University of Strathclyde Glasgow

Does Serum from People with Type 2 Diabetes Cause Insulin Resistance in Cultured Cells?

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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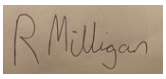
Covid-19 impact statement

Previous work demonstrated that serum from people with T2D (PWT2D) induced insulin resistance in cultured cells. The overarching aim of this project was to identify the factor(s) responsible for this effect and their mode(s) of action. To this end, we recruited 20 PWT2D and 20 matched controls for serum donation. At the outset of this project, this process was estimated to take between three to six months, but due to Covid-19 related disruption, it took a year and nine months to complete. This had a significant impact on how much could be achieved during this 3-year project. I ask that these facts are considered when this PhD thesis is judged.

Signed:  - Roderick Stewart Milligan (PhD Student).

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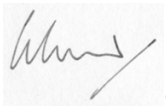
Signed:  - Professor Gwyn Gould (Principal Investigator).

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Abstract

Type 2 diabetes (T2D) is a chronic metabolic condition characterised by insulin resistance that affects millions worldwide, leading to significant health complications like heart disease, kidney failure, and vision loss if not properly managed. Previous studies have shown that serum from people with T2D (PWT2D) can induce insulin resistance when applied to cultured cells, yet the identity and mechanism of action of the responsible circulating factor(s) remain unclear. The aim of this PhD project was to investigate the insulin-promoting capacity of serum from PWT2D using two complimentary functional assays and to explore the nature of the mediating humoral factor(s).

Insulin-mediated repression of *Pck1* transcript accumulation was assessed in H4-II-E hepatoma cells, and insulin-stimulated radiolabelled glucose uptake was measured in 3T3-L1 adipocytes. Both assays were validated and shown to be sensitive to experimentally induced insulin resistance. However, pooled serum from individuals with established, lifestyle-managed T2D did not induce detectable insulin resistance in either model. In contrast, preliminary experiments using serum from a small cohort of people with pre-T2D revealed a selective impairment in insulin-stimulated glucose uptake in 3T3-L1 adipocytes, without corresponding changes in proximal signalling through AKT or AS160.

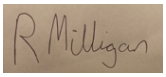
In a parallel study, age-related changes in 3T3-L1 adipocytes were characterised. As the cells aged, they adopted a more white adipose tissue-like morphology accompanied by selective reductions in several proteins involved in insulin signalling and GLUT4 trafficking. However, maximal insulin-stimulated glucose uptake remained unchanged, indicating functional resilience despite morphological remodelling.

Overall, this thesis highlights that the insulin resistance-inducing activity of serum from PWT2D is highly dependent on donor disease stage. The selective

impairment observed in adipocytes but not hepatomas raises questions about the potential tissue- or pathway-specific nature of the mediating humoral factor(s). The work provides a validated experimental platform for future identification of circulating mediators and contributes to our understanding of how humoral factors propagate insulin resistance in T2D.

Declaration of Authenticity and Author's Rights

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: 

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1 Introduction

1.1 Overview of glucose homeostasis

Glucose is the human body's main energy source, but it must be carefully regulated to prevent disease. Glucose homeostasis refers to the dynamic mechanisms that keep circulating blood glucose levels stable, usually between 4-6 mmol/L in the fasting state and below approximately 10 mmol/L after meals (postprandially) (Röder *et al.*, 2016) (Figure **1-1**). This precise control is essential to ensure adequate glucose supply to obligate glucose-utilising tissues such as the brain while avoiding the acute dangers of hypoglycaemia and the long-term glucotoxic complications associated with sustained hypoglycaemia (Röder *et al.*, 2016).

1.1.1 Key hormones and their actions

The maintenance of glucose homeostasis depends primarily on the opposing actions of insulin and glucagon, secreted by the pancreatic ß cells and α cells, respectively (Bansal and Wang, 2008). Insulin, released in response to rising blood glucose (particularly postprandially), acts as the principle anabolic hormone by promoting glucose uptake into insulin-sensitive tissues (via GLUT4 translocation in muscle and adipose tissues), stimulating glycogen synthesis and lipogenesis, and suppressing hepatic glucose production through inhibition of glycogenolysis and gluconeogenesis (Petersen and Shulman, 2018). In contrast, glucagon, secreted during fasting or hypoglycaemia, functions as the main counter-regulatory hormone by elevating blood glucose via hepatic glycogenolysis and gluconeogenesis (Röder *et al.*, 2016). Incretin hormones (glucose-dependent insulinotropic peptide (GIP) from intestinal K cells and glucagon-like peptide (GLP-1) from L cells) further enhance postprandial glucose control in a nutrient-dependent manner: they potentiate glucose-stimulated insulin secretion (accounting for 50-70% of the insulin response to oral glucose), suppress glucagon release, slow gastric emptying, and promote satiety (Baggio and Drucker, 2007).

Additional counter-regulatory hormones, including catecholamines (e.g., adrenaline), cortisol, and growth hormone, support glucose availability during stress or prolonged fasting by opposing insulin action and stimulating hepatic glucose output, ensuring robust defence against hypoglycaemia (Lager, 1991).

1.1.2 Major organs/tissues and inter-organ crosstalk

Glucose homeostasis emerges from dynamic inter-organ communication, with the pancreas serving as the central endocrine regulator that integrates signals from peripheral tissues to orchestrate glucose disposal and production (Error! Reference source not found.) (Röder *et al.*, 2016). In the postprandial state, rising glucose and incretins stimulate insulin secretion from pancreatic ß cells, which suppresses hepatic glucose output (via inhibition of glycogenolysis and gluconeogenesis) while promoting glucose uptake primarily in skeletal muscle (around 70-80% of postprandial disposal through insulin-stimulated GLUT4 translocation) and, to a lesser extent, adipose tissue (for lipogenesis and suppression of lipolysis (Dimitriadis *et al.*, 2021). The liver acts as a key buffer, storing excess glucose as glycogen and modulating portal glucose influx. In contrast, during fasting, declining insulin and rising glucagon drive hepatic glucose production to maintain euglycaemia, with reduced peripheral uptake as muscle and adipose shift toward fatty acid oxidation; additional contributions come from renal gluconeogenesis and glucose reabsorption ((via sodium-glucose linked transporters (SGLT) transporters), as well as gut-derived incretins and central (hypothalamic) sensing of nutrient states. This coordinated crosstalk (facilitated by humoral factors, neural inputs, and direct metabolic signalling) ensures stable glycaemia but becomes disrupted in type 2 diabetes (T2D), where impaired insulin action in liver, muscle, and adipose tissues leads to excessive hepatic glucose release, reduced peripheral disposal, and elevated circulating free fatty acids (FFAs) (Röder *et al.*, 2016; Petersen and Shulman, 2018; Dimitriadis *et al.*, 2021).

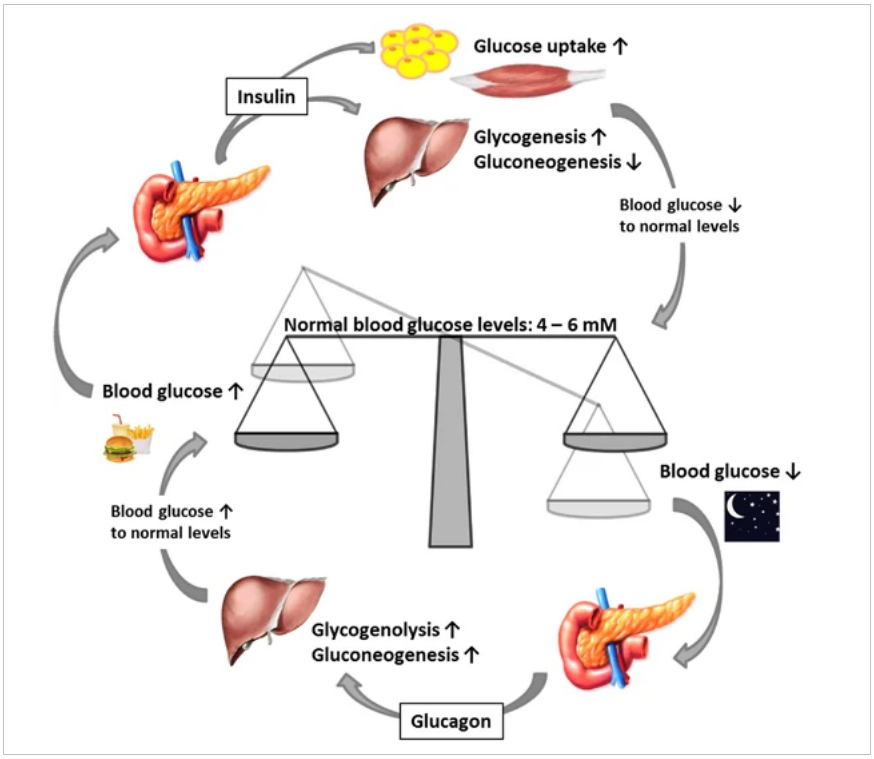

Figure 1-1: Pancreatic regulation of glucose homeostasis
Diagram shows the pancreatic regulation of glucose homeostasis, as described in the main text. Taken from Röder *et al.* (2016).

1.2 Insulin

Insulin is a 51 amino acid peptide hormone that is synthesised and secreted by the ß cells of the pancreas (Rahman *et al.*, 2021), in response to elevated blood glucose levels and levels of other metabolites, to coordinate systemic glucose homeostasis (James, Stockli and Birnbaum, 2018). Insulin does this by driving multiple processes in several organs, including, the attenuation of glucose release from the liver, the increase of glucose uptake into muscle and fat, the suppression of free fatty acid release from adipocytes, and the increase of lipid accumulation in liver and adipocytes (James, Stockli and Birnbaum, 2018). In short, insulin is a potent anabolic agent, promoting the cellular uptake, storage, and synthesis of nutrients, while blocking nutrient breakdown and release into the circulation. For this report, at least, we are primarily interested in insulin's metabolic roles in suppressing glucose release in the liver, and driving glucose uptake in fat, however, it is

important to note that insulin also plays in mitogenesis and cell differentiation (Petersen and Shulman, 2018). This is achieved, at least in part, by an overlap in the signalling networks of insulin and other insulin-like peptides, such as the insulin-like growth factors and relaxins (Petersen and Shulman, 2018).

1.3 Counter-regulatory hormones in glucose homeostasis

While insulin serves as the primary anabolic hormone that lowers blood glucose by promoting uptake, storage, and suppressing production, glucose homeostasis relies on a redundant system of counter-regulatory (glucose-raising) hormones that oppose insulin action, particularly during fasting, stress, or hypoglycaemia. These hormones defend against hypoglycaemia by stimulating hepatic glucose output (glycogenolysis and gluconeogenesis), reducing peripheral glucose utilisation and mobilising alternative fuels (e.g., FFAs via lipolysis). The response is hierarchically organised: rapid-acting factors dominate acute defence, while slower-acting ones sustain recovery during prolonged challenges (Cryer, 1993; Cryer, 1996).

The most critical and rapid counter-regulatory hormone is glucagon, secreted by pancreatic α cells in response to falling glucose (threshold around 3.8-4.0 mmol/L in healthy individuals). Glucagon directly stimulates hepatic glycogenolysis and gluconeogenesis (via cAMP/PKA activation of phosphorylase kinase and *Pck1*/*G6Pase* expression), increasing glucose production within minutes and accounting for most of the initial recovery from hypoglycaemia. In the absence of glucagon ((e.g., advanced type 1 diabetes (T1D)), counter regulation is severely impaired unless other mechanisms compensate (Ramnanan *et al.*, 2011; Verhulst *et al.*, 2022).

Next in the hierarchy is adrenaline, released from the adrenal medulla (located atop the kidneys) via sympathoadrenal activation (threshold around 3.4-3.8mmol/L) (Nedoboy, Cohen and Farnham, 2021). Adrenaline synergises

with glucagon by enhancing hepatic glucose output, stimulating muscle and adipose lipolysis (providing glycerol/FFA substrates for gluconeogenesis), and limiting peripheral glucose uptake (via ß-adrenergic effects). It also generates autonomic warning symptoms (sweating, tremors, anxiety). Noradrenaline contributes modestly but overlaps with adrenaline's actions (Sprague and Arbeláez, 2011).

Slower-acting hormones include cortisol (from the adrenal cortex) and growth hormone (from the anterior pituitary), which rise at lower glucose thresholds (around 3.2-3.5 mmol/L for cortisol; around 3.2-3.8 mmol/L for growth hormone). Cortisol promotes gluconeogenesis by inducing key enzymes (e.g. PEPCK) and mobilising amino acids from protein breakdown, while also exerting permissive effects on other hormones. Growth hormone stimulates lipolysis (elevating FFAs for hepatic use) and reduces insulin sensitivity in muscle/ adipose tissues, limiting glucose disposal. These hormones become physiologically relevant during prolonged (>1-2 hours) hypoglycaemia or fasting, contributing to sustained euglycaemia but with delayed onset (hours) (Cryer, 1996; Verhulst *et al.*, 2022)

In healthy physiology, suppression of endogenous insulin secretion during falling glucose is the first defence, unmasking full counter-regulatory effects (Sprague and Arbeláez, 2011). This redundant, hierarchical system (glucagon > adrenaline > cortisol/growth hormone) ensures robust protection against neuroglycopenia (shortage of glucose to the brain) (Cryer, 1996). In T2D, counter-regulatory responses are generally preserved or only mildly attenuated compared to T1D (where glucagon and adrenaline responses often fail progressively), but chronic hyperglycaemia may shift thresholds or blunt efficacy indirectly through glucotoxicity or autonomic changes (Bokhari *et al.*, 2014; Verhulst *et al.*, 2022).

1.4 Tissue-specific actions of insulin in skeletal muscle and tissue-specific insulin resistance

Skeletal muscle is the primary site of insulin-stimulated glucose disposal in humans, accounting for around 70-80% of postprandial glucose uptake under euglycaemic hyperinsulinaemic conditions. Insulin binds the insulin receptor (IR) on the sarcolemma, triggering autophosphorylation and recruitment of insulin receptor substrate (IRS-1). This activates the PI3K/AKT pathway, leading to the phosphorylation of TBC1D4 (AS160) and TBC1D1, which releases GLUT4 storage vesicles (GSVs) from intracellular retention. GLUT4 translocates to the sarcolemma and T-tubules, facilitating glucose entry. Within the myocyte, insulin also promotes glycogen synthesis (via inhibition of glycogen synthase kinase-3 (GSK3), supresses proteolysis, and stimulates protein synthesis through mechanistic target of rapamycin complex 1 (mTORC1). These coordinated actions convert skeletal muscle into a major anabolic sink for glucose, storing it as glycogen or directing it towards oxidative metabolism (Petersen and Shulman, 2018; Merz and Thurmond, 2020; Sylow *et al.*, 2021) (**Figure 1-2**).

Unlike in liver (where insulin suppresses gluconeogenesis and glycogenolysis) or adipose tissue (where it inhibits lipolysis and promotes lipogenesis), insulin action in skeletal muscle is highly specialised for rapid, high-capacity glucose clearance. This tissue specificity arises from high expression of GLUT4, the muscle-specific hexokinase II isoform, and a dense microvascular network that enhances insulin delivery. Exercise further sensitises muscle to insulin via (AMPK)-dependent GLUT4 translocation, providing an insulin-independent route that remains intact even when classical insulin signalling is impaired. In T2D and obesity, skeletal muscle exhibits tissue-specific insulin resistance characterised by selective defects in glucose metabolism while other effects (e.g., amino acid transport or mitogenic signalling via mitogen-activated protein kinase (MAPK) may be preserved or even enhanced. The hallmark is impaired insulin-stimulated GLUT4 translocation

and reduced glucose uptake, resulting from multiple convergent mechanisms: (1) lipid overload leading to intramyocellular accumulation of diacylglycerol and ceramides, which activate protein kinase C-theta PKCθ and c-Jun N-terminal kinase (JNK) to serine-phosphorylate IRS-1 (inhibiting PI3K recruitment); (2) mitochondrial dysfunction and oxidative stress elevating reactive oxygen species (ROS); (3) low-grade inflammation with elevated tumour necrosis factor-alpha (TNF) and Interleukin-6 (IL6) activating inhibitor of nuclear factor kappa-B kinase subunit beta/ nuclear factor kappa-light-chain-enhancer of activated B cells (IKK-ß/ NF-κB); and (4) endoplasmic (ER) reticulum stress. These post-receptor defects blunt AKT phosphorylation and TBC1D4 inactivation, Trapping GLUT4 intracellularly. Consequently, postprandial hyperglycaemia ensues, while hepatic glucose production remains inadequately suppressed and adipose lipolysis continues, creating a vicious cycle of elevated circulating FFAs and glucolipotoxicity that further exacerbates muscle resistance (Petersen and Shulman, 2018; Merz and Thurmond, 2020; Sylow *et al.*, 2021).

Despite detailed knowledge of these intracellular pathways, the precise blood-borne factors present in the serum of people with T2D (PWT2D) that confer skeletal muscle insulin resistance (particularly the failure of insulin to stimulate GLUT4 translocation and glucose uptake in myocytes) remain incompletely defined. While elevated FFAs, pro-inflammatory cytokines, and chronic hyperglycaemia have been implicated individually, their relative contributions, synergistic interactions, and direct effects when presented together in human T2D serum are not fully understood. Experimental models that expose cultured myotubes or primary skeletal muscle cells to patient-derived serum could therefore represent a valuable strategy to dissect these humoral mediators and identify the circulating factors responsible for conferring the muscle-specific defects observed in T2D.

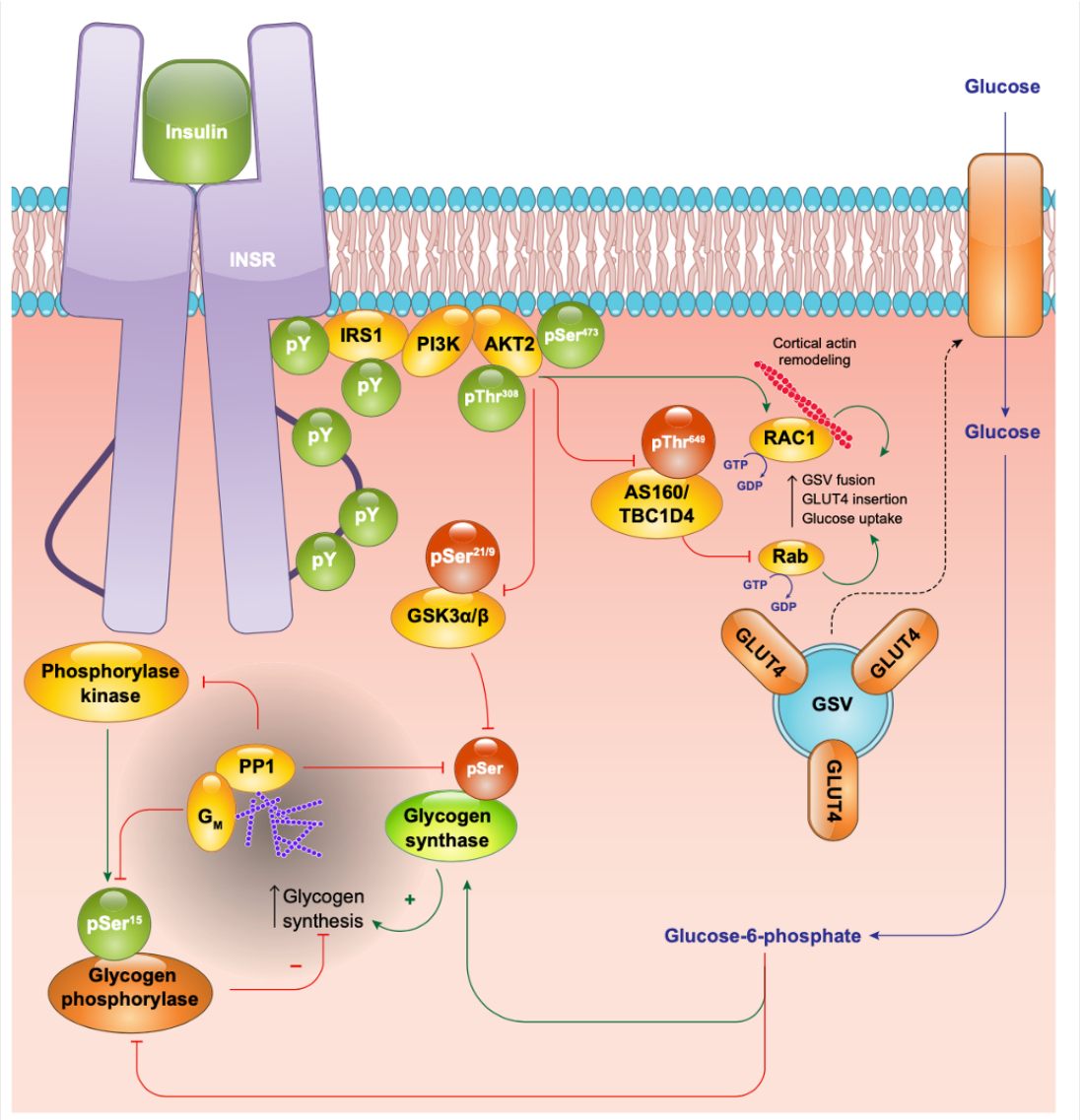

Figure 1-2: The insulin signalling cascade in skeletal muscle
Diagram shows the insulin signalling cascade in skeletal muscle, as described in the main text. Taken from Petersen and Shulman, (2018).

1.5 Tissue-specific actions of insulin in the liver and tissue-specific hepatic insulin resistance

The liver is the central regulator of endogenous glucose production and a key buffer for postprandial glucose excursions, contributing to the balance between cellular glucose uptake and hepatic glucose release that maintains blood glucose within a narrow physiological range (Hatting *et al.*, 2018)

(**Figure 1-3**). In the postprandial state, rising glucose and incretins stimulate insulin secretion, which suppress hepatic glucose production (HGP) while promoting glycogen storage and *de novo* lipogenesis. In liver, insulin binds the insulin receptor, triggering autophosphorylation and IRS-1/IRS-2 recruitment. This activates the PI3K/AKT pathway, leading to the phosphorylation of FOXO1 at Thr24, Ser256, and 319, promoting its nuclear exclusion (via 14-3-3 binding) and preventing transcription of gluconeogenic genes such as *Pck1* (encoding phosphoenolpyruvate carboxykinase, PEPCK) and *G6pc* (encoding glucose-6-phosphatase, G6Pase) (Hatting *et al.*, 2018; Petersen and Shulman, 2018; Bo *et al.*, 2024). These are the rate-controlling enzymes of gluconeogenesis: PEPCK catalyses the conversion of oxaloacetate to phosphoenolpyruvate, while G6Pase hydrolyses glucose-6-phosphate to free glucose for release (Zhang *et al.*, 2018). Insulin also inhibits glycogenolysis through mTORC1/SREBP-1c activation of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Approximately 50-80% of portal insulin is cleared by the liver via receptor-mediated endocytosis, modulating systemic exposure (Hatting *et al.*, 2018; Petersen and Shulman, 2018).

Hepatic insulin action is unique compared with skeletal muscle (primarily GLUT4-driven uptake) or adipose tissue (mainly antilipolytic). It combines direct hepatocyte-autonomous effects (e.g., glycogen synthesis, lipogenesis, and transcriptional repression of *Pck1*/*G6pc*) with indirect extrahepatic effects (e.g., suppression of adipose lipolysis to reduce glycerol/FFA substrate supply for gluconeogenesis and inhibition of glucagon secretion) (Han *et al.*, 2016; Petersen and Shulman, 2018; Bo *et al.*, 2024). *Pck1* and *G6pc* share transcriptional similarities: both are induced by glucagon (via cAMP/PKA >CREB) and glucocorticoids, but dominantly repressed by insulin through at least three transcription factors (FOXO1(a master regulator of gluconeogenesis), hepatocyte nuclear factor 4α (HNFα), and CREB) as well as the coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Granner *et al.*, 1983; Mithieux *et al.*, 1996;

Hatting *et al.*, 2018). Under short-term fasting, HGP is met primarily by glycogenolysis; after prolonged fasting (when glycogen stores are depleted), gluconeogenesis becomes essential to avoid hypoglycaemia. In the postprandial state, insulin inhibits HGP to prevent unnecessary glucose synthesis (Zhang *et al.*, 2019a; Petersen and Shulman, 2018).

In T2D and obesity, the liver develops selective insulin resistance, a major driver of fasting hyper glycaemia. While insulin fails to adequately suppress HGP (leading to excessive glycogenolysis and gluconeogenesis, with persistent *Pck1*/*G6pc* expression), lipogenic pathways often remain sensitive or are hyper-activated, fuelling non-alcoholic fatty liver disease (NAFLD) and hypertriglyceridaemia (Petersen and Shulman, 2018; Bo *et al.*, 2024). This selective insulin resistance arises from several convergent mechanisms. Lipid overload results in intrahepatic accumulation of diacylglycerols (DAGs), which activate PCKε. PCKε then binds to and inhibits the insulin receptor kinase, thereby impairing proximal insulin signalling. This leads to reduced tyrosine phosphorylation of IRS-1, diminished PI3K recruitment, and blunted AKT activation, ultimately preventing effective nuclear exclusion of FOXO1 and allowing sustained transcription of gluconeogenic genes such as *Pck1* and *G6pc*. Low-grade inflammation, driven by cytokines such as TNF and IL-6, together with ER stress and oxidative stress, activates JNK and IKK-β pathways. These kinases serine-phosphorylate IRS-1 and IRS-2, further disrupting insulin signalling downstream of the receptor (Samuel, Petersen and Shulman, 2010; Petersen and Shulman, 2018). Extrahepatic factors also contribute where insulin resistance in adipose tissue fails to suppress lipolysis, resulting in elevated circulating FFAs that provide both additional substrates for gluconeogenesis and ongoing lipid overload to the liver (Petersen and Shulman, 2018; Sancar and Birkenfeld, 2024). Paradoxically, the lipogenic branch of insulin-signalling remains relatively preserved or even enhanced. Downstream pathways involving mTORC1, sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate-response element-binding protein (ChREBP) continue to respond to insulin (and

hyperglycaemia), driving increased de novo lipogenesis despite the impairment in glucose-regulatory control (Petersen and Shulman, 2018; Bo *et al.*, 2024).

Despite detailed knowledge of these intracellular pathways, the precise blood-borne factors present in the serum from PWT2D that confer this hepatic insulin resistance (particularly the failure of insulin to repress *Pck1* transcription in hepatomas) remain incompletely defined. While elevated FFAs, pro-inflammatory cytokines, and chronic hyperglycaemia have been implicated individually, their relative contributions, synergistic interactions, and direct effects when presented in human T2D serum are not fully understood (Samuel, Petersen and Shulman, 2010; Petersen and Shulman, 2018; Bo *et al.*, 2024). This critical knowledge gap limits our ability to target

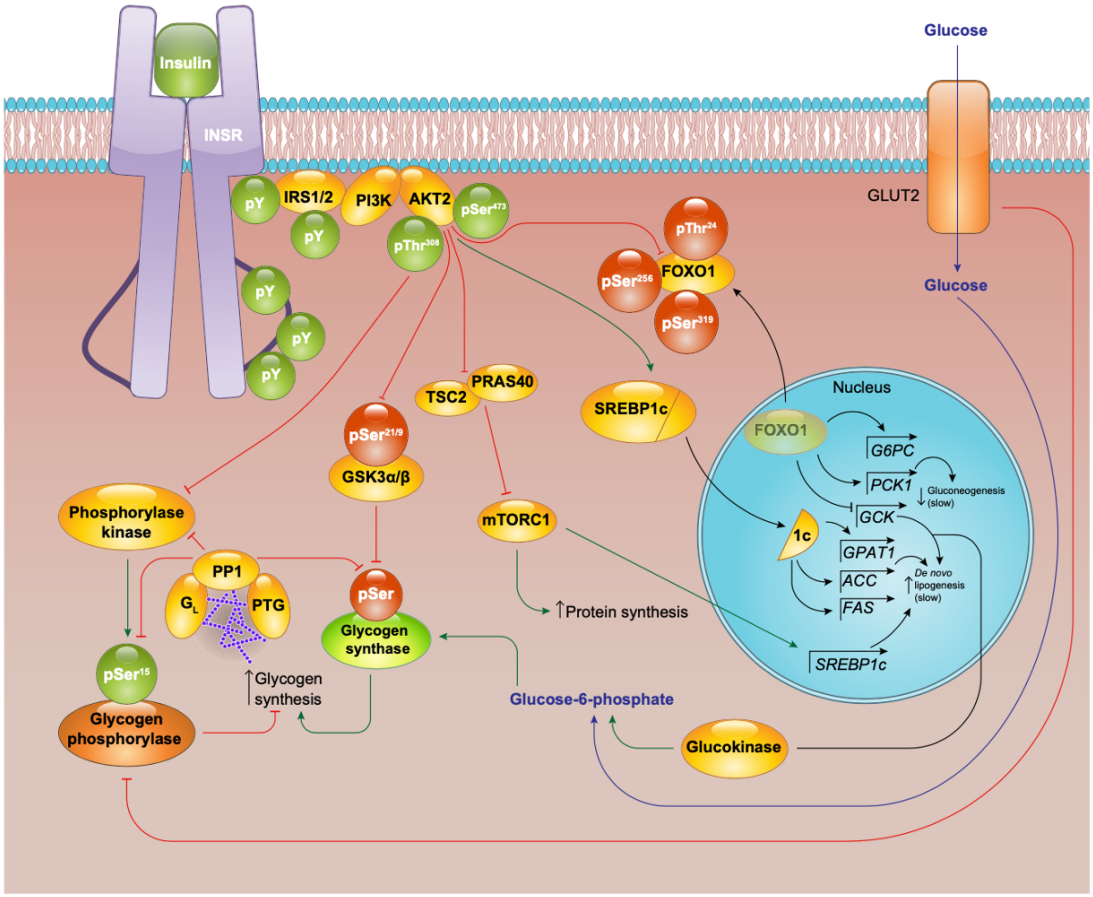

Figure 1-3: Hepatic insulin signalling
Diagram shows the insulin signalling cascade in liver, as described in the main text. Taken from Petersen and Shulman, (2018).

the circulating mediators that drive persistent gluconeogenic gene expression and hepatic glucose overproduction in T2D. Experimental models that directly expose to patient-derived serum therefore could offer a powerful approach to dissect these serum-induced molecular defects and identify the key humoral factors responsible.

