand between the dashed lines. Score 1 point for hands connecting at the pivot.

🕒 ***Timing*** One minute.

To begin…

Give the sheet of paper with the pre-drawn circle and a pencil to the patient. Say **"Now put in the numbers like the face of a clock."** Then say **"Set the hands to show ten past eleven." Place the numbers and hands as carefully as you can."**

You may prompt at each stage…"**put in the numbers…. put the time as ten past eleven**".

Score:

Numbers Correct + _____/ 12

Errors - _____

Hands + _____/ 2

Pivot + _____/ 1

Total + _____/ 15

1.4 Delayed Recall

✎ ***Scoring***. Score 4 points for each word recalled. Subjects may recall words in any order.

🕒 ***Timing*** 10 sec.

To begin say…

A few minutes ago I named five words. Name as many of those words as you can remember.

dog	**rain**	**butter**	**love**	**door**

Score _____ / 20

Alternate word groups include…

cat	**Dark**	**pepper**	**fear**	**bed**
rat	**Heat**	**bread**	**round**	**chair**

1.5 Verbal Fluency

Instructions and Scoring Guide

✎ ***Scoring***. Give ½ point for each correct word recalled to a maximum of 40 words. Round up the final score. Do not count words with different suffixes twice (e.g. fish / fishes, mouse / mice, etc.). Accept alternate species (e.g. blue jay, robin, sparrow, duck, etc.). Alternate forms include fruits and vegetables, cities and towns.

🕒 ***Timing.***60 sec. Write down each word the patient says. (You may need to develop some kind of "shorthand" for the speedier patients, such as writing the first 3 letters of each word and then completing them later.)

To begin say…

"Name as many *animals* as you can in one minute. Ready? Go."

Score _____ / 20

1.6 Logical Memory

✎ ***Scoring.*** Give 2 points for each correct word item recalled verbatim. All bolded words within each section must be recalled for score 2 points. Otherwise score 0. Recall may be in any order.

🕒 ***Timing***. 30 sec. Check off each word unit recalled.

To begin say…

"I am going to read you a short story. After I have finished reading I want you to tell me as much of the story as you can. OK?" [patient signifies agreement, then begin reading the paragraph at about 1 second for each word unit] **"The red… fox… ran across..... the bushes."**

6. Logical Memory			
The **red**	The **brown**	The **white**	2 / 0
fox	**Dog**	**hen**	2 / 0
ran across	**ran across**	**walked across**	2 / 0
the **ploughed**	the **metal**	the **concrete**	2 / 0
field.	**bridge**.	**road**.	2 / 0
It was **chased** by	It was **hunting**	It was **followed** by	2 / 0
a **brown**	a **white**	a **black**	2 / 0
dog.	**rabbit**.	**cat**.	2 / 0
It was a **hot**	It was a **cold**	It was a **warm**	2 / 0
May	**October**	**September**	2 / 0
morning.	**day**.	**afternoon**.	2 / 0
Fragrant	**Ripe**	**Dry**	2 / 0
blossoms	**apples**	**leaves**	2 / 0
were **forming** on	were **hanging** on	were **blowing** in	2 / 0
the **bushes**.	the **trees**.	the **wind**.	2 / 0

Score _____ / 30

QMCI Total Score	_____	/ 100