1.6 Tissue-specific actions of insulin in adipose tissue and tissue-specific adipose insulin resistance

Adipose tissue is a major insulin-sensitive organ that plays a critical role in whole body energy homeostasis and glucose regulation. In the postprandial state, insulin promotes glucose uptake into adipocytes (primarily via GLUT4 translocation) and suppresses lipolysis, shifting the tissue from fatty acid release to storage as triglycerides (Samuel and Shulman, 2016; Czech, 2017; Petersen and Shulman, 2018). Insulin binds the insulin receptor on adipocytes, triggering autophosphorylation and recruitment of IRS-1 and IRS-2. This activates the Pi3K/AKT signalling pathway. AKT then phosphorylates AS160 (TBC1D4), which releases GSVs from intracellular retention and promotes GLUT4 translocation to the plasma membrane for glucose entry. Within the adipocyte, insulin stimulates lipogenesis through activation of mTORC1, which in turn activates SREBP-1c and upregulates FAS and ACC for triglyceride synthesis. Critically, insulin inhibits hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) activity through AKT-mediated phosphorylation of perilipin-1 and other regulatory proteins, thereby blocking triglyceride hydrolysis and the release of FFAs and glycerol into the circulation (Samuel and Shulman, 2016; Czech, 2017; Petersen and Shulman, 2018) (**Figure 1-4**).

Adipose tissue insulin action differs from skeletal muscle (high capacity GLUT4 uptake for glycogen storage and oxidation) and liver (suppression of hepatic glucose production combined with lipogenesis). Adipose tissue is quantitatively the smallest contributor to postprandial glucose disposal

(around 5-10% of total) but it is qualitatively the most important regulator of circulating lipids. By suppressing lipolysis, insulin prevents excess FFA flux to the liver and muscle, indirectly supporting hepatic glycogen synthesis and peripheral glucose uptake. This antilipolytic effect is highly sensitive to insulin and occurs at low physiological concentrations, making adipose tissue a primary determinant of systemic insulin sensitivity (Samuel and Shulman, 2016; Czech, 2017; Petersen and Shulman, 2018).

In T2D and obesity, adipose tissue develops adipose insulin resistance, a key early defect that exacerbates systemic hyperglycaemia and dyslipidaemia. The hallmark is failure of insulin to suppress hydrolysis, leading to elevated circulating FFAs and glycerol. This occurs through several convergent mechanisms. Lipid overload and ceramide accumulation activate PKCε and protein phosphatase 2A (PP2A), which inhibit AKT signalling and reduce phosphorylation of perilipin-1 and HSL, resulting in persistent triglyceride hydrolysis and FFA release. Low-grade inflammation, driven by pro-inflammatory cytokines (TNF, IL-6, IL-1β) from infiltrating macrophages, activates JNK and IKK-β pathways. These kinases then serine/threonine-phosphorylate IRS-1 and IRS-2, impairing PI3K/AKT activation and defective GLUT4 translocation. ER stress and oxidative stress further activate JNK, leading to additional IRS serine phosphorylation and disrupted insulin signalling. Dysregulation of adipokines also contributes; reduced adiponectin (which normally enhances insulin sensitivity) and increased leptin or resistin impair antilipolytic and glucose-uptake responses (Samuel and Shulman, 2016; Czech, 2017; Petersen and Shulman, 2018).

The resulting chronic elevation of circulating FFAs provides excess substrate for hepatic gluconeogenesis (glycerol as a direct precursor; FFAs oxidised to acetyl-CoA, which allosterically activates pyruvate carboxylase) and causes lipid overload in the liver (FFAs converted to triglycerides, DAGs, and ceramides), while also inducing lipotoxicity in skeletal muscle (ceramide/ PKCθ activation leading to IRS-1 serine phosphorylation and peripheral

insulin resistance). This creates a vicious cycle of systemic insulin resistance, fasting hyperglycaemia, and postprandial glucose intolerance. Adipose insulin resistance often appears early in T2D pathogenesis, preceding significant hepatic and muscle defects in many individuals (Samuel and Shulman, 2016; Czech, 2017; Petersen and Shulman, 2018).

Despite substantial knowledge of these intracellular pathways, the precise blood-borne factors present in the serum from PWT2D that confer insulin resistance (particularly the failure of insulin to suppress lipolysis and promote glucose uptake in adipocytes) remain incompletely defined (Samuel and Shulman, 2016; Czech, 2017; Petersen and Shulman, 2018). While elevated FFAs, pro-inflammatory cytokines, and chronic hyperglycaemia have been implicated individually, their relative contributions, synergistic interactions, and direct effects when presented in human T2D serum are not fully understood. Experimental models that expose adipocyte cell lines to patient-derived serum therefore offer a valuable approach to dissect these humoral mediators and identify the key circulating factors responsible for conferring the adipose-specific defects observed in T2D.

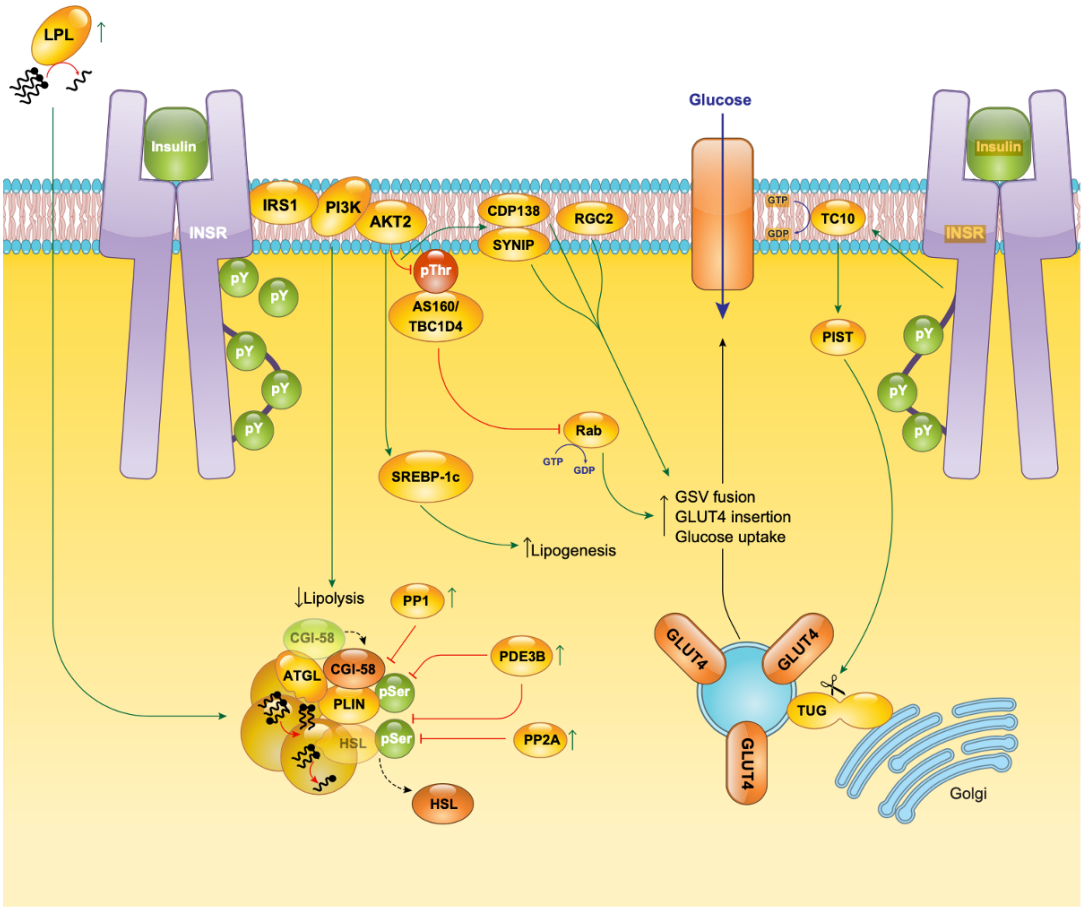

Figure 1-4: Insulin signalling in white adipose tissue
Diagram shows the insulin signalling cascade in white adipose tissue, as described in the main text. Taken from Petersen and Shulman, (2018).

1.7 Insulin signalling

Insulin exerts pleotropic effects through activation of its receptor, which is expressed in tissues that are sensitive to insulin. The insulin receptor is a tyrosine kinase, consisting of glycosylated, disulphide-linked $\alpha_2\beta_2$ tetramers, which is mainly found at the plasma membrane, although a fraction can be found in the nucleus (Saltiel, 2021). Each insulin receptor has two binding sites, but due to negative cooperativity, it is thought that at physiological concentrations, one molecule of insulin binds and activates one receptor (Petersen and Shulman, 2018). Binding, causes conformational changes, resulting in the phosphorylation and activation of the ß subunits of the

receptor, which can then recruit and phosphorylate substrates to perpetuate signalling (Petersen and Shulman, 2018).

Insulin signalling can be broadly divided into two categories – mitogenic, and metabolic (Petersen and Shulman, 2018; Le *et al.*, 2023; Burchfield, Diaz-Vegas and James, 2025). Mitogenic signalling is perpetuated by the recruitment and phosphorylation of an Shc adapter protein, leading to a cascade of phosphorylation events, involving the mitogen-activated protein kinases, the primary mediators of mitogenic insulin signalling (Petersen and Shulman, 2018; Le *et al.*, 2023; Burchfield, Diaz-Vegas and James, 2025). This project primarily focuses on metabolic signalling which will now be described.

Most of the physiological metabolic actions of insulin occur through the insulin-AKT axis, which can be arbitrarily divided into proximal and distal sections. (James, Stockli and Birnbaum, 2018; Burchfield, Diaz-Vegas and James, 2025). Proximal signalling is perpetuated by recruitment and phosphorylation of the insulin receptor substrate (IRS) proteins (James, Stockli and Birnbaum, 2018; Burchfield, Diaz-Vegas and James, 2025). These tyrosine-phosphorylated IRS proteins form a scaffold, to which phosphoinositide-3-kinase is recruited, catalysing the formation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3), a phospholipid at the inner leaflet of the plasma membrane (James, Stockli and Birnbaum, 2018; Burchfield, Diaz-Vegas and James, 2025) (**Figure 1-5**). Net accumulation of PIP_3 propagates and amplifies insulin signalling (Petersen and Shulman, 2018). PIP_3 recruits Ser/Thr protein kinases, such as PDK1 and AKT. This allows PDK1 to phosphorylate AKT at position Thr308, partially activating it. AKT phosphorylates and activates mammalian target of rapamycin 2 (mTORC2) which in turn phosphorylates AKT at position Ser473, fully activating it (James, Stockli and Birnbaum, 2018; Burchfield, Diaz-Vegas and James, 2025). Proximal insulin signalling can be thought of as ending at this point. Numerous distal pathways, resulting in a multitude of biological

functions, lie downstream of activated AKT, including glycogen synthesis, anti-lipolysis, *de novo* lipogenesis, protein synthesis, and glucose uptake (**Figure 1-5**). Two of these pathways; glucose uptake, and the suppression of hepatic glucose production are related to this project and will now be described.

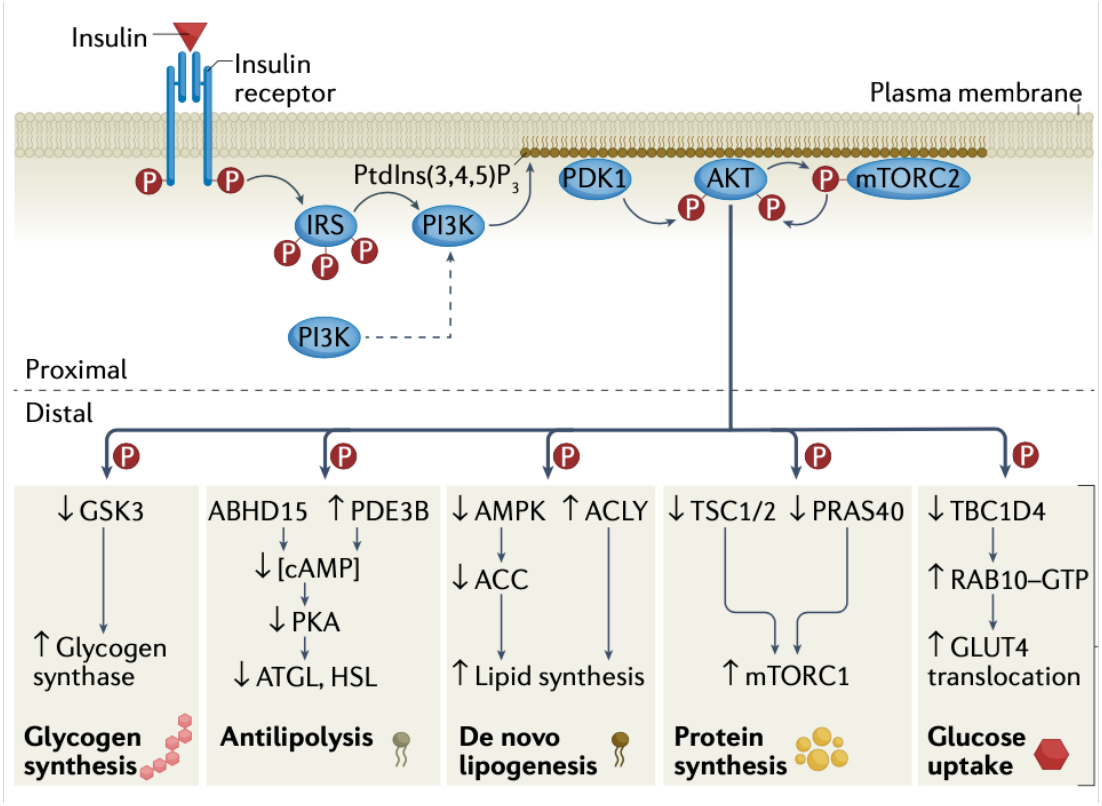

Figure 1-5: AKT is a key node in the insulin signalling network.
Diagram shows the proximal events of insulin signalling, described in the main text, leading to some of the distal physiological consequences. Taken from James, Stockli and Birnbaum,(2018).

1.8 Glucose uptake

A pivotal metabolic function of insulin is the stimulation of glucose uptake into muscle and adipose tissue (Klip, McGraw and James, 2019). This process involves the trafficking of the facilitative glucose transporter, GLUT4 to the plasma membrane, enabling it to provide the bulk of cellular glucose uptake (Chadt and Al-Hasani, 2020) (**Figure 1-6**). The precise molecular mechanism

by which GLUT4 is recruited to the cell surface is incompletely understood (James, Stockli and Birnbaum, 2018; Drobiova *et al.*, 2025), although AKT plays an important role, at least in part, by phosphorylating AKT substrate of 160 kDa (AS160), also known as TBC1 domain family member 4 (TBC1D4) on T642 (Cartee, 2015). AS160 controls the Rab GTPase Rab10 which is a mediator of membrane transport (Babbey *et al.*, 2006). Deletion of AS160 and Rab10 does not completely block the insulin-mediated transport response, implying that other AKT substrates are involved (James, Stockli and Birnbaum, 2018) (depicted as the purple protein X on **Figure 1-6**). Other molecular components involved in this process, are the clathrin machinery, the retromer protein complex, and the docking/fusing machinery necessary for GLUT4 exocytosis (James, Stockli and Birnbaum, 2018). Work is ongoing to fully elucidate how these, and other components work together to enable this key process that is misregulated in disease states such as diabetes (Klip, McGraw and James, 2019). Defective glucose metabolism is a central component of T2D, and defective insulin stimulated glucose uptake is a well-documented manifestation of the disease (James, Stockli and Birnbaum, 2018; Burchfield, Diaz-Vegas and James, 2025). Hence, it has been the focus of this section.

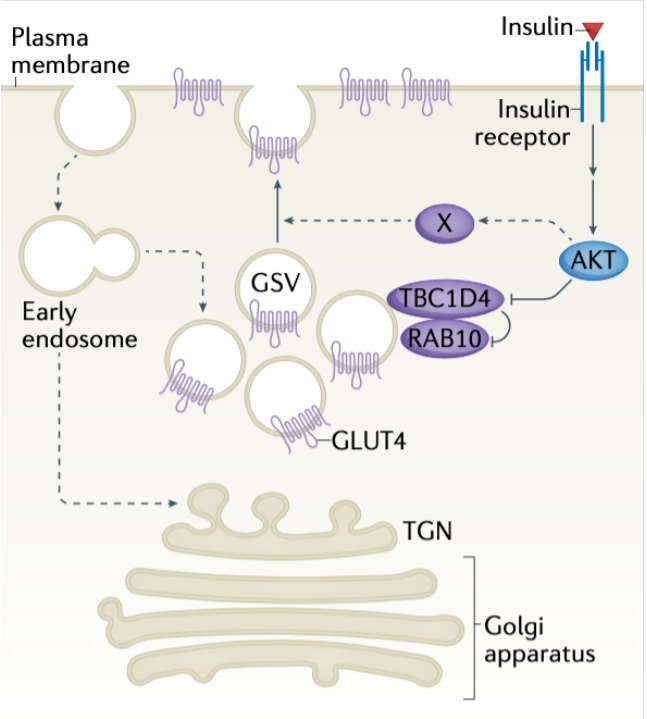

Figure 1-6: Insulin promotes glucose uptake in muscle and fat.
Diagram shows some of the molecular mechanisms involved in the insulin-mediated trafficking of GLUT4 to the plasma membrane of muscle and adipose cells. Taken from Taken from James, Stockli and Birnbaum,(2018).

1.9 Suppression of hepatic glucose production

Blood glucose concentrations are maintained within a healthy narrow range by the balance of cellular glucose uptake and endogenous glucose production (Hatting *et al.*, 2018). The liver contributes to this process by altering levels of glucose release through controlling the processes of *de novo* glucose production (gluconeogenesis) and the breakdown of glycogen into free glucose (glycogenolysis) (Barroso *et al.*, 2024). Under short-term fasting conditions, the demand for glucose is met by glycogenolysis (Zhang *et al.*, 2018). After prolonged fasting, glycogen stores become depleted and therefore to avoid hypoglycaemia, new glucose is synthesised by gluconeogenesis (Zhang *et al.*, 2019a). In the postprandial state, synthesis and release of this glucose would cause blood glucose levels to exceed the healthy range. Thus, a key action of insulin is to inhibit hepatic gluconeogenesis.

Insulin can regulate hepatic gluconeogenesis via transcription of gluconeogenic genes such as *Pck1* and *G6pc* (Hatting *et al.,* 2018). *Pck1* and *G6pc* encode phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) respectively, which are the rate controlling enzymes of gluconeogenesis (Zhang *et al.*, 2018). Mechanistically, PEPCK catalyses the conversion of oxaloacetate to phosphoenolpyruvate and G6Pase catalyses the conversion of glucose-6-phosphate to glucose (Zhang *et al.*, 2019a). *Pck1* and *G6pc* have several transcriptional similarities, in that they are induced by glucagon and glucocorticoid signalling and dominantly repressed by insulin (Granner *et al.*, 1983; Mithieux *et al.*, 1996). Insulin-mediated repression is known to mediated through at least three transcription factors including Forkhead box protein O1 (FOXO1), a master regulator of gluconeogenesis, hepatocyte nuclear factor 4α (HNF4α), and cAMP-response element-binding protein (CREB), and also through a transcriptional coactivator called peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Barroso *et al.*, 2024). However, the exact molecular mechanisms have not yet been fully defined (Hatting *et al.*, 2018). There is interest in further elucidating the role of insulin signalling in the regulation of gluconeogenesis to develop new therapies in disease states such as diabetes (Barroso *et al.*, 2024).

1.10 Insulin resistance: definitions and implications

Insulin resistance is a central pathophysiological feature of T2D and a major focus of this thesis. Despite its importance, the term is frequently used without precise definition. The following section provides a structured overview of insulin resistance from 3 complimentary perspectives: biomedical science, physiological measurement, and pragmatic clinical application.

1.10.1 Biomedical science definition: a post-receptor defect in insulin signalling

In biomedical terms, insulin resistance is defined as a defect in the intracellular signalling pathways downstream of the insulin receptor (a "post-receptor" defect (Petersen *et al.*, 2016). As outlined above, in healthy cells, insulin binds its receptor causing autophosphorylation and recruitment of IRS-1/2. This activates PI3K and AKT, which mediate the metabolic actions of insulin (GLUT4 translocation in muscle/adipose, suppression of gluconeogenesis in liver, inhibition of lipolysis in adipose tissue). In insulin-resistant states, proximal receptor signalling may be intact, but downstream transduction is impaired, leading to reduced responsiveness to insulin at the cellular level (Petersen *et al.*, 2016; Petersen and Shulman, 2018).

The most common molecular mechanisms involve serine/threonine phosphorylation of IRS-1/2 (rather than the required tyrosine phosphorylation), which uncouples the receptor from PI3K/AKT. Key triggers include intra-tissue lipid metabolites (DAGs activating PKCε or PKCθ; ceramides activating PP2A), low-grade inflammation (TNF, IL-6 activating JNK and IKK-β), ER stress, and oxidative stress. These pathways converge to blunt AKT activation, preventing effective nuclear exclusion of FOXO1 in the liver (leading to persistent *Pck1*/*G6pc* expression), GLUT4 translocation in muscle, and perilipin/HSL inhibition in adipose tissue. The result is a selective or pathway-specific defect: glucose regulatory actions are impaired while some mitogenic or lipogenic branches (e.g., mTORC1/SREBP-1c) may remain preserved. (Samuel and Shulman, 2012; Petersen *et al.*, 2016; Petersen and Shulman, 2018).

This post-receptor paradigm distinguishes insulin resistance from rare receptor-level defects (e.g., mutations in the IR causing extreme syndromes) (Pliszka and Szablewski, 2025). In common T2D and obesity, the defect is acquired and reversible initially through weight loss or exercise, highlighting its post-receptor nature. Understanding insulin resistance at this molecular

level is essential because it explains why (compensatory) hyperinsulinaemia can coexist with hyperglycaemia and why tissue-specific manifestations (hepatic glucose overproduction, muscle glucose under-utilisation, adipose lipolysis) drive the metabolic syndrome.

1.10.2 Physiological definition: a reduced response to a given concentration of insulin

Physiologically, insulin resistance is defined as a subnormal biological response to a given concentration of insulin (Tam *et al.*, 2012). The gold-standard method for quantifying this in humans is the euglycaemic-hyperinsulinaemic clamp, developed by DeFronzo *et al.* (1979). During the clamp, insulin is infused at a fixed rate (typically 40-120 mU/m^2/min) to achieve steady state euglycaemia (around 5 mmol/L). Under these conditions, hepatic glucose production is fully suppressed, so the glucose infusion rate (GIR) required to maintain euglycaemia directly equals whole-body glucose disposal (primarily muscle uptake). A lower GIR indicates insulin resistance (Tam *et al.*, 2012).

Insulin resistance is therefore operationally defined as a rightward shift in the insulin concentration-response curve (reduced sensitivity) and/or a decrease in maximal response (reduced responsiveness. (Gastaldelli, 2022). For example, a GIR <5.6mg/kg fat-free mass/min (or <4.9 mg/kg/min when normalised to body weight) at a standard insulin infusion rate is commonly used as a threshold for insulin resistance in white populations (Tam *et al.*, 2012; Gastaldelli, 2022). This definition is tissue-agonistic at the whole-body level but can be refined with tracers (e.g., to separate hepatic versus peripheral contributions). The clamp remains the most precise and reproducible method because it isolates the effect of insulin under controlled conditions, free from confounding variables such as fasting glucose or endogenous insulin secretion (Tam *et al.*, 2012; Gastaldelli, 2022).

1.10.3 Pragmatic clinical definitions

In everyday clinical practice, insulin resistance is pragmatically inferred from surrogate markers of compensatory hyperinsulinaemia in the face of normal or elevated glucose. The most widely used index is the homeostasis model assessment of insulin resistance (HOMA-IR), calculated as (fasting glucose [mmol/L] x fasting insulin [μU/mL]/22.5. Values >2.5-3.0 (depending on population) indicate insulin resistance (Tam *et al.*, 2012; Gastaldelli, 2022). Other surrogates include the quantitative insulin sensitivity check index (QUICKI), elevated fasting insulin alone (.10-15 μU/mL), or the presence of the metabolic syndrome features (central obesity, dyslipidaemia, hypertension). (Tam *et al.*, 2012; Gastaldelli, 2022). These definitions are convenient for large epidemiological studies and clinical risk stratification but are less precise than clamp-derived measures because they rely on fasting steady-state assumptions and do not distinguish hepatic from peripheral resistance (Tam *et al.*, 2012; Gastaldelli, 2022).

1.10.4 Link to study rationale

Collectively, these definitions frame insulin resistance as a core, proximal defect in T2D pathogenesis. The biomedical (post-receptor signalling failure), physiological (impaired glucose disposal during clamp), and clinical (compensatory hyperinsulinaemia) perspectives converge on the same underlying problem: target tissues fail to adequately respond to insulin. As we shall see, the purpose of this PhD project was to identify blood-borne factor(s) in serum from PWT2D that mediate these phenomena.

1.11 Inflammation and metabolic stress in insulin resistance

Chronic low-grade inflammation and metabolic stress are central drivers of acquired insulin resistance in T2D and obesity. The term “metaflammation” (metabolic inflammation) describes this sterile, nutrient-excess-driven

inflammatory state that differs from classical pathogen-induced inflammation by being chronic, low-grade, and localised primarily in metabolic tissues such as adipose tissue, liver, and skeletal muscle. (Wu and Ballantyne, 2020; Charles-Messance *et al.*, 2020) (**Figure 1-7**).

In obesity, adipose tissue expansion leads to adipocyte hypertrophy, hypoxia, and cell death, which trigger the recruitment and activation of pro-inflammatory macrophages (Alexaki, 2024). These immune cells, along with stressed adipocytes, secrete pro-inflammatory cytokines such as TNF, IL-6, and IL-1ß. These cytokines activate stress-sensitive kinases, notably JNK and IKK-ß. JNK and IKK-ß directly serine-phosphorylate IRS-1 at inhibitory sites (e.g., Ser307), thereby uncoupling the insulin receptor from downstream PI3K/AKT signalling and impairing GLUT4 translocation in muscle and adipose tissue, suppression of hepatic glucose production, and inhibition of lipolysis in adipose tissue (Solinas and Karin, 2010; Bensussen, Torres-Magallanes and de Alvarez-Buylla, 2023).

Metabolic stress pathways amplify this inflammatory response. Excess nutrient influx induces ER stress through accumulation of unfolded proteins, activating the unfolded protein response (UPR). The IRE1α branch of the UPR recruits and activates JNK, further promoting IRS-1 serine phosphorylation and inflammatory gene expression via NF-κB. Oxidative stress, arising from mitochondrial overload and ROS production, activates additional stress kinases (JNK, p38, MAPK) and exacerbates ER stress, creating self-reinforcing loops. Lipid metabolites such as DAGs and ceramides also directly activate PKCθ in muscle and PKCε in liver and adipose tissue, which inhibit insulin signalling upstream and downstream of AKT (Lyu *et al.*, 2021; Gusev, Sarapultsev and Zhuravleva, 2026).

These processes are not confined to one tissue. Adipose-derived cytokines and FFAs released due to unsuppressed lipolysis spill over into the circulation, inducing lipotoxicity and inflammation in distant organs. In the

liver, this promotes persistent gluconeogenic gene expression (*Pck1* and *G6pc*); in skeletal muscle, it impairs glucose uptake. The result is a vicious cycle in which insulin resistance further fuels inflammation and metabolic stress, accelerating pancreatic ß cell dysfunction and progression to overt T2D (Petersen and Shulman, 2018; Lee, Park and Choi, 2022; Sancar and Birkenfeld, 2024).

Importantly, metaflammation and metabolic stress provide a mechanistic link between obesity and the circulating factors present in T2D serum. Elevated FFAs, pro-inflammatory cytokines, and hyperglycaemia (glucotoxicity) in patient serum could induce these same stress and inflammatory pathways when applied to cultured cells, offering a plausible explanation for the serum-mediated insulin resistance observed in experimental models.

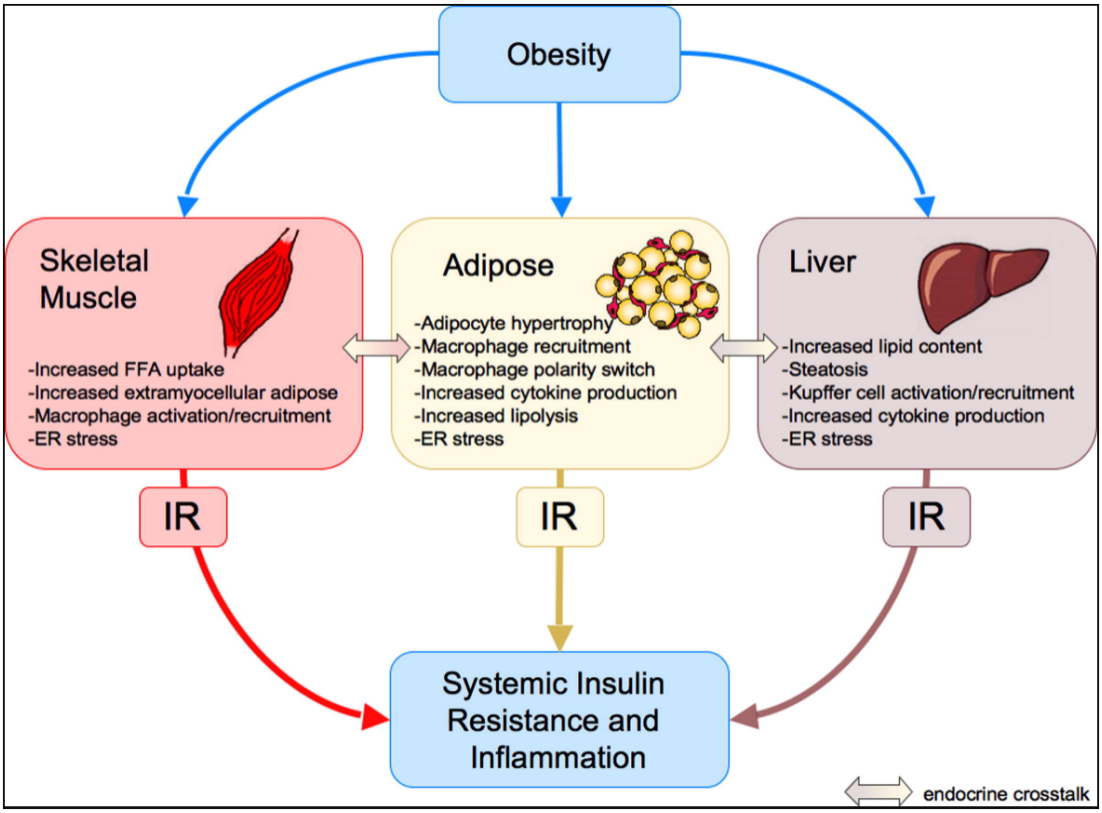

Figure 1-7: The development of inflammation and insulin resistance in obesity
Diagram shows the development of inflammation and insulin resistance in obesity, as described in the main text. Taken from de Luca and Olefsky (2008).

1.12 Diabetes

There are several diseases involving the dysfunction of insulin. Perhaps the most well-known among them is diabetes mellitus. Diabetes (from the Greek word meaning siphon or pass through) mellitus (from the Latin word meaning sweet) is a metabolic disease involving inappropriately elevated blood glucose levels in either fasting or postprandial states (Sapra, Bhandari and Wilhite Hughes, 2021). There are several categories of diabetes, including gestational diabetes, neonatal diabetes, and steroid-induced diabetes. The two main categories of diabetes have been designated type 1 (T1D) and type 2 (T2D) (Sapra, Bhandari and Wilhite Hughes, 2021).

1.13 T1D

T1D, also known as autoimmune diabetes, is a chronic condition characterised by insulin deficiency due to the destruction of the pancreatic ß-cells by T cells of the immune system (Burrack, Martinov and Fife, 2017). Treatment for T1D involves the supply of exogenous insulin (now most commonly by self-administered injection of biosynthetic human insulin). However, even when carefully managed, this treatment can still lead to fluctuations in blood glucose levels outside the normal physiological range, eventually resulting in severe and life-threatening complications such as cardiovascular disease (CVD) (Burrack, Martinov and Fife, 2017). To address this, continuous glucose monitoring devices and high precision insulin pumps have recently been developed and may soon be the best option for long-term management of T1D (Sherwood, Russell and Putman, 2020).

1.14 T2D

1.14.1 Background and epidemiology

T2D is a chronic metabolic disorder typified by hyperglycaemia caused by insulin resistance and impaired insulin secretion (DeFronzo *et al.*, 2015). Over time, these elevated blood glucose levels can lead to damage to the

heart, vasculature, eyes, kidneys, and nerves. T2D accounts for 90% of all diabetic cases, making it the commonest form of the disease (DeFronzo *et al.*, 2015). In 2019, diabetes caused 4.2 million deaths, worldwide. At the same time, there were 463 million adults, aged between the ages of 20 and 79, living with diabetes (Galicia-Garcia *et al.*, 2020). This number could rise to 700 million by 2045 (Galicia-Garcia *et al.*, 2020). Patients with T2D have a 15% increased risk of all-cause mortality compared to people without diabetes (Galicia-Garcia *et al.*, 2020). Moreover, cardiovascular disease is the greatest cause of morbidity and mortality associated with T2D. The estimated worldwide direct healthcare expenditure for diabetes was 760 billion US dollars (USD) in 2019 (Williams *et al.*, 2020). This figure is projected to rise to 845 USD by 2045 (Williams *et al.*, 2020). The bulk of this expenditure will be T2D-related. In short, T2D is a serious disease with large associated morbidities, mortalities, and economic costs.

1.15 Risk factors and pathophysiology

T2D is a heterogeneous disease that is caused by a complex interplay of genetic, epigenetic, and environmental factors (Prasad and Groop, 2015). The heritability of T2D has been reported to range from 30 to 70% (Laakso and Silva, 2022). Consequently, genome wide association studies, and meta-analyses of these studies, have been conducted to identify the genetic risk factors involved. Several candidate alleles have been identified by these means (Xue *et al.*, 2018), although the clinical insights provided by GWAS as a whole have been described as limited (Cano-Gamez and Trynka, 2020). This is perhaps due to many genes being involved, each only contributing a small effect, making the analysis of overall risk and the elucidation of specific mechanisms a complex task. Importantly, aside from genetics, the development and progression of T2D is influenced by lifestyle and environmental factors, such as obesity, lack of exercise, high fat diets, and even environmental pollution, (Leahy, 2005). These factors could be tackled to potentially ameliorate the disease.

The biggest single risk factor for developing T2D is obesity, [defined as having a body mass index of 30 (kg/m2) or above]. Consequently, a twin-cycle hypothesis outlining how obesity could lead to T2D has been developed (**Figure 1-8**) (Taylor, 2019). In this model, a positive calorie balance causes excess fat to accumulate in the liver of people with insulin resistance (determined by genetic and/or lifestyle factors) due to higher plasma insulin levels. This fat causes a relative resistance on the ability of insulin to suppress hepatic glucose production (potentially through an attenuation of the repression of *Pck1* transcription, for example), resulting in an increase in fasting plasma levels. Over time this results in an increase of basal insulin secretion as a homeostatic response. This insulin further facilitates the conversion of excess calories into more liver fat. Thus, a vicious cycle of hyperinsulinemia and inadequate suppression of hepatic glucose production is perpetuated. Excess fat in the liver leads to increased export of low-density lipoprotein triacylglycerol. This leads to increased fat delivery to all tissues including the pancreatic ß cells which secrete insulin. Increased fat in these cells impairs insulin secretion in response to ingested food. This eventually leads to dedifferentiation of the ß cells and post-prandial hyperglycaemia. This hyperglycaemia then leads to further hyperinsulinemia and increased hepatic lipogenesis which will feed forward into the pancreatic cycle again. Eventually this leads to a catastrophic level of ß-cell de-differentiation, such that a sudden onset of clinical diabetes occurs (Taylor, 2019).

This model emphasises the importance of crosstalk between different tissues in the development of insulin resistance in the progression of T2D. Much work is going on to elucidating these networks of communication as a route to developing new therapies (Rosa *et al.*, 2020; Romero and Eckel, 2021; Yang *et al.*, 2022; Xourafa, Korbmacher and Roden, 2024). It is also important to note that the underlying molecular lesion or lesions that initiate and then promote insulin resistance are different in different people (Woods, Petrie and Sutherland, 2009). That is, T2D is a heterogeneous disease. Therefore,

people with T2D may require specific treatments – a precision (also known as personalised or stratified) medical approach. To enable this, the identification of biomarkers which indicate underlying specific molecular lesions are needed.

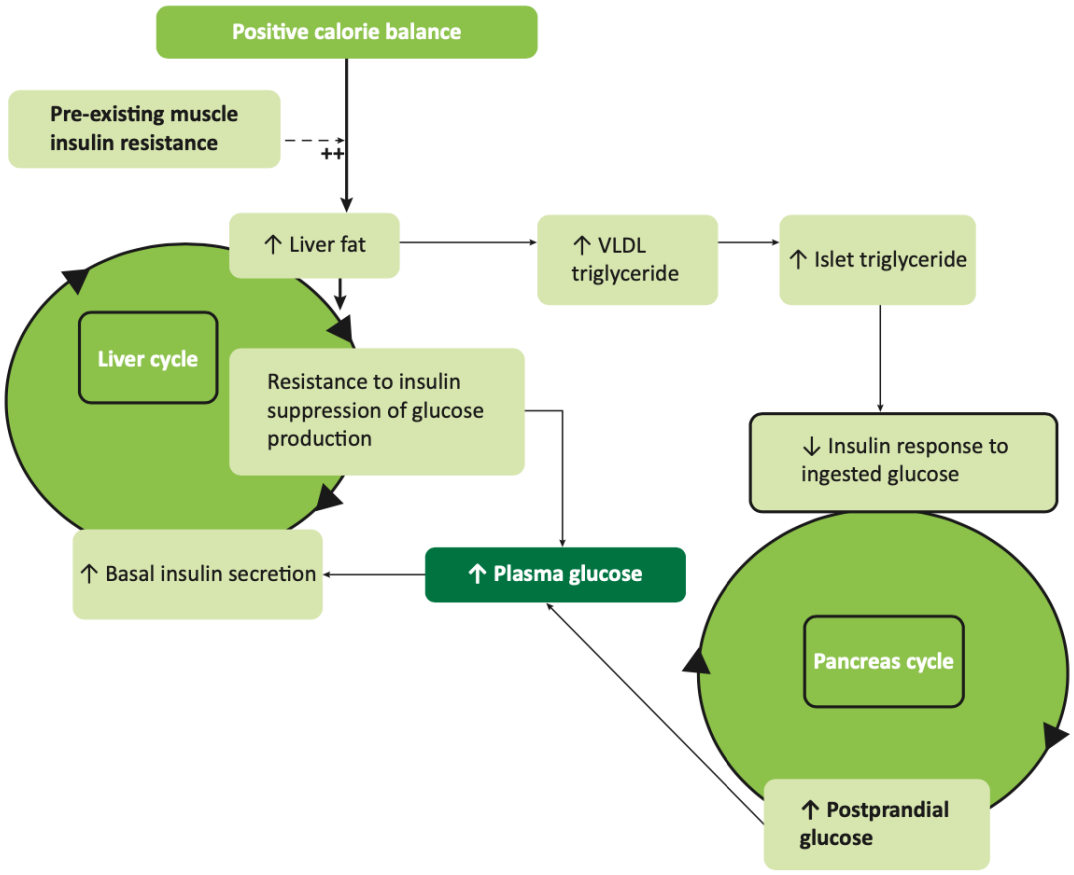

Figure 1-8: Diagram of the twin cycle hypothesis as described in the main text. Taken from Taylor(2019).

Regarding treatments for T2D, strict calorie control resulting in substantial weight loss has been shown to delay, prevent the onset, and even to generate remission of the disease in recently diagnosed individuals (60% remission for those who have had T2D for less than 4 years, falling to 21% remission for those with duration greater than 8 years) (Taylor, 2019). This speaks to the strong correlation between obesity and T2D but what about the 10-15% of people with a BMI of less than 27 kg/m^2 who are diagnosed with T2D? Taylor

(2019) suggested that these people may just have acquired too much fat than they could personally cope with. This hypothesis was later tested with the Reversal of Type 2 Diabetes upon Normalisation of Energy Intake in the Non-obese) (ReTUNE) study, showing that 70% of people with T2D and a BMI of less than 27 kg/m^2 at diagnosis achieved remission through weight loss (with an average loss of 8% of bodyweight needed for remission) (Taylor *et al.*, 2023). Therefore, a key takeaway from these studies is that weight loss should be prioritised as a key treatment for people with T2D. However, most people fail when they try to lose weight (Poraj-Weder, Wasowicz and Pasternak, 2021). Incretin-based medications could help in this regard as they have been shown to be highly effective in treating obesity and T2D (Nauck *et al.*, 2026). Correspondingly, use of these medications is becoming widespread as more efficacious next-generation versions are being developed and generic older (but still effective) versions are becoming available (Nauck *et al.*, 2026).

Given that the twin cycle hypothesis (**Figure 1-8**) elucidates the central role of intra-organ fat in driving hepatic insulin resistance and ß cell dysfunction, the search for novel, non-invasive biomarkers (such as circulating metabolites or lipid species reflective of these ectopic fat cycles) holds promise for advancing precision medicine approaches in T2D by enabling earlier diagnosis, personalised risk stratification, and tailored remission strategies. A potential strategy that could lead to the identification of such biomarkers will now be discussed.

1.16 Serum from people with T2D

A foundational study that underpins this PhD project is Logie *et al.* (2010). Using the rat hepatoma cell line H4-II-E, the authors demonstrated that culture in serum obtained from PWT2D resulted in a significant average reduction in insulin sensetivity. This was evidenced by attenuaton of insulin-mediated repression of *Pck1* gene expression, a key regulator of hepatic gluconeogenesis. Importantly, substantial inter-individual variability was

observed: while some T2D serum samples produced only modest impairment, others markedly blunted insulin's ability to repress *Pck1*, far exceeding the response seen with control serum (Logie *et al.*, 2010).

This PhD project extended this line of investigation by incubating both H4-II-E hepatoma cells (assessing insulin-mediated repression of *Pck1* transcript accumulation by qPCR) and 3T3-L1 adipocytes (assessing insulin-stimulated radiolabelled glucose uptake) with serum from PWT2D. These complimentary functional readouts were used to evaluate the insulin resistance-promoting properties of patient serum. The findings of Logie *et al.* (2010) stongly suggest the presence of one or more circulating factor(s) in the blood of people with T2D that can directly induce insulin resistance and can potentially contribute to disease progression. Identification and characterisation of this factor (or factors) could therefore open several therapeutic avenues: (1) development of new treatments aimed at reducing or blocking its activity, (2) stratification of T2D patients according to the level or presence of the factor(s) for more personalised therapy, and (3) use as an early biomarker to identify individuals with incipient insulin resistance before overt T2D develops, potentially allowing intervention to halt or reverse disease progression.

The first critical step towards these goals is to identify and characterise the nature of the responsible circulating factor(s) in serum from PWT2D.

1.17 The serum factor(s) as biomarkers

Building upon the demonstration that serum from PWT2D can directly induce insulin resistance in cultured cells (Logie *et al.*, 2010), relatively little attention has been paid to the identity of the responsible blood-borne factor(s). We were therefore interested in whether these circulating factor(s) could serve as novel biomarkers for insulin resistance and T2D. Several biomarkers are already in clinical use for T2D, including inflammatory markers such as C-reactive protein (CRP), IL-6, and TNF, as

well as HbA1c, HOMA-IR, the oral glucose test, and lipid profiles (Stanimirovic *et al.*, 2022; Mukherjee and Im, 2025). The gold-standard research tool for quantifying insulin sensitivity remains the hyperinsulinaemic-euglycaemic clamp technique (DeFronzo, Tobin and Andres, 1979; Tam *et al.*, 2012).

Nevertheless, T2D is a highly heterogeneous condition with diverse underlying risk factors and complications, and current biomarkers such as HbA1c do not fully capture this complexity (Adams *et al.*, 2021; Munns, Brown and Buckberry, 2025). There is therefore a clear need for more robust biomarkers capable of identifying individuals at risk, stratifying patients for personalised therapy, and revealing new therapeutic targets (Adams *et al.*, 2021; Munns, Brown and Buckberry, 2025).

Logie *et al.* (2010) observed marked inter-individual variation in the ability of serum from PWT2D to provoke insulin resistance in cultured cells. This variability implies that the proposed blood-borne factor(s) differ between patients. Identification and quantification of such factor(s) could therefore enable subclassification of PWT2D and support more personilised treatment strategies. In this context, the blood-borne factor(s) themselves could function as novel biomarkers.

1.18 Evidence for circulating mediators and biomarkers of insulin resistance

Supporting the existence of circulating mediators of insulin resistance, experimental models have demonstrated inter-tissue communication in this process. For example, adipose-specific GLUT4 knockout in mice induced systemic insulin resistance in muscle and liver, leading to the identification of retinol-binding protein 4 (RBP4) as a candidate humoral factor. Circulating RBP4 is elevated in obese and insulin-resistant humans, and experimental elevation of RBP4 induces insulin resistance through macrophage-derived pro-inflammatory cytokines and JNK-mediated inhibition of GLUT4

translocation (Santoro and Kahn, 2023). Thus, RBP4 exemplifies both a validated biomarker and a mechanistic driver, raising the possibility that Logie *et al.* (2010)'s serum factor(s) could include proteins such as RBP4.

More recent biomarker discovery has increasingly employed omics-based approaches. Metabolomic ans lipidomic studies have identified branch-chain amino acids, GlcA (glycoprotein acetylation), specific lysophosphatidylcholines, gangliosides, cholesterol esters, phosphatidylethanolamines, and phosphatidic acid as potential biomarkers, some with sex-specific associations (Liu *et al.*, 2025; Simón *et al.*, 2025; Qi *et al.*, 2025). Proteomic analyses have highlighted elevated α2-macroglobulin and complement protein C3 (Wang *et al.*, 2025), while targeted assays have implicated angiopoietin-like 5 (Alghanim *et al.*, 2019). RNA-based biomarkers, including multiple miRNAs that target insulin signalling components, as well as certain lncRNAs and circRNAs, are also altered in T2D serum (Wan *et al.*, 2017; Aljaibeji *et al.*, 2022; Ning *et al.*, 2022; Greco *et al.*, 2023; Su *et al.*, 2024). Exosomes have also emerged as potential mediators; Fuchs *et al.* (2021) showed that exosomes from individuals with non-alchoholic fatty liver disease induced insulin resistance when incubated with human myotubes and mouse hepatocytes, suggesting that Logie *et al.* (2010)'s serum factor(s) may be exosome-associated.

Known blood-borne factors associated with insulin resistance can broadly be categorised as drivers or biomarkers. FFAs are among the strongest causal drivers. In adipose insulin resistance, failure to suppress lipolysis leads to chronic FFA spillover, which induces lipotoxicity in liver and muscle through DAG and ceramide accumulation, activating PKCε and PKCθ and impairing insulin signalling (Petersen and Shulman, 2018; Sancar and Birkenfeld, 2024). Inflammatory cytokines such as TNF and IL-6 act as both drivers and biomarkers: TNF directly activates JNK and IKK-β to serine phosphorylate IRS-1, while chronic IL-6 promotes Suppressor of Cytokine Signalling 3 expression and hepatic insulin resistance (Hotamisligil, 2017). Among

adipokines, low adiponectin is both a biomarker and a protective driver, whereas elevated leptin primarily reflects adipose mass and leptin resistance rather than directly involving insulin resistance (Turer and Scherer, 2012; Ye and Scherer, 2013; Petersen and Shulman, 2018).

A systems biology perspective is increasingly recognised as essential for moving beyond single-factor studies in insulin resistance research (Li *et al.*, 2022). Insulin resistance is a complex, multi-organ phenomenon involving dynamic crosstalk between adipose tissue, liver, skeletal muscle, and beta pancreatic cells., mediated by a network of metabolites, cytokines, adipokines, exosomes and other humoral signals (Yang *et al.*, 2022; Petersen and Shulman, 2018). Systems approaches integrate multi-omics datasets (genomics transcriptomics, proteomics, metabolomics, lipidomics) with computational network modelling and machine learning to identify emergent patterns and predictive signatures that single-molecule studies could miss c(Bragg *et al.*, 2022; Cheng *et al.*, 2023; Xie *et al.*, 2025). In the context of circulating mediators, such approaches could help map how combinations of factors (rather than any single molecule) interact to propagate insulin resistance, and how these signatures differ across disease states or patient subgroups. While powerful, systems-level studies currently face challenges including high cost, the need for large well-phenotyped cohorts, and difficulty distinguishing correlation with causation (Li *et al.*, 2022; Abel *et al.*, 2024). Functional bioassays such as the serum-exposure models used in this thesis provide a valuable reductionist complement, allowing causal testing of candidate mediators or serum fractions within a systems framework.

Classic parabiosis experiments provided clear evidence for circulating satiety factors (e.g., leptin in ob/ob mice), but analogous studies for insulin resistance have largely been inconclusive. Pairing insulin resistant obese mice with lean partners does not consistently rescue insulin sensitivity or transfer resistance, suggesting that insulin resistance is predominantly

tissue-autonomous or requires chronic, multifactorial exposure rather than a single dominant humoral factor (Coleman, 1973). This contrasts with the monogenic nature of leptin deficiency and highlights why identifying circulating mediators of insulin resistance has proven more challenging.

In summary, while several blood-borne candidates (FFAs TNF/IL-6, low adiponectin, RBP4, specific lipids, miRNAs, and exosomes) have been implicated, their relative contributions, synergistic interactions, and direct effects when presented together in serum from PWT2D remain incompletely defined. This critical gap directly motivates this PhD project, which assessed the insulin resistance-promoting properties of serum from PWT2D. The overall aim was to identify blood-borne factor(s) in serum from PWT2D that mediate proximal post-receptor defects in insulin signalling, thereby providing new insight into the humoral mediators responsible for conferring insulin resistance in T2D.

1.19 Hypothesis

The literature reviewed above highlights that while serum from PWT2D can induce insulin resistance in cultured cells, the identity and mechanism of action of the responsible circulating factor(s) remain poorly defined. To address this gap, we hypothesise that serum from PWT2D contains co-factors that promote insulin resistance that may contribute to the ongoing disease. We will test the hypothesis by asking the following research questions:

1. Does serum from PWT2D induce insulin resistance in cultured cells, by impairing insulin-mediated repression of *Pck1* transcript accumulation in H4-II-E hepatomas?

2. Is the insulin resistance conferred by the serum factor(s) cell-type or pathway specific?

3. What is the nature of the blood-borne factor(s) present in serum from PWT2D that confer insulin resistance to cultured cells?

By answering these questions, the study aimed to provide new insight into the humoral mediators of insulin resistance and lay the foundation for future identification and therapeutic targeting of the responsible circulating factor(s).

2 Materials and Methods

2.1 Human serum

2.1.1 Original cohort details and recruitment

3 cohorts of people – people with established T2D (PWT2D), people with pre-T2D (PWPT2D) and healthy volunteers (controls)– were recruited to provide the blood serum used for this project. PWT2D (HbA1c over 50 mmol/mol) and PWPT2D (HbA1c between 42-49 mmol/mol) were controlling their condition through diet and told us they had not used diabetes medications for at least 3 months prior to collection. Controls were people without diabetes or pre-diabetes (HbA1c less than 42 mmol/mol). All volunteers were aged 18 or over and were recruited via the following 5 means. First, PWT2D and PWPT2D were recruited from the NHS Research Scotland Diabetes Research Network. This register has a large group of PWT2D and some PWPT2D who have expressed a willingness to be involved in research and given permission to be contacted. Second, PWT2D were approached by their care teams in diabetes clinics or for PWPT2D by their care teams as part of a weight management service/Tayside diabetes prevention programme. Third, PWPT2D within the Tayside diabetes programme may have been approached in writing using a "clinic letter". Fourth, posters were placed in diabetes clinics and eye screening clinics (for diabetes patients) and in workplace corridors or notice boards (for controls). Fifth, social media posts were used to engage individuals in the local (Tayside) community.

2.1.2 Second cohort details and recruitment (people with pre-T2D)

3 months before the end of the project, we received serum samples from 6 people with pre-T2D which were recruited as follows: recently diagnosed people with pre-T2D within the Tayside area were referred to the Tayside diabetes prevention programme and given an invitation to participate in a

study assessing levels of environmental toxins conducted by Professor Calum Sutherland. The 6 people who expressed an interest to participate had HbA1cs of between 42-47 mmol/mol and were not taking medication for glucose control. Healthy age and sex matched controls were recruited as above.

2.1.3 Blood donation procedure

Volunteers were asked to fast overnight before donation. Their height, weight, and body composition (using bioimpedance scales) were measured. Blood was drawn for an HbA1c test to confirm pre-T2D/T2D (or lack thereof) and a larger volume drawn to generate serum. For this purpose, the blood was allowed to clot before being centrifuged (to pellet the clot and blood cells). The supernatant (serum) was then transferred to 5 mL aliquots, snap frozen and stored at -80 °C. Blood was collected in clinics and surgeries and transferred initially to the University of Dundee for initial storage. Serum samples were shipped to Glasgow on dry ice and stored at -80 °C prior to use.

2.2 Ethics and approval

The relevant ethical approval for this project was given to the University of Dundee. The Integrated Research Application System form and ethical approval letters are in Appendix **Sections 1.1 and 1.2**, respectively. All protocols described in these documents were adhered to

2.3 Cell culture

2.3.1 H-4-II-E cells

H-4-II-E rat hepatoma cells were used in this study as we were trying to replicate the work of Logie *et al*. (2010) who used these cells. They were obtained from European Collection of Authenticated Cell Cultures (ECACC**,** 87031301**)**, were maintained in Dulbecco's Modified Eagle Medium (DMEM),

low glucose (1 gram of D-glucose per litre), pyruvate (Gibco, 31885-023), supplemented with 10% (v/v) foetal bovine serum (FBS) (Gibco, A3840402), and 1% (v/v) penicillin-streptomycin (Sigma-Aldrich, P4458), in 75 cm^2 flasks (Corning, CLS430641U), in a 37 °C, 5% CO_2 incubator. Cells were grown to around 90% confluency, then, after removing the medium and rinsing with sterile phosphate buffered saline (PBS), consisting of one PBS tablet (Fisher Scientific, BR014G) dissolved in 100 mL of ultra-pure water (giving 1X PBS), passaged using 3 mL TrypLE Express Enzyme to detach the cells (GibcoTM,12604013). By these means, the cells were split at a 1:4 ratio 3 times per week.

2.3.2 3T3-L1 fibroblasts/adipocytes

3T3-L1 mouse fibroblast cells, obtained from American Type Culture Collection (CRL-1548) were maintained in what we call 'NCS media', consisting of DMEM, high glucose (4.5 grams of D-glucose per litre), pyruvate, no glutamine (Gibco, 21969-035), supplemented with 10% (v/v) new-born calf serum (Gibco, 26010-066), 1% (v/v) penicillin-streptomycin, and 1% (v/v) L-glutamine (Sigma-Aldrich, G7513) in 75 cm^2 flasks in a 37 °C, 10% CO_2 incubator. Cells were grown until around 70% confluent and then passaged as described for the H-4-II-E cells above.

2.3.2.1 3T3-L1 differentiation

The differentiation process was carried out as described in Bremner *et al.* (2022). Briefly, two days after the 3T3-L1 fibroblasts became 100% confluent, at now termed Day 0 (D0), NCS media was removed and replaced with FBS-media (DMEM, high glucose, pyruvate, no glutamine (Gibco, 21969-035), with 10% (v/v) foetal bovine serum (Gibco, A3840402), 1% (v/v) penicillin-streptomycin, 1% (v/v) L-glutamine (Sigma-Aldrich, G7513)) supplemented with 1 µg/mL porcine insulin (Sigma-Aldrich, I5523), 5 µM troglitazone, 0.25 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. At Day 3 (D3), the D0 media was replaced with D3 differentiation media consisting of FBS

media, supplemented with 1 µg/mL porcine insulin (Sigma-Aldrich, I5523), and 5 µM troglitazone. From Day 6 (D6) onwards, the cells were maintained in FBS media with no other additions, refreshed on D6, D7, D10, and 3 times per week after D10. For all experiments, 10 to 15-day old 3T3-L1 adipocytes were used, other than those specifically indicated otherwise in **Chapter 5**. Before experiments, the cells were viewed with a Leica TCS SP8 confocal microscope to confirm they had differentiated into adipocytes (which are larger than fibroblasts and contain lipid droplets, not seen in fibroblasts).

2.4 Treatments

2.4.1 Human serum

For qPCR, H-4-II-E cells were grown for three weeks in DMEM, low glucose (1 gram of D-glucose per litre), pyruvate, supplemented with 1% (v/v) penicillin-streptomycin and either 5% or 10% (v/v) human serum (as indicated in the legends). Regarding these concentrations, 5% was used to replicate Logie *et al*. (2010) and 10% was used to determine if an increased concentration would show an effect where 5% did not. The cells were passaged as described in the cell culture section.

2.5 Preparation of cell lysates

Media was removed and the cells were washed in ice-cold PBS. The cells were lysed by scraping them into ice-cold lysis buffer, consisting of 50 mM Tris-HCL, 50 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 250 mM mannitol, and 1 mM DTT, pH 7.4, with the addition of one protease inhibitor tablet (Fisher Scientific, A32955), dissolved per 10 mL, and 100 µL of phosphatase inhibitor cocktail (Sigma-Aldrich, P5726) per 10 mL. Note: for this purpose, the cells were grown in 12-well plates. 50 µL of lysis buffer was used per well. The lysate from each well was transferred to a 1.5 mL microfuge tube, incubated on ice for 20 mins, then centrifuged at

21,910 x g for 5 mins at 4 °C. The supernatant was collected and stored at -20 °C.

2.6 Measurement of protein concentration in lysates

Total protein concentration was calculated by spectrophotometric analysis using a bicinchoninic acid (BCA) assay kit (Fisher Scientific, 23225) following the manufacturer's instructions. Briefly, in duplicate, 2 µL of cell lysate (or 10 µL of protein standard, consisting of 0, 0.03125, 0.0625, 0.125, 0.25, 0.5, 0.75, 1, 1.5, and 2 mg/mL of bovine serum albumen (BSA) dissolved in lysis buffer) was added to 200 µL of a (50:1) BCA Reagent A / BCA Reagent B mixture and incubated at 37 °C for 30 minutes. Absorbance was measured at 562 nm using a WPA S2000 spectrophotometer. A curve of absorbance over known concentrations of the protein standards was plotted. After fitting a linear trendline, the equation of the line (y = mx + c) was rearranged and the lysate absorbance readings inputted to determine the protein concentrations of the lysates.

2.7 SDS-PAGE

Lysate proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), carried out using 1.5 mm thick 10% (v/v) acrylamide gels, each containing a 2 cm 5% (v/v) acrylamide stacking portion. Samples (containing 40 µg of protein as determined by BCA assay) (or 20 µg and 80 µg for 1X and 4X in the case of GLUT4) were mixed with Laemmli Sample Buffer at pH 6.8 (giving final concentrations of 50 mM Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% (m/v) bromophenol blue, 62.5 mM DTT) and heated at 60 °C for 5 minutes for GLUT1/GLUT4 blots or 95 °C for 5 minutes for blots of all other proteins. Electrophoresis was carried out at 100 V until the dye front migrated from the gel. Approximate molecular weights of proteins were determined with reference to a molecular weight marker (New England Biolabs, P7718).

2.8 Western blotting

2.8.1 Electrophoretic transfer of proteins onto nitrocellulose membranes

After SDS-PAGE, each gel was placed on a nitrocellulose membrane with a 0.25 µm pore size, between four sheets of blotting paper (two on each side). A sponge was placed on each side of this, and the resulting sandwich was encased in a gel holder cassette. Electrophoretic transfer was carried out at 60 V for 2 h and 15 mins. Post-transfer, the nitrocellulose membrane was stained with Ponceau (0.2% (w/v) Ponceau S, 1% (v/v) acetic acid) to check the homogeneity of transfer and the equal loading of protein between lanes, and then de-stained by washing in Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.5, 137 mM NaCl) 3 times for 5 minutes.

2.8.2 Blocking of membranes and incubation with primary antibodies

To reduce non-specific attachment of antibodies, membranes were incubated at room temperature, on a shaker, in 5 % (w/v) dried skimmed milk powder in TBS for 40 minutes. After washing in TBS, membranes were incubated with primary antibodies in TBS + Tween 20 (TBST) [20 mM Tris-HCl, pH7.5, 137 mM NaCl, 0.1% (v/v) Tween 20] and 1 % BSA, at 4 °C, overnight on a shaker.

2.8.3 Primary antibodies used for western blotting

Epitope	Supplier	Product Number	Dilution	Host Species
ACC	Cell Signalling	#3662	1:1000	Rabbit
AKT (pan)	Cell Signalling	#2920	1:1000	Mouse

AMPK α subunit	Cell Signalling	#2532	1:1000	Rabbit
AS160	Cell Signalling	#2670	1:1000	Rabbit
CREB	Cell Signalling	#9104	1:1000	Mouse
GLUT1	Abcam	ab115730	1:1000	Rabbit
GLUT4	Home-made using a combination of rabbit polyclonal antibodies raised against the N and C termini of GLUT4 (Bremner *et al.*, 2022)	N/A	1:1000	Rabbit
Insulin Receptor ß-subunit	Abcam	ab69508	1:1000	Mouse
IRAP	Cell Signalling	#3808	1:1000	Rabbit
Phospho-AKT (Ser473)	Cell Signalling	#4058	1:1000	Rabbit

Phospho-AS160	Cell Signalling	#8881	1:1000	Rabbit
Phospho-CREB (Ser133)	Cell Signalling	#9198	1:1000	Rabbit
Sortilin	Abcam	ab16640	1:1000	Rabbit
Syntaxin-16	Abcam	ab134945	1:1000	Rabbit
β-Actin	Cell Signalling	#3700	1:1000	Mouse

Table 2-1: Primary antibodies used for western blotting

2.8.4 Incubation with secondary antibodies

Membranes were washed in TBST then incubated using a secondary antibody matching the epitope of the host species of the primary antibody (from the list below) in TBST and 5% BSA with gentle shaking in the dark.

2.8.5 Secondary antibodies used for western blotting

Epitope	Supplier	Product Number	Dilution	Host Species	Conjugated Molecule
Mouse IgG	LI-COR Biosciences	926-68022	1:10000	Donkey	IRDye 680LT
Rabbit IgG	LI-COR Biosciences	926-32213	1:10000	Donkey	IRDye 800CW

Table 2-2: Secondary antibodies used for western blotting

2.8.6 Visualisation and densitometric quantification of protein bands

Membranes were washed in TBST 3 times for 5 minutes. The fluorescence of the secondary antibodies was imaged using a LI-COR Odyssey SA system. The intensities of the protein bands were quantified using Image Studio Lite software.

2.9 qPCR

2.9.1 RNA extraction

Total cellular RNA was extracted from H-4-II-E cells using a Monarch Total RNA Miniprep Kit (New England Biolabs, T2010S), according to the manufacturer's instructions. Briefly, the cells were lysed in 300 µL of lysis buffer per well of a 12-well plate. The contents of each well were transferred to a gDNA removal column and centrifuged for 30 seconds. Note: all centrifugation steps in this procedure were carried out at 16, 000 *g*. An equal volume of 99.8% ethanol was added to the flow through and mixed. This mixture was added to an RNA purification column, centrifuged for 30 seconds, and the flow through discarded. 500 µL RNA priming buffer was added to the column which was centrifuged for 30 seconds and the flow through discarded. 500 µL wash buffer was added to the column which was centrifuged for 30 seconds and the flow through discarded. Another wash step was carried out, this time centrifuging for 2 minutes and the flow through discarded. 50 µL nuclease free water was added to the column which was centrifuged for 30 seconds. The flow through containing the RNA was then collected in a 1.5 mL microfuge tube.

2.9.2 gDNA removal

Residual genomic DNA was removed using a TURBO DNA-free Kit (Invitrogen, AM1907), according to the manufacturer's instructions. Briefly, 0.1 volume TURBO DNAse buffer and 1 µL TURBO DNAse enzyme was mixed with the RNA and incubated at 37 °C for 30 minutes (to degrade any residual genomic DNA). To inactivate the DNAse enzyme, 0.1 volume of DNAse Inactivation Reagent was added, mixed, and incubated at room temperature for 5 minutes (with gentle flicking to keep the contents mixed). To remove the DNAse Inactivation Reagent, the samples were centrifuged at 10 000 *g* for 5 minutes and the supernatant was transferred to a fresh tube.

2.9.3 Quantification of extracted RNA

The concentration and purity (via 260/280 and 260/230 ratios) of the RNA was measured using NanoDrop2000c Spectrophotometer. RNA samples were stored at – 70 °C.

2.9.4 cDNA synthesis

RNA was transcribed into cDNA using a LunaScript reverse transcriptase (RT) SuperMix Kit (New England Biolabs, E3010L) according to the manufacturer's instructions. Briefly, in 20 µL reactions, 750 ng of RNA and a 1X final concentration of LunaScript RT SuperMix (or a SuperMix minus RT to provide a genomic DNA control for the qPCR reaction) were mixed in nuclease free water and incubated in a thermal cycler for the following incubation steps:

Step	**Temperature**	**Time**
Primer annealing	25 °C	2 minutes
cDNA synthesis	55 °C	10 minutes

Heat inactivation	95 °C	1 minutes

Table 2-3: cDNA synthesis thermocycling protocol

After these incubation steps, 80 µL of TE buffer (Fisher Scientific, AM9849), was mixed with each sample before being stored at – 20°C. For ease of explanation, going forward, I will refer to these samples as 'cDNA template' for the ones incubated with RT and 'cDNA control' for the ones incubated minus RT.

2.9.5 qPCR reaction

Each qPCR reaction was performed using Luna Universal Probe qPCR Master Mix (M3004L, New England Biolabs) and a Taqman probe (Fisher Scientific) for the specific mRNA in question (*Pck1*, *G6pc*, *Actb* or *Rplp1*) in a 96-well reaction plate (Fisher Scientific, 4346907), sealed with optical adhesive film (Fisher Scientific, 4360954). Further details of the Taqman probes used can be found in the table below.

Target	**Amplicon size (bp)**	**Probe type**	**Assay ID**
Pck1	87	FAM-MGB	Rn01529014_m1
G6pc	64	FAM-MGB	Rn00689876_m1
Rplp1	96	VIC-MGB	Rn03467157_gH
Actb	91	VIC-MGB	Rn00667869_m1

Table 2-4: Taqman probes used for qPCR

For each qPCR reaction, a master mix was made for each Taqman probe needed depending on the experiment in question. Typically, this would be for the gene of interest (either *Pck1* or *G6pc*) and a housekeeping gene (either *Rplp1* or *Actb*) for normalisation purposes. A 20 µL total volume was added, per well, consisting of a 1X final concentration of Luna Universal Probe qPCR Master Mix, 0.4 µM forward primer, 0.4 µM reverse primer 0.2 µM TaqMan probe and 1 µL of cDNA template (or cDNA control). The reaction was performed in a StepOne Real-Time PCR System (Fisher Scientific, 4376357) for the following steps:

Cycle step	Temperature	Time	Cycles
Initial denaturation	95 °C	60 seconds	1
Denaturation	95 °C	15 seconds	40
Extension	60 °C	60 seconds (+ plate read)	

Table 2-5: qPCR thermocycling protocol

2.9.6 qPCR controls and analysis

For each condition (basal, + insulin, etc.), cDNA from 2 technical replicates was used. For each of these technical replicates, 8 qPCR reactions were conducted. The first was a gDNA control, consisting of qPCR master mix (including Taqman probe for the GOI) and 1 µL of cDNA control (RNA and SuperMix minus RT, from the cDNA synthesis reaction). The next 3 wells provided 3 technical replicates, consisting of qPCR master mix (including Taqman probe for the GOI) and 1 µL of cDNA template. The pattern of 4 wells just described was then repeated, this time using qPCR master mix including

Taqman probe for the housekeeping gene (instead of the GOI), for normalisation purposes. The 3 GOI cycle threshold (Ct) values generated for each of the 2 technical replicates for each condition were averaged and then these two average figures were themselves averaged, giving a single GOI Ct value per condition. These steps were then repeated, for the housekeeping gene Ct values, giving a single housekeeping Ct value per condition.

2.9.6.1 ΔΔCt method (normalising to basal)

In most cases, the GOI Ct values were normalised to the housekeeping Ct values using the ΔΔCt method (Livak and Schmittgen, 2001). Briefly, the single average housekeeping Ct value for each condition was subtracted from the single average GOI Ct value for the same condition. This provided ΔCt values. To provide ΔΔCt values (normalising to basal), the basal ΔCt was subtracted from itself and the ΔCt figures for all other conditions. To provide the $2^{-\Delta\Delta Ct}$ values, 2 was raised to the power of the negative ΔΔCt values. Statistics, if shown, were performed on data from at least 3 biological replicates. Before performing statistical tests, the average $2^{-\Delta\Delta Ct}$ values were base 2 log-transformed to avoid potential skewing on the linear scale.

2.9.6.2 $2^{(40-Ct)}$ method

In some cases, we analysed our qPCR data following the method of Logie *et al*. (2010). For this, the GOI and housekeeping Ct values for each condition were inserted into the formula $2^{(40-Ct)}$. Then the GOI value for each condition was divided by the housekeeping value for that same condition.

2.10 Glucose uptake assay

3T3-L1 adipocytes were incubated in serum-free DMEM for 2 hours (to make them quiescent to maximise the effects of insulin) before being washed in Krebs-Ringer Phosphate (KRP) buffer at pH 7.4 (128 mM NaCl, 4.7 mM KCl, 5 mM NaH_2PO_4, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$) then incubated in KRP for a

further 20 minutes. Cells were stimulated with insulin as described in the appropriate figure legend. Assays were initiated by the addition of 50 µM 2-deoxy-D-glucose and 0.5 µCi 2-[3H]-deoxy-D-glucose (Revvity, NET328A250UC). Cells were incubated at 37 °C for 3 minutes (or 27 °C for 30 or 60 minutes for the experiments presented in **Figure 5-3**, as described in the legends) before being washed 3 times in ice-cold PBS and then lysed with 1% (v/v) Triton X-100. After the addition of Optiphase hisafe 3 (Revvity, 1200.437). The radioactivity was determined with liquid scintillation, using a Perkin Elmer Tri-Carb liquid scintillation counter. In parallel, to determine non-specific association of 2-[3H]-deoxy-D-glucose, cells were treated with 10 µM cytochalasin B (Sigma-Aldrich, C6762), a selective inhibitor of the GLUT family.

2.11 Imaging of aged 3T3-L1 adipocytes

Aged 3T3-L1 adipocytes were imaged at x10 magnification using an EVOS FL Auto Imaging System.

2.12 Data analysis of lipid droplets

Images of aged 3T3-L1 adipocytes were uploaded to Fiji and the following steps were performed to analyse characteristics of number and area of lipid droplets:

- Image > Adjust > Threshold > B&W > Dark Background
- Process > Binary > Watershed
- Analyse > Set Measurements:

Area

Perimeter

Diameter

- Limit to Threshold > Send to the threshold image

- Analyse > Analyse Particles

  Size: 5-Infinity Circularity: 0.5-1.0 Show: Mask Display Results Exclude on Edges Overlay Add to Manager Composite ROIs

- Display of results were then transferred and analysed in PRISM

The lipid droplet volume was estimated, where, assuming sphericity, the diameter output from above was divided by 2 to give the radius then entered in the formula $V= 4/3\ \pi r^3$ (where V = volume and r = radius).

2.13 Statistical analysis

All statistical analyses were performed with GraphPad Prism Version 10.6.1 (GraphPad Software, Inc). n values and names of analyses used are stated in the figure legends. *, **, ***, ****, and ns represent $P\leq0.05$, $P\leq0.01$, $P\leq0.001$, $P\leq0.0001$, and not significant, respectively.

3 Chapter 3

3.1 Aims

Our general aims for this chapter were to establish and validate the experimental systems (assays) needed to screen serum from PWT2D to determine its ability to promote insulin resistance in cultured cells. We then sought to collect serum from PWT2D (and controls) and to test it using these validated assays.

Regarding the human serum needed to test the hypothesis that serum from PWT2D promotes insulin resistance in cultured cells, our specific aim was:

- To collect and pool serum from 20 PWT2D and 20 age and sex matched controls

Using H4-II-E hepatoma cells needed to test the hypothesis that serum from PWT2D promotes insulin resistance in cultured cells, our specific aims were:

- To show that H4-II-E cells responded to insulin
- To test two housekeeping genes, selecting the most stable one for use in our version of Logie *et al*. (2010)'s *Pck1* assay
- To show that *Pck1* mRNA accumulation can be induced with 8-CPT-cAMP/dexamethasone and then dominantly repressed by insulin
- To confirm that the insulin concentrations used by Logie *et al*. (2010) would be suitable for use in our version of the *Pck1* assay
- To validate the *Pck1* assay by showing that it could detect synthetic insulin resistance induced by TNF or hyperinsulinemia

- To use the *Pck1* assay to determine if pooled serum from PWT2D promoted insulin resistance

Using 3T3-L1 adipocytes to test the hypothesis that serum from PWT2D promotes insulin resistance in a different cell line to H4-II-Es, our specific aims were:

- To show that 3T3-L1 adipocytes responded to insulin

- To establish a glucose uptake assay to detect insulin-mediated glucose uptake

- To validate the glucose uptake assay by showing that it could detect synthetic insulin resistance induced by TNF or hyperinsulinemia

- To use the glucose uptake assay to determine if pooled serum from PWT2D promoted insulin resistance

3.2 Results

3.2.1 Delay of collection of human serum

The overarching aim of this project was to collect serum from people with T2D (PWT2D) and then to test its ability to induce insulin resistance, in cultured cells, by using the *Pck1* transcript accumulation assay described in Logie *et al*. (2010). However, due to the COVID-19 pandemic, the serum collection process was delayed by around a year and a half. During this time, I conducted tests to validate the assays that we eventually used to assess the effects of the serum from PWT2D when it arrived. These tests and the initial results from testing the serum from PWT2D form the basis of this chapter.

3.2.2 H4-II-E hepatoma cells respond to insulin

3.2.2.1 Insulin concentration response

Before conducting test *Pck1* transcript accumulation assays, we wanted to make sure that the H4-II-E cells we intended to use for these assays responded to insulin as has been reported (Liao *et al.*, 1998; Logie *et al.*, 2010; Hectors *et al.*, 2012). To do this, we carried out an insulin concentration response assay, western blotting for the S473 phosphorylation of the serine/threonine-specific protein kinase AKT, which is a marker for activation of the proximal insulin signalling pathway (Petersen and Shulman, 2018; Le *et al.*, 2023; Burchfield, Diaz-Vegas and James, 2025). As **Figure 3-1(A&B)** show, these cells responded to insulin in a concentration-dependent manner, showing significant increases in AKT phosphorylation after 20 minutes at insulin concentrations of 1, 10, and 100 nM.

We also used western blotting to detect the phosphorylation of AKT substrate of 160 kDa (AS160), also known as TBC1 domain family member 4 (TBC1D4), as a second measure of insulin-induced activation. As seen in **Figure 3-1 (C)**, in contrast to the concentration-dependent increases of phosphorylated AKT, there are similar amounts of T642 phosphorylated AS160 after treatments of 1, 10, and 100 nM insulin for both biological replicates. For technical reasons, these data are not normalised for total AS160, but nevertheless, alongside the clearer cut AKT data, this provides strong evidence that H4-II-E cells respond to insulin.

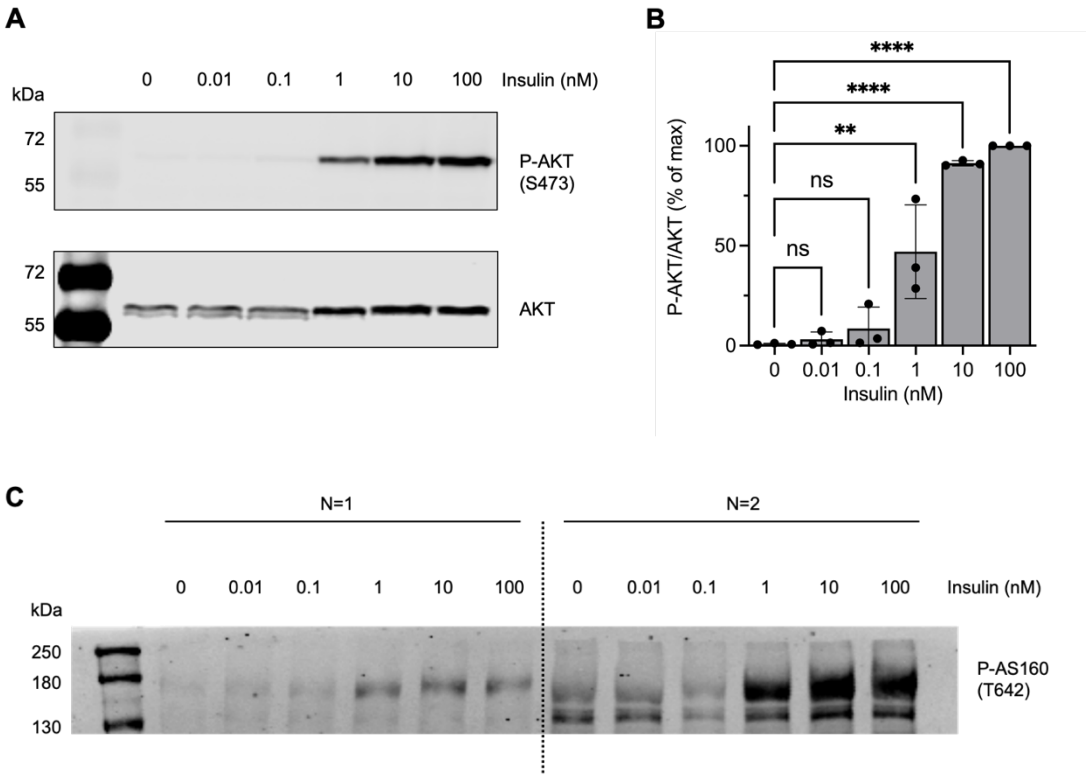

Figure 3-1: H4-II-E cells respond to insulin in a concentration-dependent manner
H4-II-E cells were serum-starved then treated with the indicated concentrations of insulin for 20 mins before being harvested. **(A)** Picture shows a representative western blot from one experiment. Lysates were probed with antibodies raised against the indicated proteins. Molecular weights shown in kilodaltons (kDa). **(B)** Graph shows the densitometric analysis of three biological replicates as per **(A)**. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by one-way ANOVA and Tukey's multiple comparisons test. **(C)** Western blot showing two biological replicates (labelled N=1 and N=2). Lysates were probed with the indicated antibodies. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.2.2 Insulin time course

To determine how quickly H4-II-E cells responded to insulin, we performed a time course using 100 nM (acute) insulin treatments. As **Figure 3-2** shows, in terms of AKT phosphorylation, there was a strong and significant response within a minute which lasted through to 60 minutes. In addition, at 60 minutes, the level of phosphorylation dropped compared to the 5-, 20-, and 40-minute time-points, (but not the 1-minute time point), indicating a potential desensitisation of the insulin signalling pathway occurred after 40 minutes these cells.

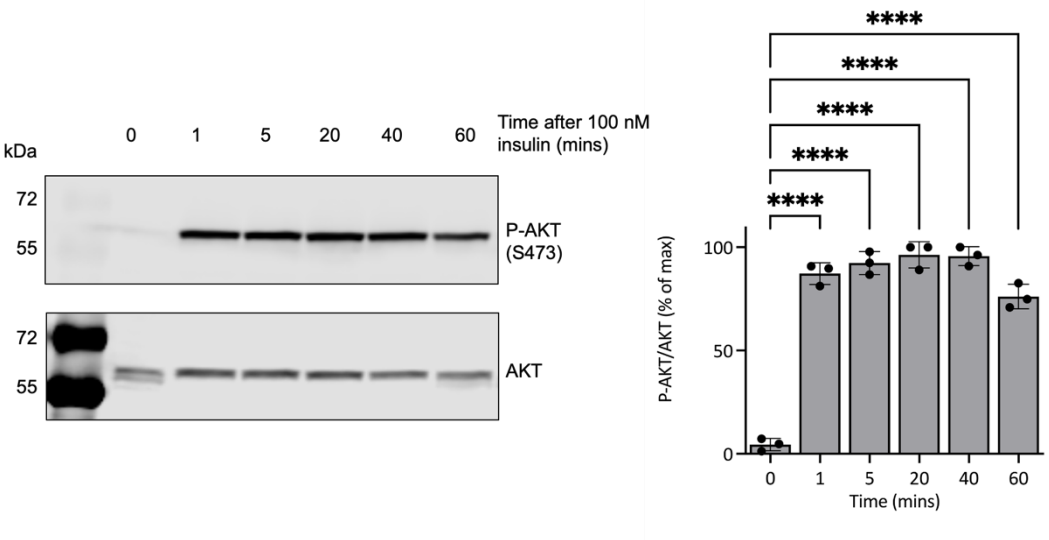

Figure 3-2: AKT is phosphorylated in response to insulin in H4-II-E cells within minutes

H4-II-E cells were serum-starved then treated with 100 nM of insulin for the times indicated before being lysed. **(A)** Picture shows a representative western blot from one experiment. Lysates were probed with antibodies raised against the indicated proteins. Molecular weights shown in kilodaltons (kDa). **(B)** Graph shows the densitometric analysis of three biological replicates as per **(A)**. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) compared to the 0- and 60-min time points were determined by one-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.3 Establishment of *Pck1* assay

3.2.3.1 Selection of a suitable reference gene

Logie *et al*. (2010) used beta-actin (*Actb*) as a housekeeping gene for normalisation purposes in their *Pck1* assay, however, ribosomal protein lateral stalk subunit P1 (*Rplp1*), has been reported to be more stable than beta-actin in insulin-resistant rat livers (Hernandez, Curi and Salazar, 2015). We wanted to pick the most stable of these 2 genes for our assay. We, therefore, conducted the qPCR experiment (shown in **Figure 3-5**) to generate enough data to make this comparison. We then inputted the *Actb* and *Rplp1* Ct values from this experiment into RefFinder (Xie et al., 2012), a free to use web tool that integrates four different algorithms [geNorm (Vandesompele et al., 2002), Normfinder (Andersen, Jensen and Orntoft, 2004), BestKeeper (Pfaffl et al., 2004), and the comparative Delta-Ct method (Silver et al., 2006)] to compare and rank candidate genes. As seen in **Figure 3-3**, the RefFinder output data indicate that *Rplp1* is the most stable gene under our experimental conditions (that is, to similar conditions that we would use to test the serum from PWT2D).

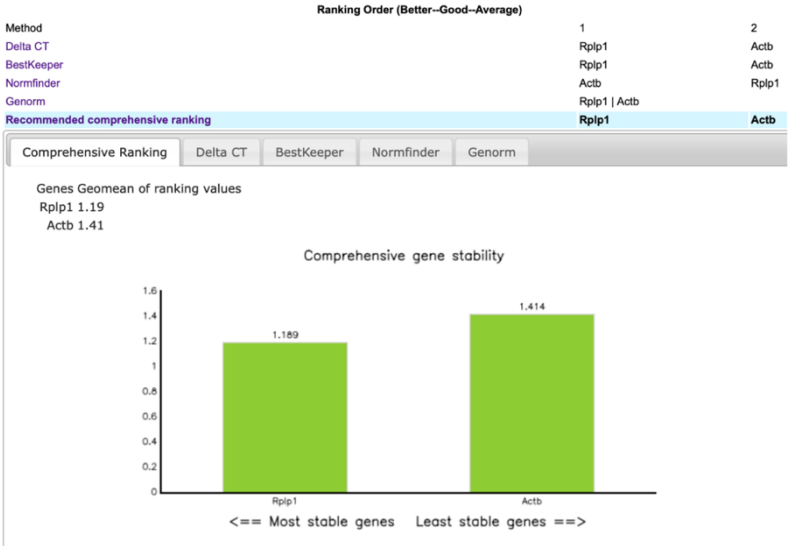

Figure 3-3: *Rplp1* is a more stable housekeeping gene than *Actb*
Rplp1 and *Actb* Ct values were inputted into RefFinder. The resultant output of this is shown. The top section of the figure shows the ranking order using the following four methods: Delta CT, BestKeeper, Normfinder, and Genorm. The bottom section of the figure shows the comprehensive RefFinder ranking based on the integration of these four ranking methods, displayed in graphical format.

To better understand the RefFinder output we graphed the reference gene candidate Ct values, to enable a manual comparison. As **Figure 3-4** shows, the *Rplp1* Ct values are more tightly clustered around the mean (that is, they have a smaller standard deviation) compared to the *Actb* Ct values. This further indicates that *Rplp1* is the most stable gene under these experimental conditions. Therefore, we opted to use *Rplp1* as the reference gene for our experiments going forward.

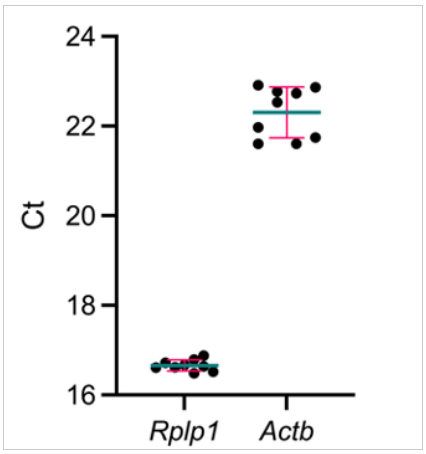

Figure 3-4: *Rplp1* is a more stable housekeeping gene than *Actb*
Graph of the *Rplp1* and *Actb* Ct values generated for **Figure 3-3**. Mean values are shown as turquoise lines and standard deviation error bars are shown in pink.

3.2.3.2 First test experiment

Due to low basal expression, Logie *et al*. (2010) induced (or boosted, in other words) *Pck1* transcription with the cyclic AMP analogue, 8-CPT-cAMP, and the glucocorticoid receptor agonist, dexamethasone, in order to better quantify changes in insulin-mediated repression of transcript accumulation (with the thinking that the larger a given value is, the more accurately a potentially small reduction of that value can be measured). We employed the same strategy going forward, and, as **Figure 3-5** shows, our initial practice experiment (which was also the same one we used to generate the housekeeping gene data, shown in **Figure 3-3**) was successful, in that we were able induce *Pck1* transcript accumulation and then repress this

(induced) transcription back to near the basal level again with 10 nM insulin. We chose this concentration of insulin to potentially give clear and robust results for my first attempt at this assay. However, we next wanted to test the assay using concentrations of insulin that we intended to use to test the serum from PWT2D.

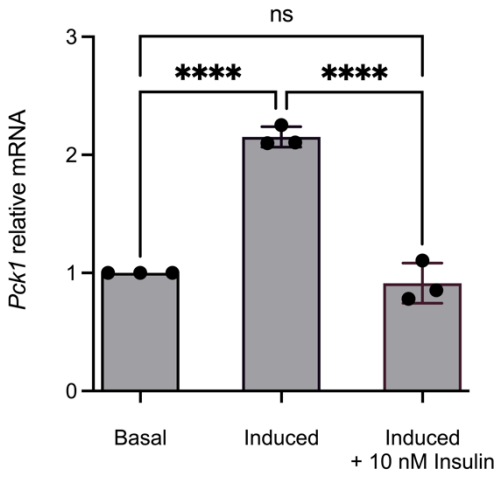

Figure 3-5: Induced *Pck1* transcription is inhibited by insulin
H4-II-E cells were serum starved prior to 3 h exposure to 500 nM dexamethasone and 0.1 mM 8-CPT-cAMP (labelled as induced) ± insulin, as indicated. Total cellular RNA was isolated, and reverse transcribed into cDNA before being subjected to qPCR analysis. *Pck1* mRNA levels relative to that of the *Rplp1* housekeeping gene are shown. Statistical significance or lack thereof was determined by one-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.3.3 Insulin concentration response

We wanted to make sure that the concentrations of insulin (0.1 and 0.5 nM) that Logie *et al*. (2010) used to detect the effects of serum from PWT2D would be suitable in our version of the assay. To do this, we performed an insulin concentration response, including a range of concentrations, including the same concentrations Logie *et al*. (2010) used, alongside concentrations of 10-fold less, and 10-/20-fold greater. Importantly, this experiment was only performed this experiment once which is a limitation, however, as **Figure 3-6** shows, insulin concentrations of 0.01 and 0.05 nM

did not reduce *Pck1* mRNA to below induced levels, indicating that these concentrations may not have been strong enough to produce a reliable effect. Contrastingly, insulin concentrations of 0.1 and 0.5 nM reduced *Pck1* transcripts to levels in-between induced and basal levels. That is, there was still enough induction pressure to keep the repression by insulin above the basal level, indicating these concentrations could be suitable going forward. Finally, according to this one experiment, at least, insulin concentrations of 1 and 10 nM reduced *Pck1* transcripts to below basal level, which may have overpowered any potential insulin resistance caused by the serum from PWT2D. Therefore, we ruled them out.

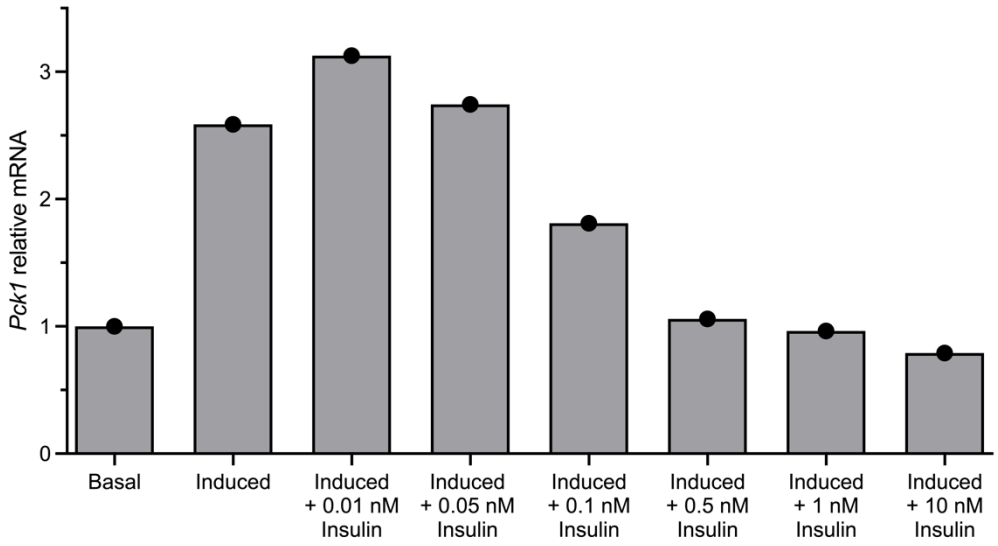

Figure 3-6: *Pck1* assay insulin concentration response
H4-II-E cells were serum starved prior to 3 h exposure to 500 nM dexamethasone and 0.1 mM 8-CPT-cAMP (labelled as induced) ± insulin, as indicated. Total cellular RNA was isolated, and reverse transcribed into cDNA before being subjected to qPCR analysis. *Pck1* mRNA levels relative to that of the *Rplp1* housekeeping gene are shown.

3.2.3.4 TNF inhibits insulin-mediated repression of *Pck1*

We wanted to determine if our *Pck1* assay could detect insulin resistance generated by means other than by serum from PWT2D. A paper (Fazakerley *et al.*, 2023) published during my PhD project described 5 methods to induce insulin resistance in 3T3-L1 cells (in their case, to enable downstream

comparative phosphoproteomic analysis). Reasoning that H4-II-E and 3T3-L1 cells would behave similarly, we chose 2 of these methods to test our *Pck1* assay with. The first of these methods was a chronic Tumour Necrosis Factor alpha (TNF) treatment. As **Figure 3-7 (A)** shows, we were able to detect a significant reduction in the insulin-mediated repression of induced *Pck1* transcripts in TNF-treated H4-II-E cells, at insulin concentrations of 0.1 and 0.5 nM. That is, insulin was less able to repress induced *Pck1* transcription in TNF-treated cells compared to controls, indicating that there was insulin resistance in these cells, and we could detect it. Interestingly, there was also a modest but significant increase in the induction of *Pck1* by dexamethasone and 8-CPT-cAMP in TNF-treated cells.

I explained in **Section 3.2.3.2** the rationale for the induction of *Pck1* in our assay by dexamethasone and 8-CPT-cAMP, however, this was received wisdom and we wanted to determine if we could detect TNF-mediated insulin resistance in cells not subjected to induction treatments. As **Figure 3-7 (B)** shows, although insulin at both concentrations tested was less able to repress basal levels of *Pck1* in TNF treated cells, this was not a statistically significant difference. That is, we could not detect insulin resistance in these cells. This result, therefore, supports the need for *Pck1* induction in the assay.

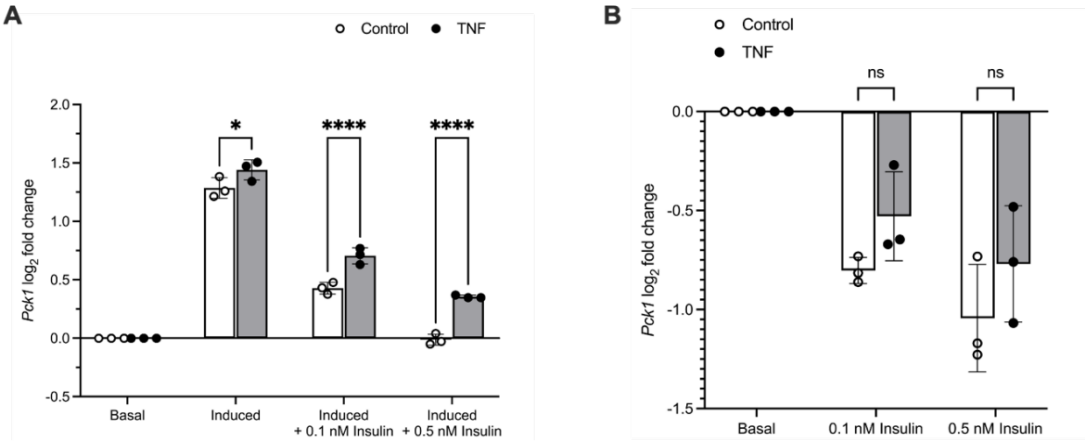

Figure 3-7: Chronic TNF inhibits insulin-mediated repression of induced (but not basal) *Pck1*
H4-II-E cells were cultured in media containing 4 ng/mL of TNF (labelled TNF) or in normal media (labelled Control) for 4 days, before being serum starved. **(A)** Cells were exposed to 500 nM dexamethasone and 0.1 mM 8-CPT-cAMP (labelled as induced) ± insulin (as indicated) for 3 h. **(B)** Cells were exposed to ± insulin (as indicated) for 3 h without induction. **(A&B)** Total cellular RNA was isolated, and reverse transcribed into cDNA before being subjected to qPCR analysis. Log_2 fold change of *Pck1* mRNA levels relative to that of the *Rplp1* housekeeping gene are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.3.5 Analysis by copy number

The results in **Figure 3-7** were produced using the widely used ΔΔCt method of analysis, however, Logie *et al*. (2010) used a slightly different method to analyse their data. This was to use the formula $2^{(40-Ct)}$ to convert their Ct values into approximate copy numbers on a linear scale. For reasons that will become clearer in **Section 3.2.5**, we wanted to see what effects, if any, this method of analysis would have on our *Pck1*-induced TNF data. As seen in **Figure 3-8**, the copy number method gave similar results to the ΔΔCt method, in that insulin, at both concentrations tested, was significantly less able to repress induced *Pck1* transcript accumulation in TNF-treated cells, compared to controls. Similar to the ΔΔCt method, the copy number method also showed that TNF-treated cells were induced to significantly higher levels than controls. Thus, overall, there was little difference in this data set, whether the results were analysed by ΔΔCt, or the copy number method.

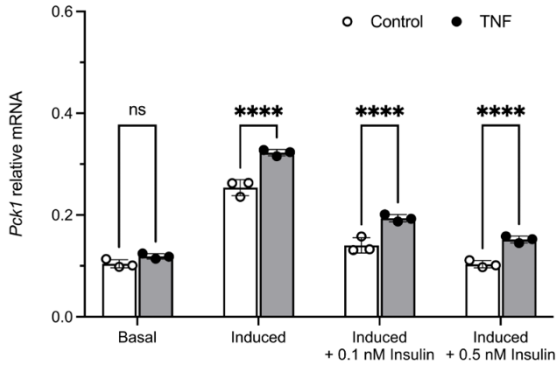

Figure 3-8: TNF-mediated insulin resistance is still detected when analysed by copy number
Data from **Figure 3-7** were reanalysed by copy number. *Pck1* mRNA levels relative to that of the housekeeping gene *Rplp1* are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.3.6 Hyperinsulinemia inhibits insulin-mediated repression of *Pck1*

The second method of generating insulin resistance from Fazakerley *et al.*, (2023) that we chose to test our *Pck1* assay with was hyperinsulinemia (HI). We first used this method in cells where *Pck1* was induced. As **Figure 3-9 (A)** shows, insulin, at both concentrations given, did not significantly inhibit induced *Pck1* mRNA accumulation in HI-treated cells, compared to controls. Moreover, looking at the general (but not statistically significant) trends, there was an inconsistent pattern of mean *Pck1* levels between HI-treated cells at the two concentrations of insulin tested. At 0.1 nM of insulin, HI-treated cells had lower levels of *Pck1* (indicating these cells were more sensitive to insulin), compared to controls. This contrasts to the results at 0.5 nM of insulin, where HI-treated cells had higher levels of *Pck1* (indicating these cells were less sensitive to insulin), compared to controls. Another point of interest here, is that *Pck1* was induced to significantly lower levels in cells given HI treatments, compared to controls (This result contrasts to the TNF-treated cells of **Figure 3-7 (A)**). Importantly, overall, we could not detect insulin resistance by this method of analysis in HI-treated cells.

We also looked for insulin resistance in HI-treated cells where *Pck1* was not induced. As **Figure 3-9 (B)** shows, insulin at 0.1 nM was less able to repress *Pck1* mRNA accumulation in HI-treated cells compared to controls, although this lack of repression was not statistically significant. However, insulin at 0.5 nM, was less able to repress *Pck1* mRNA accumulation in HI-treated cells compared to controls, to a statistically significant degree, indicating these cells were insulin resistant (at least by this one measure) and we could detect it.

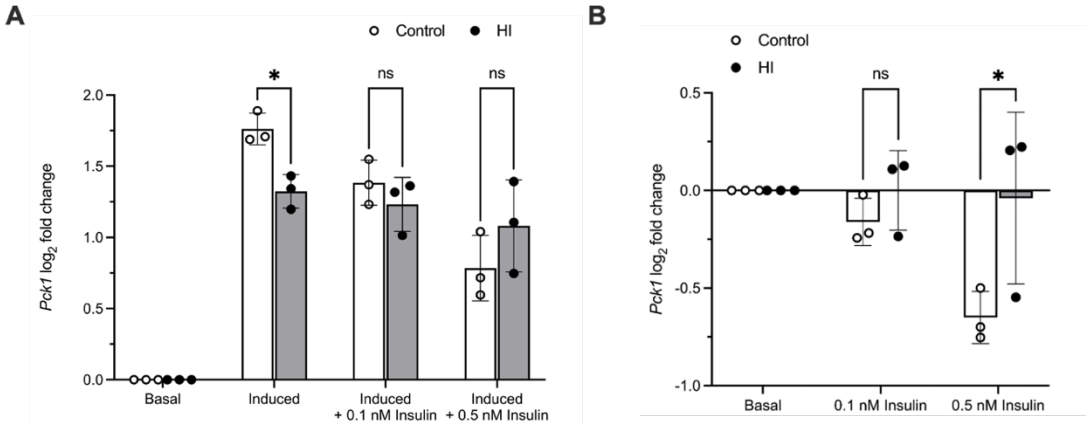

Figure 3-9: Hyperinsulinemia inhibits insulin-mediated repression of basal (but not induced) *Pck1*
(A&B) H4-II-E cells were cultured in media containing 200 nM of insulin (labelled HI) or in standard media (labelled Control) for a week, before being serum starved. **(A)** Cells were exposed to 500 nM dexamethasone and 0.1 mM 8-CPT-cAMP (labelled as induced) ± insulin (as indicated) for 3 h. **(B)** Cells were exposed to ± insulin (as indicated) for 3 h without induction. **(A&B)** Total cellular RNA was isolated, and reverse transcribed into cDNA before being subjected to qPCR analysis. Log_2 fold change of *Pck1* mRNA levels relative to that of the *Rplp1* housekeeping gene are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.3.7 Analysis by copy number

We re-analysed the qPCR data from *Pck1*-induced HI-treated cells using the copy number method. As seen in **Figure 3-10**, these results show that, although there was a trend of insulin, at both concentrations tested, to repress *Pck1* mRNA accumulation to a lesser degree in HI-treated cells, compared to controls, this difference was not statistically significant. In

addition, this method of analysis showed that in HI-treated cells, *Pck1* was induced to a greater (but not statistically significant degree) compared to controls.

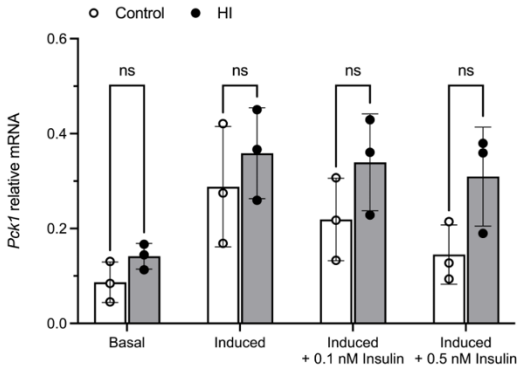

Figure 3-10: HI-mediated insulin resistance is not detected when analysed by copy number
Data from **Figure 3-9** were reanalysed by copy number. *Pck1* mRNA levels relative to that of the housekeeping gene *Rplp1* are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.4 Arrival of human serum

Our original aim was to collect serum from 20 PWT2D and 20 matched controls. However, after a year and 3 months, due to the COVID-19 pandemic, we had only managed to collect serum from 18 PWT2D and 14 age and sex-matched controls (**Figure 3-11**). Rather than waiting longer to collect the rest of the serum, we decided the best way to proceed was to test the serum we had already collected, as we were hopeful that this would give us more time to confirm that serum from PWT2D caused insulin resistance and then to make progress on identifying the factor(s) in serum which were responsible for this.

A

	Controls					
	Volunteer No	Sex	Age	BMI	HbA1c	Duration of disease
	2	M	58	24.2	35	
	3	F	58	21.6	38	
	15	M	59	25.7	32	
	16	M	64	29	39	
	18	F	55	28.2	40	
	20	F	51	23.9	35	
	21	M	66	24.7	33	
	24	M	66	27.8	34	
	26	M	76	21.3	36	
	27	M	51	26.7	34	
	28	M	78	25.5	37	
	30	M	75	31.8	39	
	31	M	50	25.4	36	
	32	F	61	30.3	39	
Mean (SD)	14	10M, 4F	62 (9.34)	26.15 (3.05)	36.21 (2.52)	

B

	Cases					
	Volunteer No	Sex	Age	BMI	HbA1c	Duration of disease
	5	M	75	24.8	51	11
	6	M	71	31.6	42	12
	7	M	70	25.8	52	10
	8	M	69	25.7	56	16
	9	M	60	28.7	60	4
	10	F	52	34.6	51	4
	11	M	75	29.9	45	9
	12	F	59	22.1	53	4
	13	M	49	29.7	42	4
	14	M	60	32.3	47	5
	19	M	61	27.3	80	5
	22	M	59	27.3	45	5
	23	F	56	36.3	54	6
	25	M	52	26.3	41	13
	29	F	66	33.3	44	12
	33	F	64	30.7	40	0.42
	34	M	66	31.3	53	6
	35	M	65	28.9	50	7
Mean (SD)	18	13M, 5F	62.72 (7.67)	29.26 (3.67)	50.33 (9.32)	7.41 (4.10) years

Figure 3-11: Initial volunteer details
The figure shows details of the healthy volunteers (Controls) (defined as having an HbA1c of less than 40 mmol/mol (**A**) and people with T2D (Cases) (defined as having an HbA1c of 40 mmol/mol or greater) (**B**) whom we pooled serum from for the experiments shown in this chapter. Age is shown in years. BMI in kg/m^2. HbA1c in mmol/mol. Mean figures, next to standard deviations (SD) in brackets, if appropriate, are shown at the bottom of columns.

3.2.5 5% serum from PWT2D has no effect on the insulin-mediated repression of *Pck1*

We formed a pool of serum from 18 PWT2D and another from 14 matched controls. Then, mirroring, Logie *et al*. (2010), we cultured our cells in 5% of either serum for 3 weeks, before testing these cells for insulin resistance.

Before describing the results of this experiment, I will point out that the cells did not grow in this human serum as well as they did in standard media (containing FBS). Specifically, we found that the cells grown in human serum would stop dividing after around 9 days (with the cells grown in control serum being affected to a greater degree, in this instance) if the stock media (DMEM and the additives described in the materials and method's section, along with human serum) was stored for any longer than a week before being replaced.

To circumvent this, we made up media with freshly defrosted human serum on a weekly basis, after this discovery, which allowed us to complete this experiment. Moreover, as **Figure 3-12 (A)** shows, we did not see any difference in the insulin-mediated repression of induced *Pck1* in cells grown in serum from PWT2D, compared to controls. That is, we did not see any serum-induced insulin resistance. As **Figure 3-12 (B)** shows, insulin at 0.1 nM (but not 0.5 nM) was significantly less able to repress non-induced *Pck1* mRNA accumulation in cells grown in serum from PWT2D, but we reasoned that this signal (if it was a true signal) was too weak and potentially too difficult to reproduce to attempt to dissect.

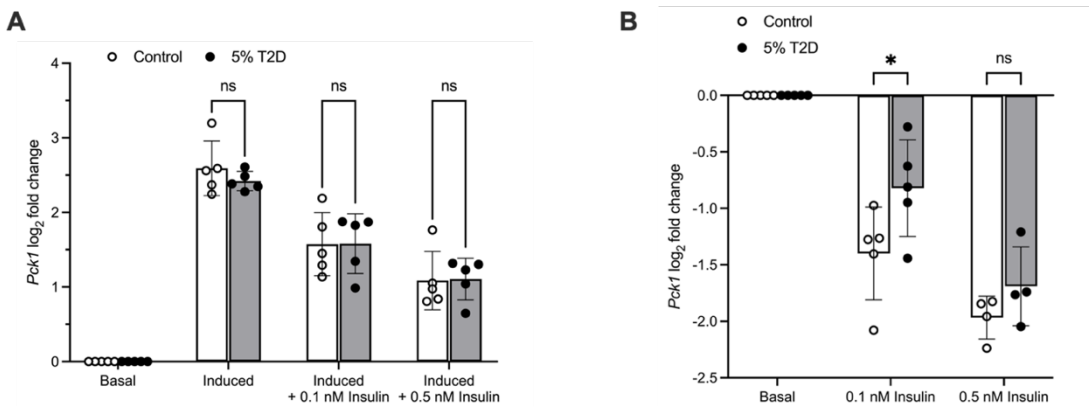

Figure 3-12: 5% serum from PWT2D has no effect on the insulin-mediated repression of induced *Pck1* but has a significant effect on the repression of basal *Pck1*
H4-II-E cells were cultured in media containing 5% serum from PWT2D (labelled 5% T2D), or 5% serum from healthy volunteers (labelled 5% Control), replenished 3 times/week for 3 weeks, before being serum starved. **(A)** Cells were exposed to 500 nM dexamethasone and 0.1 mM 8-CPT-cAMP (labelled as induced) ± insulin (as indicated) for 3 h. **(B)** Cells were exposed to ± insulin (as indicated) for 3 h without induction. **(A&B)** Total cellular RNA was isolated, and reverse transcribed into cDNA before being subjected to qPCR analysis. Log_2 fold change of *Pck1* mRNA levels relative to that of the *Rplp1* housekeeping gene are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.5.1 Analysis by copy number

We considered if any differences between our and Logie *et al*. (2010)'s experiments could potentially explain the different results we saw. There were differences in the characteristics of the cohorts of PWT2D (and volunteers) and the way the serum was tested (pooled versus individual) which, at this stage in the project, it was not feasible to change. We will consider these differences in approach more fully in the discussion sections of this and the next chapters. One hypothesis we could test immediately was that the different results were due to the different methods of qPCR analysis used (ΔΔCt vs copy number). **Figure 3-13** shows, however, analysing by copy number made little difference - 5% serum from PWT2D did not induce insulin when analysed in this way.

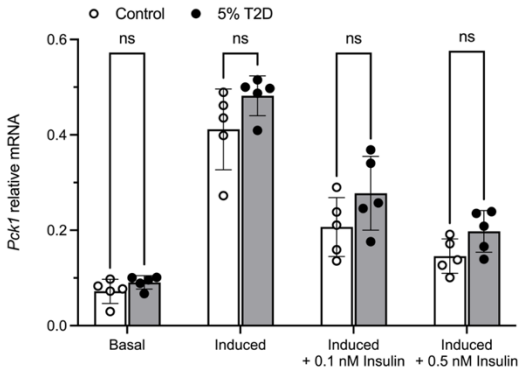

Figure 3-13: 5% serum from PWT2D does not cause insulin resistance in *Pck1*-induced cells, even if analysed by copy number
Data from **Figure 3-12** were reanalysed by copy number. *Pck1* mRNA levels relative to that of the housekeeping gene *Rplp1* are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.6 10% serum from PWT2D has no effect on the insulin-mediated repression of *Pck1*

We hypothesised that our serum from PWT2D was somehow less potent than that of Logie *et al*. (2010). To test this idea, we grew H4-II-E cells for three weeks in 10% serum from PWT2D (or from healthy volunteers) before testing them for insulin resistance. These cells divided similarly quickly (and we saw obvious differences, by light microscope) compared to cells grown under standard conditions, although we continued to make up media with freshly defrosted human serum on a weekly basis. As **Figure 3-14** shows, we saw no evidence of insulin resistance in these cells. This was true for cells where *Pck1* was induced **(Figure 3-14 (A))** and for cells given no induction treatment **(Figure 3-14 (B))**.

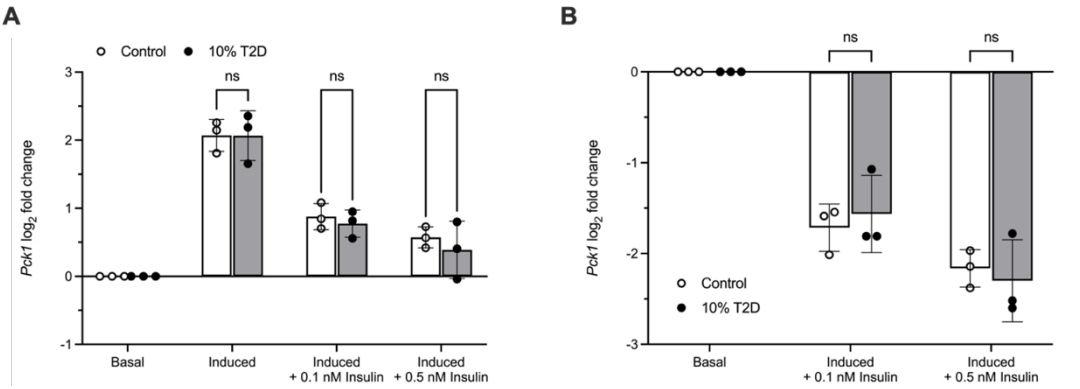

Figure 3-14: 10% serum from PWT2D has no effect on the insulin-mediated repression of induced (or basal) *Pck1*
H4-II-E cells were cultured in media containing 10% serum from PWT2D (labelled 10% T2D), or 10% serum from healthy volunteers (labelled 10% Control), replenished 3 times/week for 3 weeks, before being serum starved. **(A)** Cells were exposed to 500 nM dexamethasone and 0.1 mM 8-CPT-cAMP (labelled as induced) ± insulin (as indicated) for 3 h. **(B)** Cells were exposed to ± insulin (as indicated) for 3 h without induction. **(A&B)** Total cellular RNA was isolated, and reverse transcribed into cDNA before being subjected to qPCR analysis. Log_2 fold change of *Pck1* mRNA levels relative to that of the *Rplp1* housekeeping gene are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.6.1 Analysis by copy number

We reanalysed our 10% serum data by copy number. As **Figure 3-15** shows, although there was a trend of insulin, at both concentrations tested, to inhibit *Pck1* mRNA accumulation to a lesser degree in cells grown in serum from PWT2D compared to controls, this difference was not statistically significant. Therefore, insulin resistance caused by 10% serum from PWT2D was not evident here either. One difference shown by copy number analysis was that *Pck1* in cells that were grown in 10% serum from PWT2D was induced to a statistically significantly greater degree than in control cells.

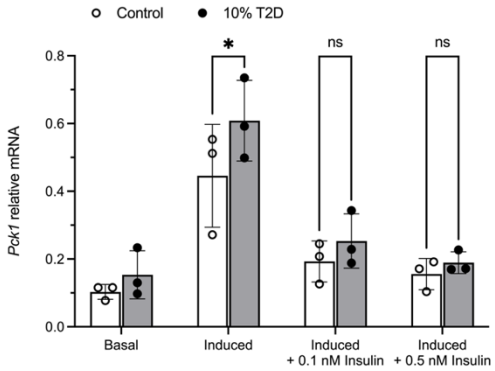

Figure 3-15: 10% serum from PWT2D does not cause insulin resistance in *Pck1*-induced cells, even if analysed by copy number
Data from **Figure 3-14** were reanalysed by copy number. *Pck1* mRNA levels relative to that of the housekeeping gene *Rplp1* are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.7 3T3-L1 adipocytes respond to insulin

3.2.7.1 Insulin concentration response

Logie *et al*. (2010) showed that growing H4-II-E cells in serum from PWT2D caused them to become resistant to insulin. Specifically, insulin was less able to repress the transcript accumulation of *Pck1*, the encoder of a rate-limiting gluconeogenic enzyme. One of the aims of this project was to test the hypothesis that serum from people with T2D could cause insulin resistance in other pathways in other cell types. That is, that the insulin resistance-promoting properties of serum from people with T2D is not specific to just one pathway (gluconeogenesis) in one cell type (H4-II-E cells). Although we did not see insulin serum-induced insulin resistance in H4-II-E cells, we decided to test our serum from PWT2D in another cell type. A classic feature of insulin resistance in T2D is the blunting of the insulin-mediated uptake of glucose into adipocytes and muscle (Minokoshi, Kahn and Kahn, 2003). We, therefore, intend to test whether serum from PWT2D has any measurable inhibitory effects on this insulin-mediated glucose uptake. We chose 3T3-L1 adipocytes, in which to test for this, as they possess a robust insulin-responsive glucose uptake system (Klip, McGraw and James, 2019). This

system involves the insulin- mediated trafficking of the glucose transporter, GLUT4 to the plasma membrane, enabling it to provide what has been described as the bulk of cellular glucose uptake (Chadt and Al-Hasani, 2020). Before performing a test glucose uptake assay, we wanted to make sure that our 3T3-L1 adipocytes responded to insulin. To do this, we performed an insulin concentration response, western blotting for S473 phosphorylated AKT as a readout, as we had done in H4-II-E cells. As **Figure 3-16** shows, the 3T3-L1 adipocytes responded to insulin in a concentration-dependent manner, with significant increases in the phosphorylation of AKT in response to insulin at 1, 10, and 100 nM. These data have been normalised to 100% as the maximum responses vary from batch to batch of cells.

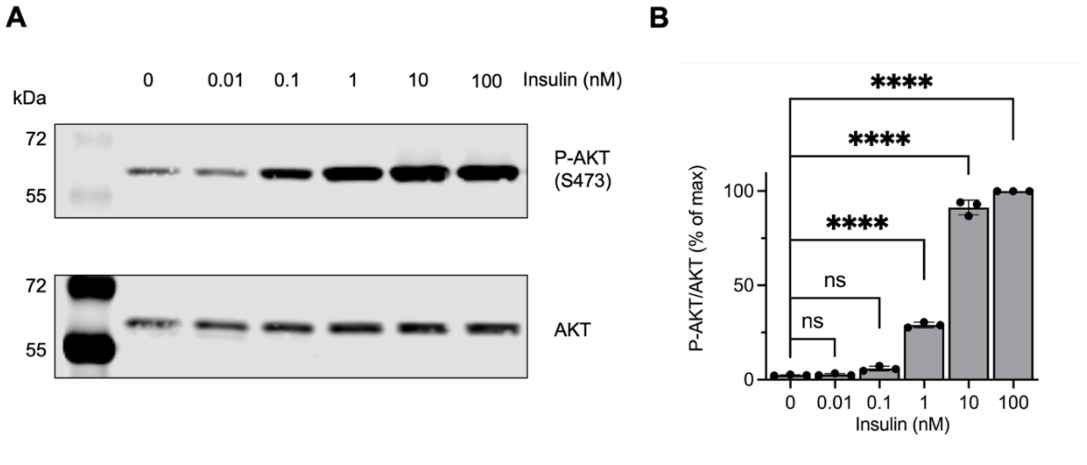

Figure 3-16: AKT is phosphorylated in response to insulin in a concentration-dependent manner in 3T3-L1 cells
3T3-L1 adipocytes were serum starved then treated with the indicated concentrations of insulin for 20 minutes, and lysed. **(A)** Picture of a representative western blot from one experiment shown. Lysates were probed with antibodies raised against the indicated proteins. Molecular weights shown in kilodaltons (kDa). **(B)** Graph shows the densitometric analysis of three biological replicates as per **(A)**. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by one-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

We also western blotted for the phosphorylation AS160 as a second measure of insulin-induced activation. As **Figure 3-17** shows, AS160 was also phosphorylated in a concentration-dependent manner to a significant degree in response to insulin at 1, 10, and 100 nM in these cells. We normalised

P-AS160 to actin instead of AS160, in this instance, as the AS160 antibody gave inconsistent results (see **Figure 3-17** and **Figure 3-18**), even after several optimisation attempts.

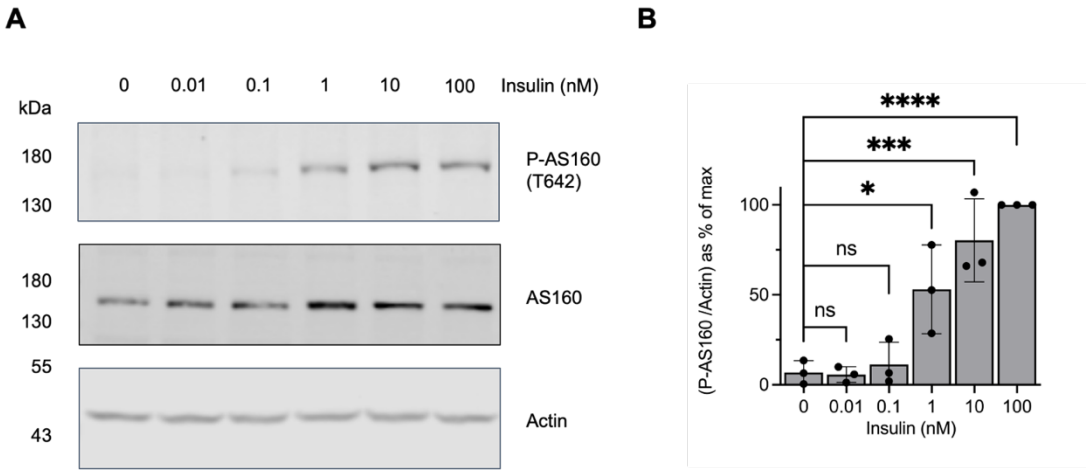

Figure 3-17: AS160 is phosphorylated in response to insulin in a concentration-dependent manner in 3T3-L1 cells
3T3-L1 Adipocytes were serum starved then treated with the indicated concentrations of insulin for 20 minutes, and lysed. **(A)** Picture of a representative western blot from one experiment shown. Lysates were probed with antibodies raised against the indicated proteins. Molecular weights shown in kilodaltons (kDa). **(B)** Graph shows the densitometric analysis of three biological replicates as per **(A)**. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by one-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.7.2 Insulin time course

To determine how quickly the 3T3-L1 adipocytes responded to insulin in our hands, be performed a time course, using acute (100 nM) insulin treatments. As seen in **Figure 3-18**, AKT was phosphorylated to a significant degree at all time points tested, after these treatments. Interestingly, AKT phosphorylation did not lower after 40 minutes as we had seen in H4-II-E cells.

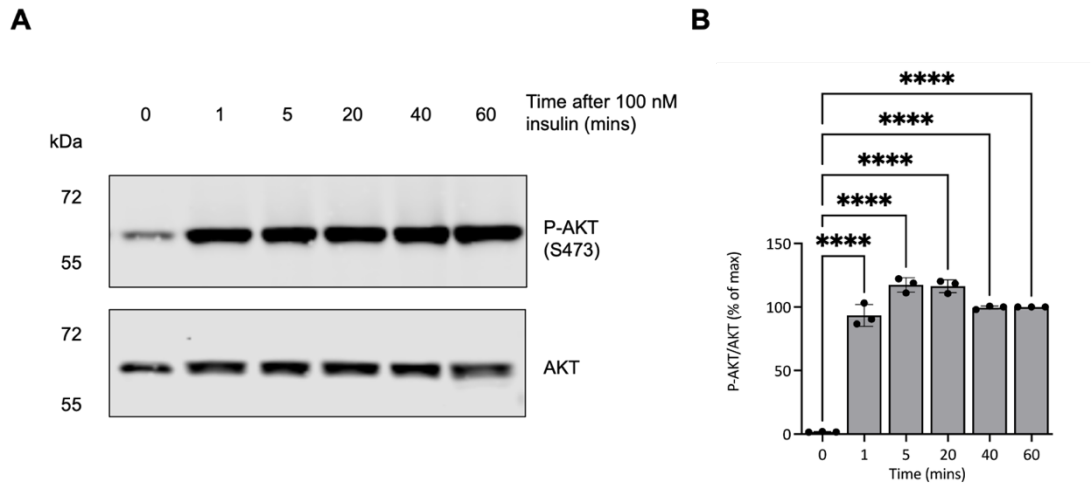

Figure 3-18: AKT is phosphorylated in response to insulin in 3T3-L1 cells within minutes.
3T3-L1 adipocytes were serum starved then treated with 100 nM of insulin for the times indicated before being lysed. **(A)** Picture shows a representative western blot from one experiment. Lysates were probed with antibodies raised against the indicated proteins. Molecular weights shown in kilodaltons (kDa). **(B)** Graph shows the densitometric analysis of three biological replicates as per **(A)**. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by one-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

We western blotted for the phosphorylation of AS160 as part of the same time course experiment as a second measure of response to insulin. As **Figure 3-19** shows, AS160 was significantly phosphorylated in response to 100 nM insulin at all time points tested. In addition, levels of AS160 phosphorylation started to drop after 40 mins, indicating that some desensitisation to insulin had taken place. Taken together, these data indicate that the 3T3-L1s responded to insulin.

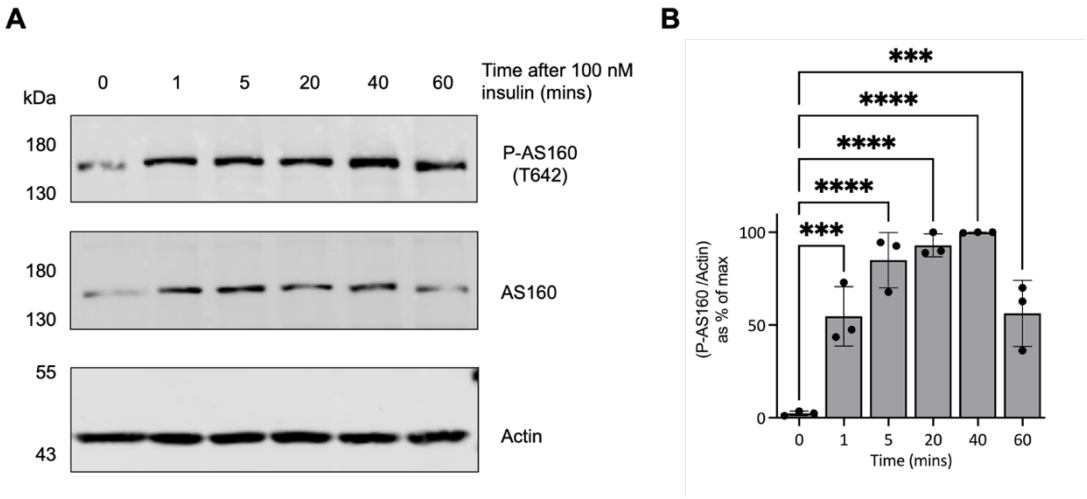

Figure 3-19: AS160 is phosphorylated in response to insulin in 3T3-L1 cells within minutes.
3T3-L1 adipocytes were serum starved then treated with 100 nM of insulin for the times indicated before being lysed. **(A)** Picture shows a representative western blot from one experiment. Lysates were probed with antibodies raised against the indicated proteins. Molecular weights shown in kilodaltons (kDa). **(B)** Graph shows the densitometric analysis of three biological replicates as per **(A)**. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by one-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.8 Establishment of glucose uptake assay

3.2.8.1 First test experiment

Having confirmed that 3T3-L1 adipocytes responded to insulin in our hands, we conducted a test glucose uptake assay. In this assay, cells are given insulin to promote glucose uptake, along with a radioactive form of the glucose analogue, 2-deoxy-D-glucose (2-DOG). 2-DOG is taken up in the same way as normal glucose, via GLUT4, but once in the cell it is phosphorylated, by hexokinase, into 2-DOG-P, which cannot be metabolised further, trapping it. The amount of 2-DOG taken up can then be determined by scintillation counting. By these means, potential defects in the insulin-mediated glucose uptake system can be measured. As **Figure 3-20** shows, in our test assay, we were able to measure a concentration-dependent increase of insulin-mediated glucose uptake in 3T3-L1 adipocytes, with significant increases

seen after treatments of insulin at 10 and 100 nM. We next wanted to determine if we could use this assay to measure insulin resistance.

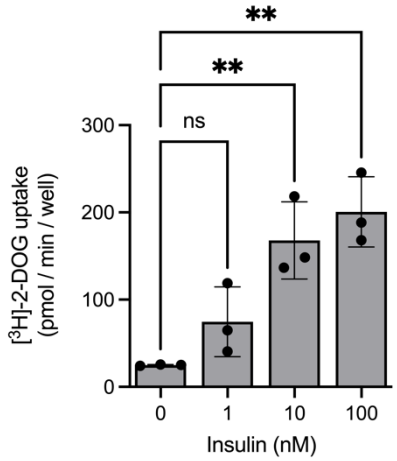

Figure 3-20: Insulin stimulates glucose uptake in 3T3-L1 adipocytes
3T3-L1 adipocytes were serum starved and treated with the indicated concentrations of insulin for 20 minutes. Glucose uptake was measured by the accumulation of [^{3}H]-2-deoxyglucose ([^{3}H]-2-DOG). Data from three independent experiments shown. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by one-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.8.2 Detection of TNF-mediated insulin resistance

We wanted to determine if our glucose uptake assay could detect insulin resistance generated by means other than by serum from PWT2D. We, therefore, exposed 3T3-L1 adipocytes to what has been described as a chronic low dose of TNF (Fazakerley *et al.*, 2023) for 4 days before testing for insulin resistance. As **Figure 3-21 (A)** shows, there was a significant reduction in insulin-mediated glucose uptake after 10 and 100 nM insulin treatments in cells exposed to TNF, compared to control cells. These data indicate that the cells grown in TNF became resistant to insulin and we could detect this with our assay.

3.2.8.3 Detection of hyperinsulinemia-mediated insulin resistance

We chose hyperinsulinemia (HI) as a second method of generating insulin resistance to test the glucose assay with. To this end, we exposed 3T3-L1 adipocytes to 200 nM insulin for 24 hours before testing in the assay. As **Figure 3-21 (B)** shows, there was a significant reduction in insulin-mediated glucose uptake after 10 and 100 nM insulin treatments in cells exposed to HI, compared to control cells. That is, these cells were resistant to insulin, and we could detect it. Interestingly, **Figure 3-21 (B)** also shows that glucose uptake was significantly higher in HI-treated cells given no (or 1 nM) insulin treatments, which was an expected result (Talior *et al.*, 2003; Fazakerley *et al.*, 2023). The rationale explaining these phenomena, is that, in insulin resistant adipocytes, GLUT1 protein levels are increased, facilitating a higher basal glucose uptake, whereas GLUT4 protein levels are decreased, reducing insulin-mediated glucose uptake (Talior *et al.*, 2003). Taken together, these results gave us confidence that our assay worked as intended and would be suitable for testing the serum from PWT2D with.

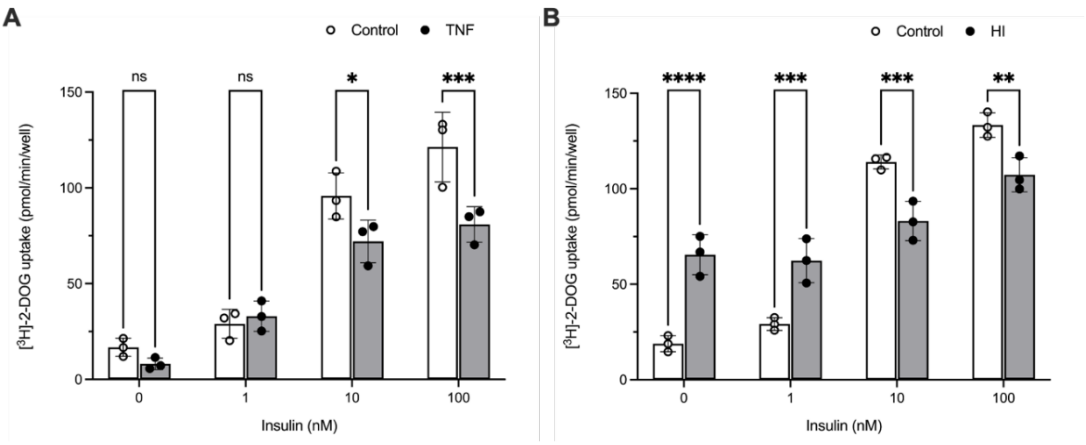

Figure 3-21: TNF and hyperinsulinemia both inhibit insulin-mediated glucose uptake in 3T3-L1 adipocytes
3T3-L1 adipocytes were cultured in media ± 2 ng/mL of TNF, (labelled as TNF or Control), replenished daily for 4 days **(A)** or ± 200 nm insulin (labelled HI or Control) for 24 h **(B)**. All cells were serum starved then treated with the indicated concentrations of insulin for 20 minutes. Glucose uptake was measured by the accumulation of [^{3}H]-2-deoxyglucose ([^{3}H]-2-DOG). Data from three independent experiments shown. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.2.9 10% serum from PWT2D has no effect on insulin-mediated glucose uptake in 3T3-L1 adipocytes

We tested pooled serum from 18 PWT2D (against pooled serum from 14 healthy volunteers) in our glucose uptake assay, looking for signs of serum induced-insulin resistance. Normally, 3T3-L1 adipocytes are cultured in media containing 10% FBS. For this reason, we chose 3-day incubations with 10% human serum. As **Figure 3-22 (A)** shows, there were no significant differences in insulin-mediated glucose uptake in cells treated with serum from PWT2D compared to controls.

3.2.10 25% serum from PWT2D has effect on insulin-mediated glucose uptake in 3T3-L1 adipocytes

We hypothesised that 10% serum from PWT2D was not high enough to generate an effect in insulin-mediated glucose uptake. We, therefore, increased the concentration to 25% for our next experiment. As **Figure 3-22 (B)** shows, there was no evidence that 25% serum from PWT2D caused insulin resistance in these cells, either. A caveat to this, is that this experiment was only carried out once, so the conclusions that can be drawn from this should be weighted accordingly. The reason that this experiment was only carried out once, was, that, at this point, we had pooled and used half of our initial batch of serum and seen no effects of insulin resistance in either the *Pck1* assay or the glucose uptake assay. Moreover, we had generated a new hypothesis to stratify our volunteers with T2D. Therefore, instead of pooling our remaining serum to produce another 2 repeats of this experiment, we chose to selectively pool the serum for further testing. This topic will be discussed more fully in the next chapter.

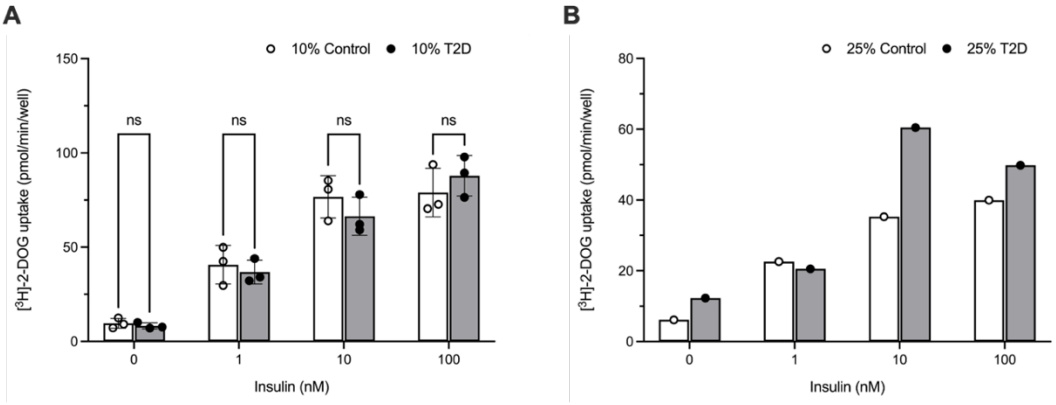

Figure 3-22: 10% serum from PWT2D has no effect on insulin-mediated glucose uptake in 3T3-L1 adipocytes

3T3-L1 adipocytes were cultured in media containing serum from people with T2D (T2D) or from healthy volunteers (Control) at concentrations of 10% **(A)** or 25% **(B)** without replenishment for 3 days. All cells were serum starved for 2 h and treated with the indicated concentrations of insulin for 20 minutes. Glucose uptake was measured by the accumulation of [^{3}H]-2-deoxyglucose ([^{3}H]-2-DOG). Data from three independent experiments shown in **(A)** and data from one experiment in **(B)**. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

3.3 Discussion

The primary aim of this chapter was to establish and validate two complementary in vitro models for detecting insulin resistance: repression of *Pck1* transcript accumulation in H4-II-E hepatomas and insulin-stimulated radiolabelled glucose uptake in 3T3-L1 adipocytes. Both assays demonstrated clear insulin responsiveness under control conditions and were sensitive to experimentally induced insulin resistance using established stimuli. However, when these validated systems were applied to pooled serum from PWT2D, no significant induction of insulin resistance was observed in either cell type. This negative outcome contrasts with previous reports and requires careful consideration of technical, methodological, and biological factors.

The successful validation of both platforms provides confidence that the absence of a serum effect was not due to inadequate assay sensitivity or poor cellular responsiveness. The H4-II-E *Pck1* repression assay showed robust induction by cAMP and dexamethasone followed by dominant repression by physiological concentrations of insulin. Similarly, the 3T3-L1 glucose uptake assay exhibited clear concentration-dependent insulin responsiveness. Both models also readily detected insulin resistance when challenged with known inducers such as TNF and chronic hyperinsulinaemia. These positive control experiments strengthen the interpretation that the lack of effect with serum from PWT2D reflects a genuine biological result rather than a technical limitation.

This finding differs from the earlier report by Logie *et al*. (2010), who observed significant attenuation of insulin-mediated *Pck1* repression in H4-II-E cells exposed to serum from PWT2D. Several factors may account for this discrepancy. Methodological differences are likely important. This PhD project employed pooled serum tested across three independent experimental runs, whereas Logie *et al*. (2010) tested individual sera once before combining the data for presentation. While pooling offers practical advantages, including greater serum volume for potential downstream

fractionation, it has a key limitation: it averages responses across donors and can therefore dilute highly active samples or obscure effects driven by a subset of individuals with potent serum. Importantly, however, pooling across three independent experimental runs yielded three biological replicates, whereas each individual serum in Logie *et al*. (2010) rested on a single observation with no replication. Our approach therefore sacrificed individual donor resolution but gained replication of the serum effect itself, providing greater confidence that the null result observed here is reproducible across independent experiments rather than an isolated finding. Additional technical variations, such as reference gene selection for qPCR normalisation, could have further influenced the outcome. These considerations highlight the inherent challenges of using complex human biofluids in long-term functional bioassays and the importance of carefully weighing the trade-offs between pooling and individual testing when studying circulating mediators.

The ability to detect insulin resistance induced by TNF and hyperinsulinaemia, but not by serum from PWT2D, carries two important implications. First, it suggests that any humoral mediator(s) present in serum from PWT2D may be relatively weak, highly context-dependent, or require coexisting factors commonly found in serum from PWT2D (such as elevated glucose or lipids) to exert measurable effects in isolated cell systems. Second, it reinforces the multifactorial and progressive nature of insulin resistance in T2D, where circulating factors likely interact with tissue-intrinsic defects and the broader metabolic environment. With the benefit of hindsight, several modifications could have increased sensitivity, including testing even higher concentrations or extending incubation periods beyond those used by Logie *et al*. (2010).

Despite the negative result, the successful establishment and validation of both assay systems constitutes a valuable technical foundation. These models are now well-characterised in our hands and provide a reliable

platform for future investigations using patient sera or candidate circulating mediators. The finding that insulin resistance was readily induced by pharmacological means but not by serum from PWT2D also serves as an important internal control, confirming the specificity and dynamic range of the systems.

In conclusion, this chapter successfully developed and validated two functional readouts of insulin action but did not detect insulin resistance-inducing activity in pooled serum from PWT2D. The discrepancy with previous literature highlights the complexity of circulating mediators in T2D and underscores key technical and biological variables that must be addressed in future serum-based investigations. These insights provide a foundation for the subsequent chapter, which examines cohort characteristics and explores potential explanations for the observed results, while also laying the groundwork for continued exploration of humoral factors in insulin resistance.

4 Chapter 4

4.1 Aims

Our general aims for this chapter were to generate and test hypotheses regarding the characteristics of the PWT2D who donated serum for this study that could explain why pooled serum from this group did not cause insulin resistance in cultured cells as expected.

Our specific aims were:

- To pool and test serum from people that had clear T2D in the *Pck1* and glucose uptake assays
- To pool and test serum from PWT2D and obesity in the *Pck1* and glucose uptake assays
- To pool and test serum from PWT2D and high HbA1cs in the *Pck1* and glucose uptake assays
- To pool and test serum from people with pre-T2D (PWPT2D) in the *Pck1* and glucose uptake assays
- To determine if serum from PWPT2D affected the insulin-mediated phosphorylation of AKT and AS160 in 3T3-L1 adipocytes

4.2 Results

4.2.1 Serum from people with clear T2D

To generate testable hypotheses that could perhaps explain why our original pooling of serum from PWT2D did not cause insulin resistance in our experiments, we considered the characteristics of our volunteers with T2D. When we did this, we saw that 3 of 18 of these volunteers had HbA1cs of 45, 47, and 44 mmol/mol when their blood was analysed after donation. For context, an HbA1c of between 42 and 47 mmol/mol is generally accepted as indicating pre-T2D. Based on this, then, the disease state of these people would be better classified as pre-T2D rather than clear T2D. In addition, 5 of the 18 people with T2D that we pooled serum from had highest measured HbA1cs on their records of 74, 66, 59, 53, and 78 mmol/mol. As an HbA1c of 48 mmol/mol or above is generally accepted as the diagnostic threshold for T2D (we will use the term 'clear T2D' for this going forward), these people had clear T2D at the time of measurement, however, their HbA1cs when measured for our study were 42, 42, 45, 41, and 40 mmol/mol. Based on this, these people would be better classified as being in remission, rather than having clear T2D. By these standards, this meant that only 10 of the 18 people with T2D that we pooled serum from had clear T2D. As all Logie *et al*. (2010)'s volunteers with T2D had clear T2D, with none being in remission or having pre-T2D, we posited that this difference could explain our different results. To test this hypothesis, we decided to pool and test the serum we had on hand from the volunteers whom we deemed to have clear T2D. By this time, the original serum collection had been completed, giving us serum samples from another 2 volunteers which met our criteria of having clear T2D. Thus, serum from 12 people with clear T2D (PWCT2D) was tested against serum from 12 healthy age and sex matched volunteers (**Figure 4-1**).

A

Controls					
Volunteer No	Sex	Age	BMI	HbA1c	Duration of disease
30	M	75	31.8	39	
42	M	69	23.4	39	
21	M	66	24.7	33	
37	M	60	23.2	35	
18	F	55	28.2	40	
3	F	58	21.6	38	
39	M	60	32.2	36	
1	F	57	29.5	30	
16	M	64	29	39	
24	M	66	27.8	34	
20	F	51	23.9	35	
41	M	63	27	41	
Mean (SD)		8M, 4F	62 (6.56)	26.86 (3.49)	36.58 (3.29)	

B

Cases					
Volunteer No	Sex	Age	BMI	HbA1c	Duration of disease
5	M	75	24.8	51	11 years
7	M	70	25.8	52	10 years
8	M	69	25.7	56	16 years
9	M	60	28.7	60	4 years
10	F	52	34.6	51	4 years
12	F	59	22.1	53	4 years
19	M	61	27.3	80	5 years
23	F	56	36.3	54	6 years
34	M	66	31.3	53	6 years
35	M	65	28.9	50	7 years
38	F	49	28.6	94	3 months
40	M	67	44.2	58	3 months
Mean (SD)		8M, 4F	62.42 (7.68)	29.86 (6.04)	59.33 (13.59)	6.13 (4.48) years

Figure 4-1: Details of volunteers with clear T2D and controls
Figure shows details of the healthy volunteers (Controls) (**A**) and people with clear T2D (Cases) (**B**) whom we pooled serum from for 'Clear T2D'' experiments. Age is shown in years. BMI in kg/m^2. HbA1c in mmol/mol. Mean figures, next to standard deviations (SD) in brackets, if appropriate, are shown at the bottom of columns.

4.2.2 5% serum from people with clear T2D has no effect on insulin-mediated repression of *Pck1*

The H4-II-E cells did not grow normally for this experiment (compared to those grown under standard conditions). We replaced the media 3-times per week with media containing freshly defrosted human serum (as we had done since experiencing the previously described growth problems), yet the cells

did not divide enough to be transferred to new flasks after the initial plating. For context, H4-II-E cells grown under standard conditions required to be passaged 3 times per week. I was able remedy this situation by adding 5% FBS to the media that these cells were grown in. After this, the cells started to divide enough (although still at a slower rate than cells in FBS) which enabled us to complete the experiment. As **Figure 4-2**, shows, however, we saw no difference in the ability of insulin to repress *Pck1* mRNA accumulation in cells grown in serum from people with clear T2D compared to controls. This was true whether *Pck1* was induced (**Figure 4-2 (A)**) or not (**Figure 4-2 (B)**).

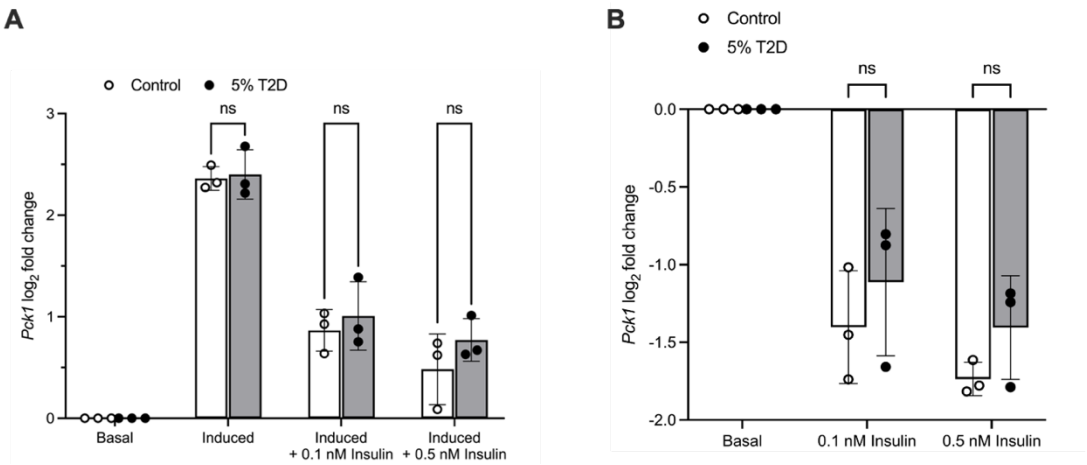

Figure 4-2: 5% serum from people with clear T2D has no effect on insulin-mediated repression of induced or basal *Pck1*
H4-II-E cells were cultured in media containing 5% FBS and 5% serum from PWT2D (labelled 5% T2D), or 5% FBS and 5% serum from healthy volunteers (labelled Control), replenished 3 times/week for 3 weeks, before being serum starved for 3h. **(A)** Cells were exposed to 500 nM dexamethasone and 0.1 mM 8-CPT-cAMP (labelled as induced) ± insulin (as indicated) for 3 h. **(B)** Cells were exposed to ± insulin (as indicated) for 3 h without induction. **(A&B)** Total cellular RNA was isolated, and reverse transcribed into cDNA before being subjected to qPCR analysis. Log_2 fold change of *Pck1* mRNA levels relative to that of the *Rplp1* housekeeping gene are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

4.2.2.1 Analysis by copy number

We re-analysed the above data by copy number to make sure that our finding was not due to the different way we analysed our qPCR data compared to

Logie *et al*. (2010). This confirmed **Figure 4-2** that insulin was equally able to repress induced *Pck1* mRNA in cells grown in serum from PWCT2D compared to cells grown in control serum (**Figure 4-3**); there was no evidence of serum-induced insulin resistance when analysed by this method.

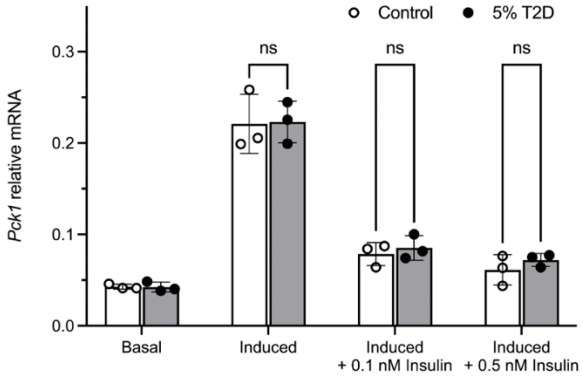

Figure 4-3: 5% serum from people with clear T2D does not cause insulin resistance in *Pck1*-induced cells, even if analysed by copy number
Data from **Figure 4-2** were reanalysed by copy number. *Pck1* mRNA levels relative to that of the housekeeping gene *Rplp1* are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

4.2.3 Serum from people with clear T2D has no effect on insulin-mediated glucose uptake in adipocytes

We tested the ability of serum from PWCT2D to induce insulin resistance in 3T3-L1 adipocytes by glucose uptake assay, firstly performing 3-day incubations with 10% serum (**Figure 4-4 (A)**). Under these conditions, there were no significant differences in insulin-mediated glucose uptake between cells grown in serum from PWCT2D and controls.

A previous study by Renstrom *et al*. (2009) reported that serum from PWT2D caused insulin resistance in primary adipocytes. This study used 24 h incubations with 25% human serum prior to glucose uptake assays to detect this effect. We therefore tested serum from PWCT2D under these conditions. However, there was again no difference in insulin-mediated glucose uptake between cells incubated in serum from PWCT2D, compared to controls; this

was evident at both submaximal and maximal insulin concentrations (**Figure 4-4 (B)**). Taken together, our data suggest that serum from people with clear T2D did not cause insulin resistance in H4-II-E cells or adipocytes under the conditions tested. Therefore, we next considered a different way to stratify our serum from PWT2D to test in our assays.

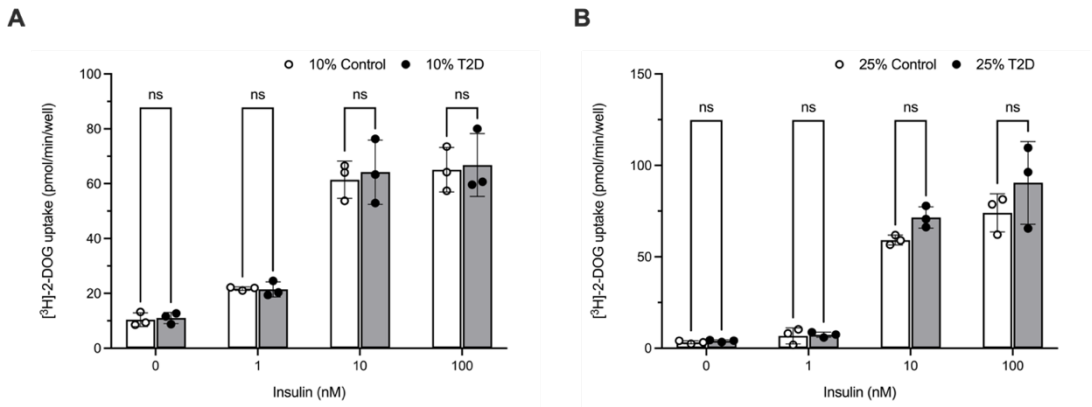

Figure 4-4: Serum from people with clear T2D has no effect on insulin-mediated glucose uptake in adipocytes
3T3-L1 adipocytes were incubated in media containing serum from people with clear T2D (T2D) or from healthy volunteers (Control) at 10% for 3 days **(A)** or 25% for 24 h **(B)**. Cells were serum starved for 2 h and treated with the indicated concentrations of insulin for 20 minutes. Glucose uptake was measured by the accumulation of [^{3}H]-2-deoxyglucose ([^{3}H]-2-DOG). Data from three independent experiments shown. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

4.2.4 Serum from people with T2D and obesity

Logie *et al.* (2010)'s volunteers with T2D had an average BMI of 34.95 and their healthy volunteers had an average BMI of 23.45. Whereas our volunteers with T2D had an average BMI of 29.26 and our healthy volunteers had an average 26.15. For context, according to the NHS, a BMI of between 18.5 and 24.9 indicates a healthy weight, a BMI of between 25 and 29.9 indicates being overweight, and a BMI of 30 and above indicates having obesity. Based on this, then, on average, Logie *et al.* (2010) tested serum from people that had T2D and obesity against serum from volunteers of a healthy weight, whereas we tested serum from PWT2D who were overweight, (although close to

having obesity) against serum from volunteers who were also in the overweight category. We hypothesised that this difference could have explained our different results, further hypothesising that the insulin resistance promoting capabilities of serum from PWT2D could correlate with obesity. To test this idea, we pooled serum from our 5 volunteers with T2D who had the highest BMIs to test against pooled serum from aged and sex-matched healthy volunteers in our assays (**Figure 4-5**).

A

	Controls					
	Volunteer No	Sex	Age	BMI	HbA1c	Duration of disease
	15	M	59	25.7	32	
	18	F	55	28.2	40	
	32	F	61	30.3	39	
	41	M	63	27	41	
	1	F	57	29.5	30	
Mean (SD)		2M, 3F	59 (3.16)	28.14 (1.86)	36.4 (5.03)	

B

	Cases					
	Volunteer No	Sex	Age	BMI	HbA1c	Duration of disease
	14	M	60	32.3	47	5 years
	23	F	56	36.3	54	6 years
	29	F	66	33.3	44	12 years
	40	M	67	44.2	58	3 months
	10	F	52	34.6	51	4 years
Mean (SD)		2M, 3F	60.2 (6.42)	36.14 (4.75)	50.8 (5.54)	5.45 (4.26) years

Figure 4-5: Details of volunteers with T2D and obesity (and controls)
Figure shows details of the healthy volunteers (Controls) (**A**) and people with T2D and obesity (Cases) (**B**) whom we pooled serum from for 'High BMI' experiments. Age is shown in years. BMI in kg/m^2. HbA1c in mmol/mol. Mean figures, next to standard deviations (SD) in brackets, if appropriate, are shown at the bottom of columns.

4.2.5 5% serum from people with T2D and obesity has no effect on insulin-mediated repression of *Pck1*

The H4-II-E cells grown in both types of human serum did not grow normally for this experiment compared to cells grown under our standard conditions. Similar to the cells grown for the 'clear T2D experiment (**Section 4.2.2, above**), they did not divide enough after their initial plating to be passaged unless the media was supplemented with 5% FBS. After this, the cells divided

enough for the experiment to be completed. However, insulin, at both concentrations tested, repressed *Pck1* mRNA accumulation to similar degrees in cells grown in serum from people with T2D and obesity, compared to cells grown in control serum. This was true when *Pck1* was induced (**Figure 4-6 (A)**) or not (**Figure 4-6 (B)**).

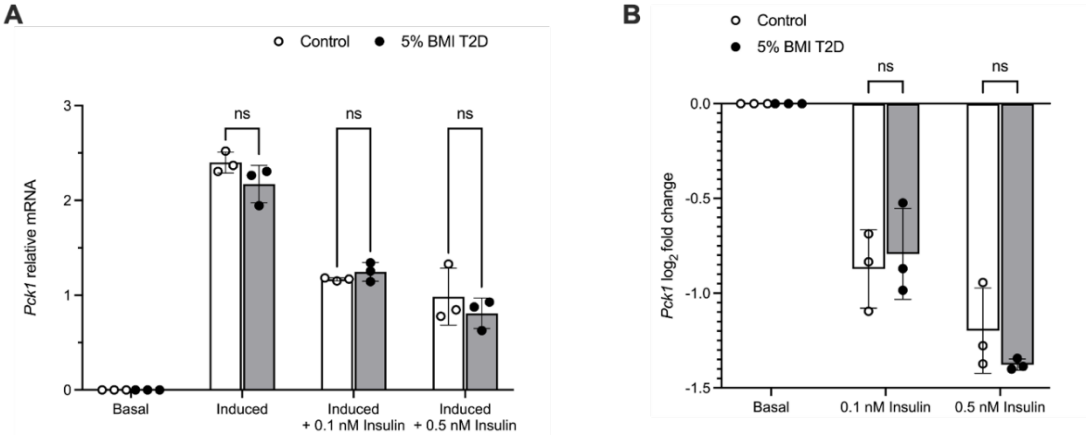

Figure 4-6: 5% serum from people with T2D and obesity has no effect on insulin-mediated repression of *Pck1*
H4-II-E cells were cultured in media containing 5% FBS and 5% serum from PWT2D and obesity (labelled 5% BMI T2D), or media containing 5% FBS and 5% serum from healthy volunteers (labelled Control), replenished 3 times/week for 3 weeks, before being serum starved. **(A)** Cells were exposed to 500 nM dexamethasone and 0.1 mM 8-CPT-cAMP (labelled as induced) ± insulin (as indicated) for 3 h. **(B)** Cells were exposed to ± insulin (as indicated) for 3 h without induction. **(A&B)** Total cellular RNA was isolated, and reverse transcribed into cDNA before being subjected to qPCR analysis. Log_2 fold change of *Pck1* mRNA levels relative to that of the *Rplp1* housekeeping gene are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. 'ns' represents not significant.

4.2.5.1 Analysis by copy number

To make sure this that result was not due to the different way to Logie *et al*. (2010) that we analysed our data by, we reanalysed by copy number. As **Figure 4-7** shows, the results are broadly similar for each method. Neither provides evidence that serum from PWT2D and obesity causes insulin resistance under the conditions tested.

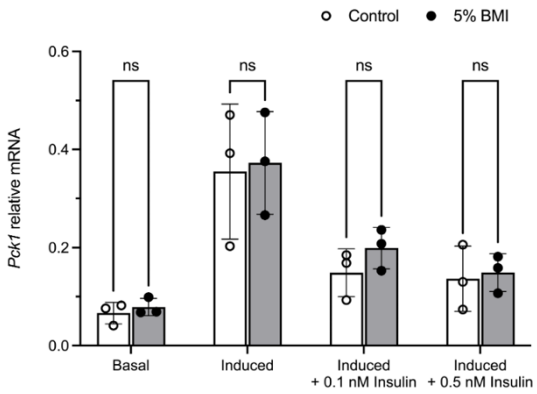

Figure 4-7: 5% serum from people with T2D and obesity has no effect on insulin-mediated repression of *Pck1* even if analysed by copy number
Data from Figure 4-6 were reanalysed by copy number. *Pck1* mRNA levels relative to that of the housekeeping gene *Rplp1* are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. 'ns' represents not significant.

4.2.6 Serum from people with T2D and obesity has no effect on insulin-mediated glucose uptake in adipocytes

We tested the ability of serum from people with T2D and obesity to generate insulin resistance in adipocytes by glucose uptake assay. We performed incubations with 25% serum for 24 h. As **Figure 4-8 shows**, at concentrations of 1, 10, and 100 nM, insulin stimulated glucose uptake to similar levels in cells incubated in serum from people with T2D and obesity, compared to controls. Therefore, this, and our other experiments, show that we did not see evidence that serum from people with T2D and obesity caused insulin resistance in H4-II-E cells and adipocytes. We next considered a different way to stratify our serum from PWT2D to test in our assays.

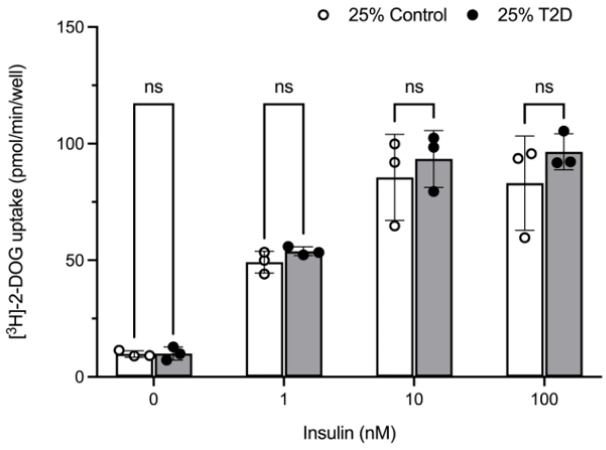

Figure 4-8: Serum from people with T2D and obesity has no effect on insulin-mediated glucose uptake in adipocytes
3T3-L1 adipocytes were incubated in media containing 25% serum from people with T2D and obesity (T2D) or from healthy volunteers (Control) for 24 h. Cells were serum starved for 2 h and treated with the indicated concentrations of insulin for 20 minutes. Glucose uptake was measured by the accumulation of [^{3}H]-2-deoxyglucose ([^{3}H]-2-DOG). Data from three independent experiments shown. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

4.2.7 Serum from people with T2D and high HbA1cs

The consensus model for the progression of T2D begins with the development of insulin resistance (Petersen and Shulman, 2018; James, Stockli and Birnbaum, 2018). To compensate, pancreatic beta cells increase production of insulin to maintain normal glucose levels in the blood (normoglycaemia) (Prentki and Nolan, 2006). Under such increased workload, the pancreatic beta cells can eventually fail to produce enough insulin to maintain function. If this occurs, then levels of glucose will rise beyond normal levels in the blood (hyperglycaemia) (Alejandro *et al.*, 2015). The majority of fasting hyperglycaemia in PWT2D has been attributed to an increased rate of gluconeogenesis (Magnusson *et al.*, 1992). During hyperglycaemia, red blood cells become glycosylated as a function of glucose concentration in the blood (Alejandro *et al.*, 2015). A measure of red blood cell glycosylation is HbA1c. With these facts in mind, we hypothesised that the insulin resistance promoting properties of serum from PWT2D might positively correlate with

the increased dysregulation of glucose homeostasis, seen as T2D progresses. To test this idea, we pooled serum from our 5 volunteers with T2D who had the highest HbA1cs to test against serum from healthy matched controls in our assays (**Figure 4-9**).

A

	Controls					
	Volunteer No	Sex	Age	BMI	HbA1c	Duration of disease
	15	M	59	25.7	32	
	18	F	55	28.2	40	
	32	F	61	30.3	39	
	41	M	63	27	41	
	1	F	57	29.5	30	
Mean (SD)		2M, 3F	59 (2.8)	28.14 (1.7)	36.4 (4.5)	

B

	Cases					
	Volunteer No	Sex	Age	BMI	HbA1c	Duration of disease
	14	M	60	32.3	47	5 years
	23	F	56	36.3	54	6 years
	29	F	66	33.3	44	12 years
	40	M	67	44.2	58	3 months
	10	F	52	34.6	51	4 years
Mean (SD)		2M, 3F	60.2 (5.7)	36.14 (4.2)	50.8 (5.0)	5.45 (3.8) years

Figure 4-9: Details of volunteers with T2D and high HbA1cs (and controls)
Figure shows details of the healthy volunteers (Controls) (**A**) and people with T2D and high HbA1cs (Cases) (**B**) whom we pooled serum from for 'High HbA1c' experiments. Age is shown in years. BMI in kg/m^2. HbA1c in mmol/mol. Mean figures, next to standard deviations (SD) in brackets, if appropriate, are shown at the bottom of columns.

4.2.8 5% serum from people with T2D and high HbA1c has no effect on insulin-mediated repression of *Pck1*

The H4-II-E cells did not grow well for this experiment. Even after supplementing with 5% FBS, the cells grown in human serum stopped dividing enough to be passaged after the second week. This made them the worst affected cells of this project. In response to this, we reduced the incubation length from 3 weeks to 2 weeks to generate the data required to test our hypothesis. As **Figure 4-10** shows, after doing so, we saw no significant differences in the ability of insulin to repress *Pck1* in cells grown in serum

from PWT2D who had high HbA1cs, compared to controls. This was true whether *Pck1* was induced (**Figure 4-10 (A)**) or not **Figure 4-10 (B)**).

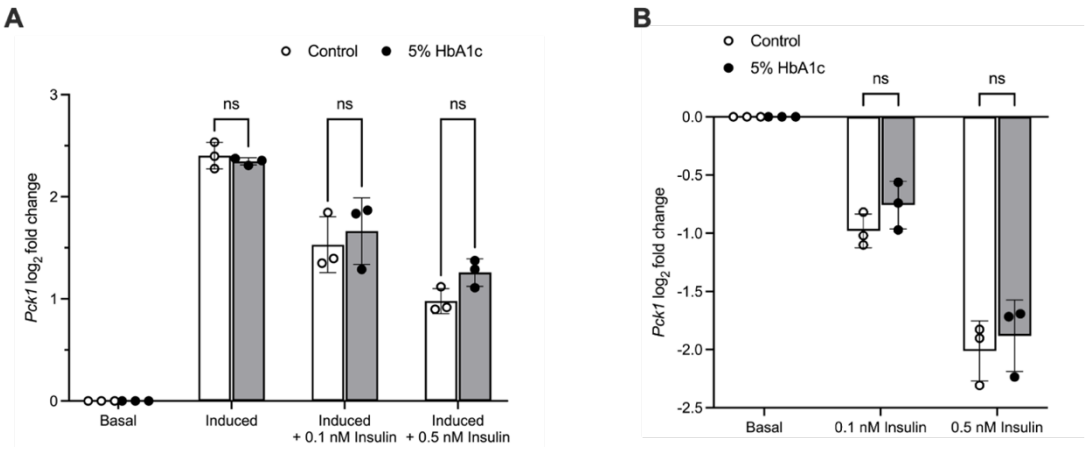

Figure 4-10: 5% serum from people with T2D and high HbA1c has no effect on insulin-mediated repression of *Pck1*
H4-II-E cells were cultured in media containing 5% FBS and 5% serum from the 5 of our volunteers with PWT2D and highest HbA1c (labelled 5% HbA1c), or 5% FBS and 5% serum from healthy volunteers (labelled Control), replenished 3 times/week for 2 weeks, before being serum starved. **(A)** Cells were exposed to 500 nM dexamethasone and 0.1 mM 8-CPT-cAMP (labelled as induced) ± insulin (as indicated) for 3 h. **(B)** Cells were exposed to ± insulin (as indicated) for 3 h without induction. **(A&B)** Total cellular RNA was isolated, and reverse transcribed into cDNA before being subjected to qPCR analysis. Log_2 fold change of *Pck1* mRNA levels relative to that of the *Rplp1* housekeeping gene are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. 'ns' represents not significant.

4.2.8.1 Analysis by copy number

To provide confidence that this result was not due to the different way compared to Logie *et al*. (2010) that we analysed our qPCR data by, we reanalysed by copy number. As **Figure 4-11** shows, this made no significant difference to our finding. We saw no clear evidence that H4-II-E cells were made resistant to insulin by being grown in serum from PWT2D who had high HbA1cs.

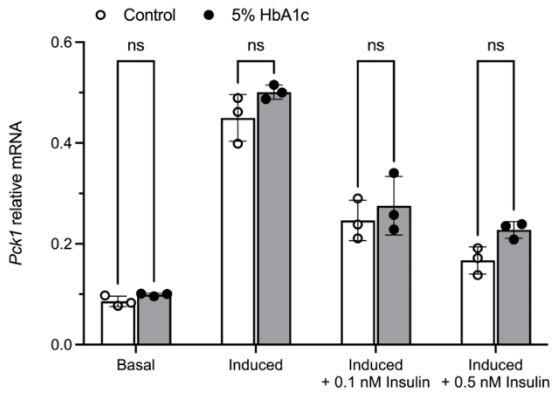

Figure 4-11: 5% serum from people with T2D and high HbA1c has no effect on insulin-mediated repression of *Pck1*, even if analysed by copy number
Data from **Figure 4-10** were reanalysed by copy number. *Pck1* mRNA levels relative to that of the housekeeping gene *Rplp1* are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. 'ns' represents not significant.

4.2.9 Serum from people with T2D and high HbA1cs has no effect on insulin-mediated glucose uptake in adipocytes

We tested the ability of serum from PWT2D who had high HbA1cs to induce insulin resistance in 3T3-L1 adipocytes, performing a glucose uptake assay after a 24 h incubation with 25% serum. As seen in **Figure 4-12**, there was no significant differences in insulin-mediated glucose uptake in adipocytes that were incubated in serum from PWT2D who had high HbA1cs, compared to controls. Having shown that serum from people with well-established T2D did not promote insulin resistance, we next considered people with the early stages of the disease.

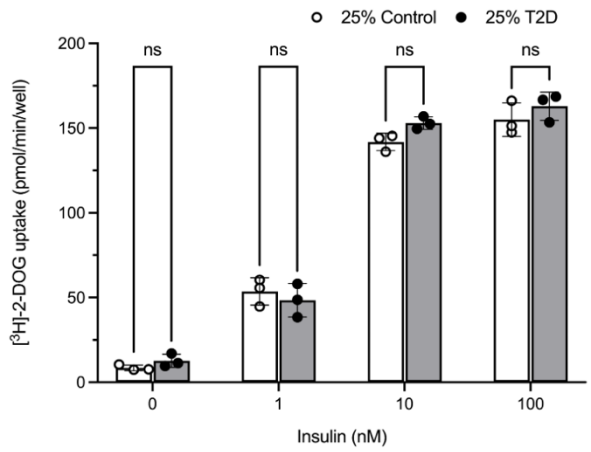

Figure 4-12: Serum from people with T2D and high HbA1cs has no effect on insulin-mediated glucose uptake in adipocytes
3T3-L1 adipocytes were incubated in media containing 25% serum from people with T2D and high HbA1cs (T2D) or from healthy volunteers (Control) for 24 h. Cells were serum starved and treated with the indicated concentrations of insulin for 20 minutes. Glucose uptake was measured by the accumulation of [^{3}H]-2-deoxyglucose ([^{3}H]-2-DOG). Data from three independent experiments shown. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. 'ns' represents not significant.

4.2.10 Serum from people with pre-T2D

Logie *et al*. (2010)'s volunteers had been recently diagnosed with T2D, whereas our volunteers had had T2D for an average of over 6.5 years (with a range of 3 months to 16 years). We hypothesised that this difference may have explained our different results and further hypothesised that the insulin promoting properties of serum from PWT2D may only be evident in people with early-stage disease. We did not have enough serum from people with pre-T2D from our original collection to pool and test this hypothesis. However, as part of a different study, assessing levels of environmental toxins, new serum samples from 6 people with pre-T2D and 6 healthy age and sex matched controls had been collected and were made available to us. To test the early disease hypothesis, we, pooled serum from these groups (separately) to examine in our assays (**Figure 4-13**).

A

	Controls			
Volunteer No	Sex	Age	BMI	HbA1c
24	M	66	27.8	34
21	M	66	24.7	33
16	M	64	29	39
42	M	69	23.4	39
31	M	50	25.4	36
41	M	63	27	41
Mean (SD)		6M	63 (6.69)	26.22 (2.09)	37 (3.16)

B

	Cases			
Volunteer No	Sex	Age	BMI	HbA1c
43	M	67	43.6	44
45	M	68	42.3	42
47	M	68	32	46
48	M	70	32.9	44
49	M	47	32.1	46
50	M	68	30.8	44
Mean (SD)		6M	64.67 (8.71)	35.62 (5.73)	44.33 (1.51)

Figure 4-13: Details of volunteers with pre-T2D and controls
Figure shows details of the healthy volunteers (Controls) and people with pre-T2D (Cases) whom we pooled serum from for 'pre-T2D' experiments. Age is shown in years. BMI in kg/m^2. HbA1c in mmol/mol. Mean figures, next to standard deviations (SD) in brackets, if appropriate, are shown at the bottom of columns.

4.2.11 Serum from people with pre-T2D has no effect on insulin-mediated repression of Pck1

We grew H4-II-E cells in media containing 10% human serum for this experiment. They divided similarly quickly (and we saw no obvious differences, by light microscope) compared to cells grown under standard conditions. Moreover, as **Figure 4-14 (A&B)** shows, H4-II-E cells grown in serum from PWPT2D were similarly sensitive to insulin as measured by insulin's ability to supress (induced and basal) *Pck1* mRNA accumulation, compared to controls.

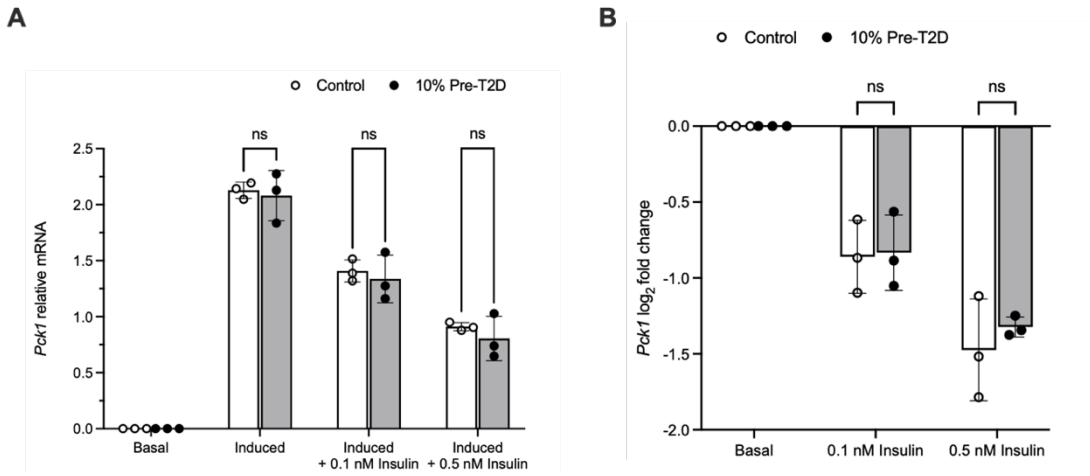

Figure 4-14: Serum from people with pre-T2D has no effect on insulin-mediated repression of *Pck1*
H4-II-E cells were cultured in media containing 10% serum from PWPT2D (labelled 10% Pre-T2D), or 10% serum from healthy volunteers (labelled Control), replenished 3 times/week for 3 weeks, before being serum starved. **(A)** Cells were exposed to 500 nM dexamethasone and 0.1 mM 8-CPT-cAMP (labelled as induced) ± insulin (as indicated) for 3 h. **(B)** Cells were exposed to ± insulin (as indicated) for 3 h without induction. **(A&B)** Total cellular RNA was isolated, and reverse transcribed into cDNA before being subjected to qPCR analysis. Log_2 fold change of *Pck1* mRNA levels relative to that of the *Rplp1* housekeeping gene are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. 'ns' represents not significant.

4.2.11.1 Analysis by copy number

We reanalysed our data by copy number to make sure the result was not due to the different analysis method we used, compared to Logie *et al*. (2010). This showed that *Pck1* was induced to a significantly greater degree in cells grown in serum from PWPT2D, compared to controls. However, the main takeaway is that the cells were similarly responsive to insulin (**Figure 4-15**) regardless of whether they were grown in serum from PWT2D or healthy volunteers.

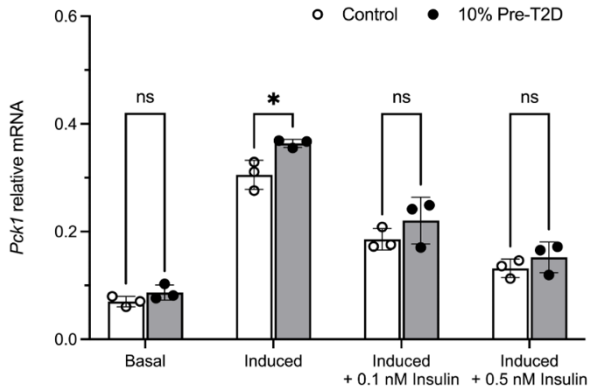

Figure 4-15: Serum from people with pre-T2D has no effect on insulin-mediated repression of *Pck1* when analysed by copy number
Data from **Figure 4-14** were reanalysed by copy number. *Pck1* mRNA levels relative to that of the housekeeping gene *Rplp1* are shown. Statistical significance or lack thereof was determined by two-way ANOVA and Tukey's multiple comparisons test. * and ns represent P≤0.05 and not significant, respectively.

4.2.12 Serum from people with pre-T2D causes insulin resistance in 3T3-L1 adipocytes

We tested the ability of serum from PWPT2D to induce insulin resistance in adipocytes by glucose uptake assay. We performed a 24 h incubation with 25% human serum. As **Figure 4-16** shows, serum from PWPT2D promoted an impairment in insulin-stimulated glucose transport at both maximal and submaximal concentrations of insulin. While preliminary, due to a volunteer number of only 6, this result supports the idea that there is a factor (or factors) present in the blood of PWT2D that promotes insulin resistance. Moreover, when considered with the preceding results of this section, and in the context of the differences between our and Logie *et al*. (2010)'s volunteers with T2D, this suggests that the factor(s) in serum which drive insulin resistance may only be present/functional in newly diagnosed individuals before the implementation of diet or drug therapies.

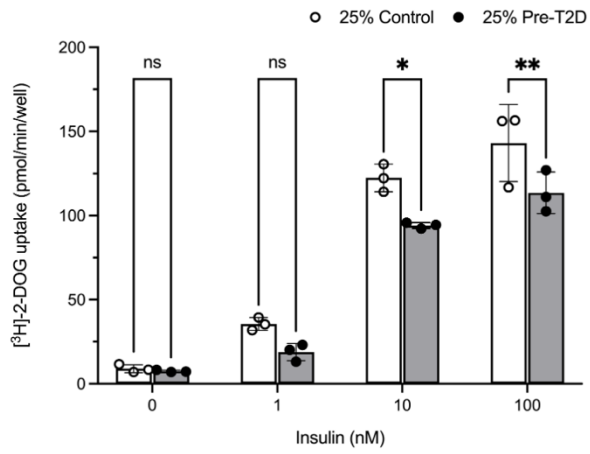

Figure 4-16: Serum from people with pre-T2D causes insulin resistance in 3T3-L1 adipocytes
3T3-L1 adipocytes were incubated in media containing 25% serum from people with pre-T2D (pre-T2D) or from healthy volunteers (Control) for 24 h. Cells were serum starved and treated with the indicated concentrations of insulin for 20 minutes. Glucose uptake was measured by the accumulation of [^{3}H]-2-deoxyglucose ([^{3}H]-2-DOG). Data from three independent experiments shown. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

4.2.13 Insulin concentration response in cells treated with serum from PWPT2D

We established that serum from PWPT2D impaired insulin-mediated glucose uptake in 3T3-L1 adipocytes. There was not enough volume from six volunteers to purify the responsible factor(s), however, we hypothesised that further defects may have been evident upstream of glucose uptake, in the context of proximate insulin signalling. To test this idea, we performed an insulin concentration response in cells treated with serum from PWPT2D, western blotting for the phosphorylation of AKT as a readout. As **Figure 4-17** shows, there were no significant differences in the phosphorylation of AKT in response to insulin at all concentrations tested in cells treated with serum from PWPT2D.

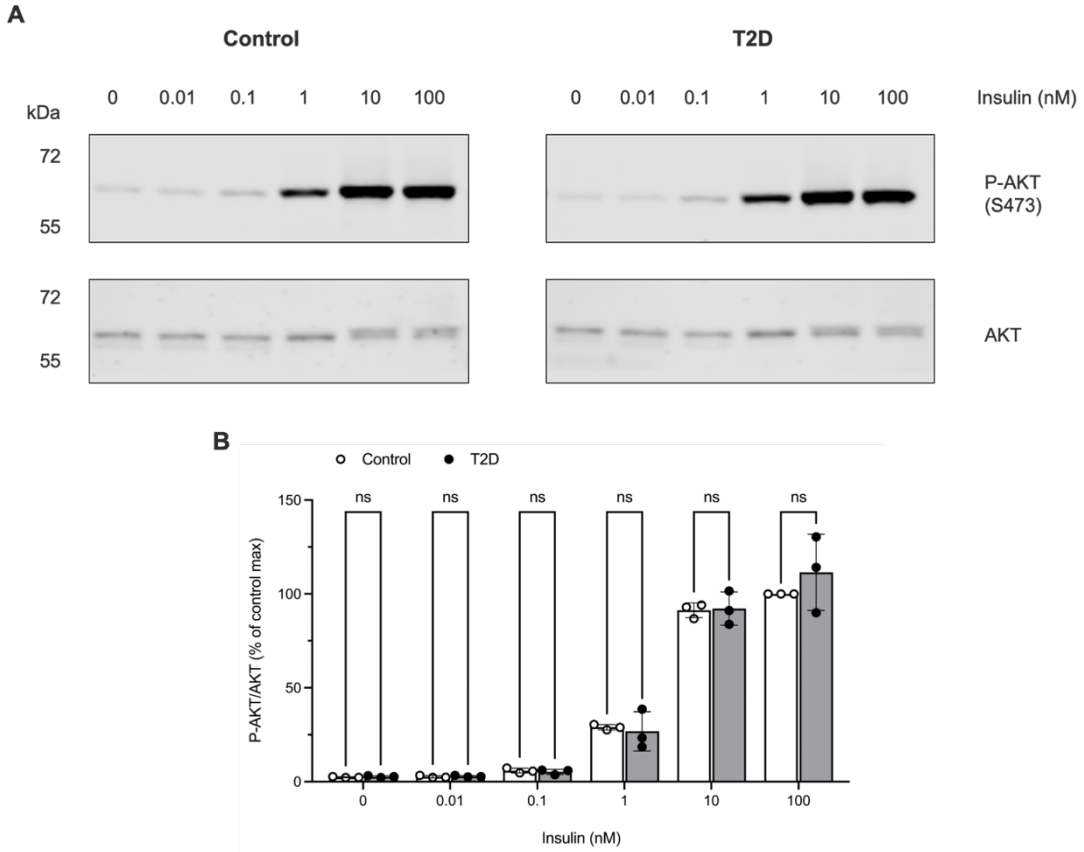

Figure 4-17: No difference in AKT phosphorylation after insulin concentration response with cells treated with serum from PWPT2D
3T3-L1 adipocytes were incubated in media containing 25% serum from people with pre-T2D (T2D) or from healthy volunteers (Control) for 24 h. Cells were serum starved for 2 h and treated with the indicated concentrations of insulin for 20 minutes, and lysed. **(A)** Picture of a representative western blot from one experiment shown. Lysates were probed with antibodies raised against the indicated proteins. Molecular weights shown in kilodaltons (kDa). **(B)** Graph shows the densitometric analysis of three biological replicates as per **(A)**. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

Having seen no difference in insulin-mediated-AKT phosphorylation after treatments with serum from PWPT2D, we considered the next molecular event in the cascade leading to glucose uptake. The AKT-dependent phosphorylation of AS160 regulates GLUT4 translocation (Tan *et al.*, 2015) and an impairment of this phosphorylation has been seen in PWT2D (Karlsson *et al.*, 2005). Thus, we blotted for the phosphorylation of AS160 as part of our insulin concentration response experiment to look for serum-mediated

impairment. As **Figure 4-18** shows, there were no significant differences in the phosphorylation of AS160 in response to four different insulin concentrations in cells treated with serum from PWPT2D.

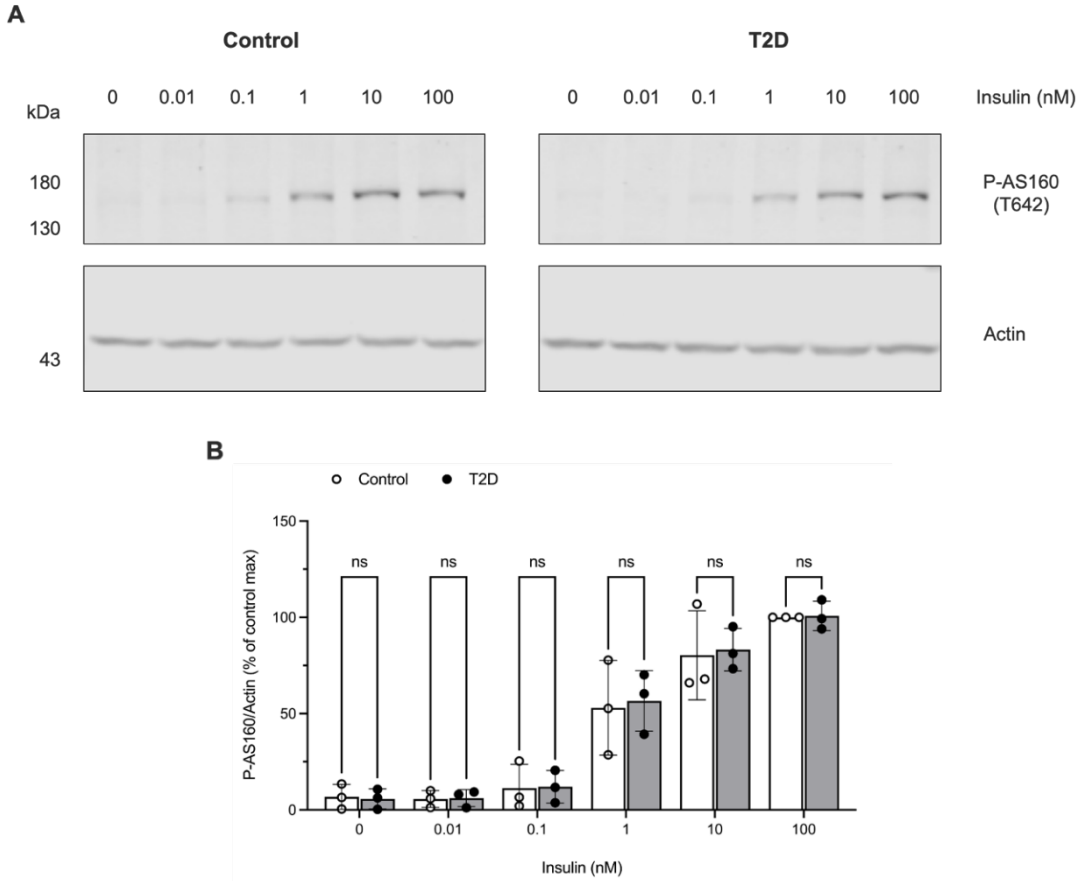

Figure 4-18: No difference in AS160 phosphorylation after insulin concentration response with cells treated with serum from PWPT2D
3T3-L1 adipocytes were incubated in media containing 25% serum from people with pre-T2D (T2D) or from healthy volunteers (Control) for 24 h. Cells were serum starved and treated with the indicated concentrations of insulin for 20 minutes, and lysed. **(A)** Picture of a representative western blot from one experiment shown. Lysates were probed with antibodies raised against the indicated proteins. Molecular weights shown in kilodaltons (kDa). **(B)** Graph shows the densitometric analysis of three biological replicates as per **(A)**. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

4.2.14 Insulin time course in cells treated with serum from PWPT2D

To further test the hypothesis that serum from PWPT2D may have induced defects in the molecular cascade leading to impaired glucose uptake, we

performed a time course in cells treated with serum from PWPT2D, western blotting for the phosphorylation of AKT in response to maximal insulin as a readout. As **Figure 4-19** shows, AKT was similarly phosphorylated in response to 100 nM insulin at all time points tested in cells treated with serum from PWPT2D, compared to controls.

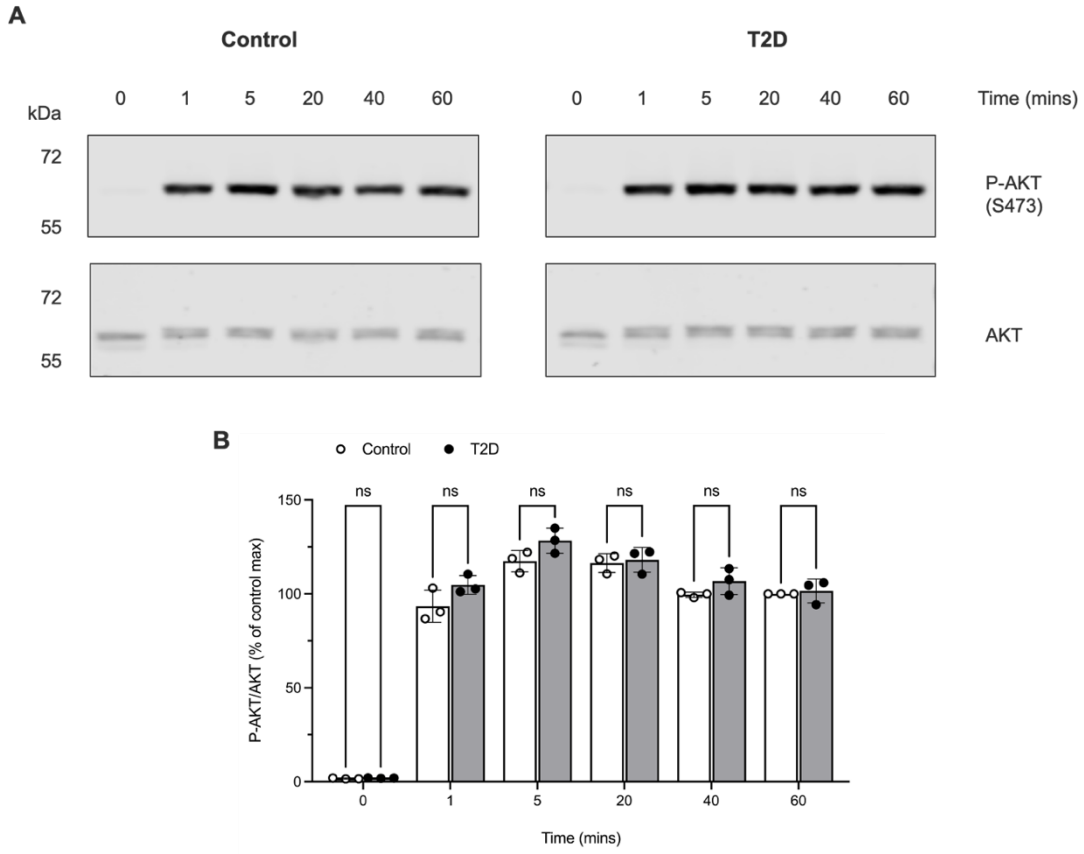

Figure 4-19: No difference in AKT phosphorylation after insulin time course with cells treated with serum from PWPT2D
3T3-L1 adipocytes were incubated in media containing 25% serum from people with pre-T2D (T2D) or from healthy volunteers (Control) for 24 h. Cells were serum starved and treated with 100 nM insulin for the indicated times, and lysed. **(A)** Picture of a representative western blot from one experiment shown. Lysates were probed with antibodies raised against the indicated proteins. Molecular weights shown in kilodaltons (kDa). **(B)** Graph shows the densitometric analysis of three biological replicates as per **(A)**. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

As part of the same time course experiment, we blotted for the phosphorylation of AS160 as a second measure of insulin sensitivity. As **Figure**

4-20 shows, AS160 was also phosphorylated to similar degrees in response to 100 nM insulin at all time points tested in cells treated with serum from PWPT2D, compared to controls.

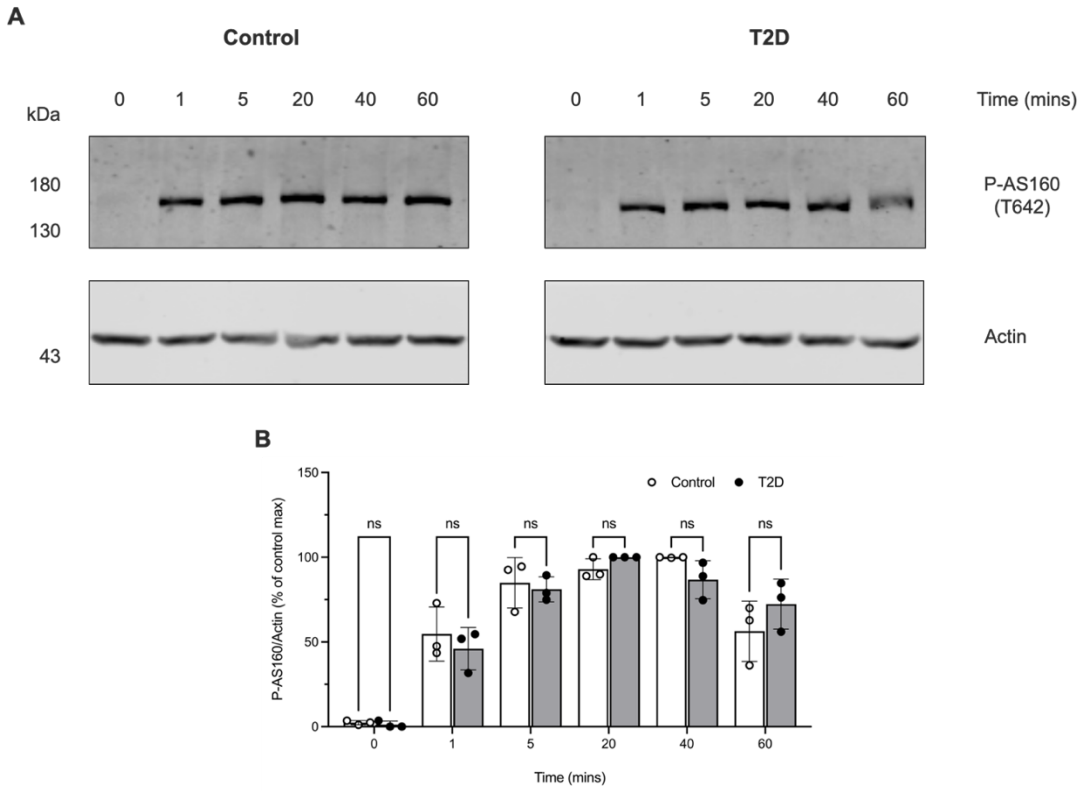

Figure 4-20: No difference in AS160 phosphorylation after insulin time course with cells treated with serum from PWPT2D
3T3-L1 adipocytes were incubated in media containing 25% serum from people with pre-T2D (T2D) or from healthy volunteers (Control) for 24 h. Cells were serum starved for 2 h and treated with 100 nM insulin for the indicated times, and lysed. **(A)** Picture of a representative western blot from one experiment shown. Lysates were probed with antibodies raised against the indicated proteins. Molecular weights shown in kilodaltons (kDa). **(B)** Graph shows the densitometric analysis of three biological replicates as per **(A)**. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

Taken together, the last 2 experiments indicate that the defect in insulin-mediated glucose uptake caused by serum from people with T2D was not evident in the insulin-mediated phosphorylation of AKT or AS160, which are key regulators of the glucose uptake process. This suggests to us that the defect may be evident in other proteins in this molecular cascade. We were

eager to test this idea, blotting for levels of GLUT4, for example, but unfortunately, there was not enough time in the project to do this.

4.2.15 Negative effects of human serum on cultured cells

As previously mentioned, we found that H4-II-E cells did not grow normally when exposed to human serum compared to cells grown under standard conditions. Moreover, as **Figure 4-21** shows, while 3-day incubations in 10% human serum, had no significant effect on insulin-mediated glucose uptake in adipocytes, compared to FBS controls (**Figure 4-21 A&B**) 24 h incubations in 25% human serum did significantly reduce such glucose uptake (**Figure 4-21 C&D**). Taken together, our results indicate that human serum may negatively affect cultured H4-II-E and 3T3-L1 cells.

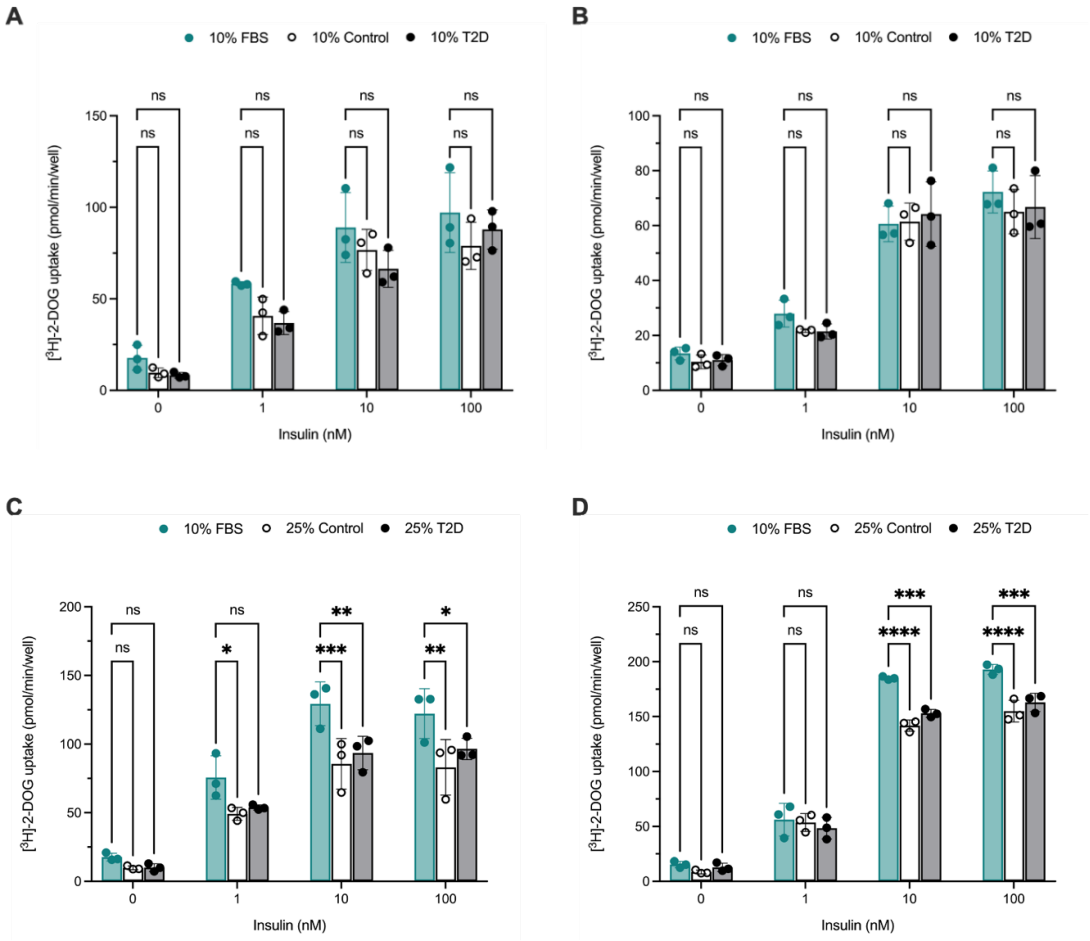

Figure 4-21: 25% human serum inhibits insulin-mediated glucose uptake in 3T3-L1 fibroblasts compared to FBS controls

Figure shows data from 4 experiments previously presented in this report, this time showing FBS controls, with the following incubation details: **A:** 10% serum from initial pooling, for 3 days. **B:** 10% serum from 'clear T2D' pooling, for 3 days. **C:** 25% serum from 'high BMI' pooling, for 24 h. **D:** 25% serum from 'high HbA1c' pooling, for 24 h. Glucose uptake was measured by the accumulation of [^{3}H]-2-deoxyglucose ([^{3}H]-2-DOG). Data from three independent experiments shown. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

4.3 Discussion

The aim of this chapter was to investigate whether differences in volunteer characteristics could explain why serum collected for this PhD project failed to induce insulin resistance, while Logie *et al*. (2010) reported clear effects. Serum was therefore stratified and pooled according to several clinically relevant criteria (clear T2D (meaning an HbA1c of 48 mmol/mol or above), T2D with high BMI, and T2D with high HbA1c) and evaluated using the validated *Pck1* repression and adipocyte glucose uptake assays. No induction of insulin resistance was observed with any of these T2D cohorts in either H4-II-E hepatomas or 3T3-L1 adipocytes. However, when pooled serum from a cohort of individuals with pre-T2D was tested, a different pattern emerged. No impairment of insulin-mediated *Pck1* repression was observed in H4-II-E hepatomas, but a selective reduction in insulin-stimulated radiolabelled glucose uptake was detected in 3T3-L1 adipocytes. These findings suggest that the insulin resistance-promoting activity of T2D serum is not uniform but may be tissue- or pathway-specific, and that such activity appears most evident in the earlier stages of disease progression.

The consistent lack of effects across multiple T2D subgroups (longer-standing disease, obesity, and elevated HbA1c) contrasts sharply with the positive findings reported by Logie *et al*. (2010). This discrepancy is unlikely to be explained by inadequate assay sensitivity, given that both models readily detected insulin resistance induced by known stimuli such as TNF and hyperinsulinaemia. Instead, the results point toward important biological differences between the cohorts. Participants in this PhD project had generally been living with T2D for several years and were mostly managing their condition through lifestyle measures, whereas Logie *et al*. (2010)'s donors were newly diagnosed, had obesity, and sampled immediately prior to pharmacological intervention. Disease duration, glycaemic trajectory, and the presence or absence of medication are known to influence circulating levels of FFAs, inflammatory cytokines, and adipokines – all of which have

been implicated in the propagation of insulin resistance. The selective impairment in adipocyte glucose uptake observed with pre-T2D serum, without a corresponding defect in the hepatic *Pck1* response, further supports the idea that circulating mediators may exert tissue- or pathway-specific effects, particularly during the earlier phases of disease.

These results carry two broader implications. First, they suggest that the capacity of T2D serum to induce insulin resistance in cultured cells may be transient or most prominent during the early, pre-clinical or newly diagnosed phase, before compensatory mechanisms or therapeutic interventions alter the circulating milieu. Second, the selective nature of the defect in adipocytes (impaired glucose uptake without corresponding changes in proximal signalling through AKT or AS160) indicates that the responsible mediator(s) may act at more distal steps of the insulin signalling cascade such as the latter steps of GLUT4 trafficking and/or vesicle fusion. This highlights the importance of using multiple functional endpoints rather than relying solely on proximal signalling markers when studying humoral factors in insulin resistance.

Several limitations should be acknowledged. The relatively small number of donors in the pre-T2D cohort and the use of pooled sera limit the ability to draw firm conclusions about prevalence and potency. While pooling offered practical advantages for downstream fractionation attempts, it may have diluted active components or masked inter-individual differences in factor activity. With the benefit of hindsight, testing a larger number of individual sera from well-characterised, newly diagnosed or pre-T2D donors would have provided greater statistical power and clearer insight into the prevalence and potency of the insulin resistance-inducing activity. Additionally, the observed negative effects of human serum on cell proliferation and, at higher concentrations, on glucose uptake in adipocytes underscore the need for careful optimisation of culture conditions when working with human biofluids.

Despite these limitations, this chapter yields two important conclusions. First, it reinforces that the capacity of serum from PWT2D to induce insulin resistance in cultured cells is not universal but appears highly dependent on donor disease stage. Second, the selective impairment in adipocyte glucose uptake induced by pre-T2D serum, without corresponding changes in proximal signalling, suggest the presence of mediators that act at distal steps of the insulin signalling cascade. These observations provide a foundation for future studies aimed at fractionating serum, identifying specific mediators, and determining whether the same factors are responsible for defects in different tissues.

In summary, this chapter demonstrates that serum from individuals with longer-standing, lifestyle-managed T2D did not induce detectable insulin resistance in the models tested, whereas preliminary evidence suggests that serum from individuals with pre-T2D can selectively impair adipocyte glucose disposal. These findings underscore the importance of donor disease stage when investigating humoral factors in insulin resistance and provide a technical platform that can be applied in future studies examining individual patent sera or candidate mediators.

5 Chapter 5

5.1 Introduction

The 3T3-L1 adipocyte cell line, originally isolated in the 1970s from a disaggregated Swiss mouse embryo (Green and Meuth, 1974), has become one of the most widely used in vitro model systems for studying adipogenesis, lipid metabolism, glucose transport, and insulin action, particularly in the contexts of obesity and T2D (Klip, McGraw and James, 2019). Its popularity stems from the ability of these cells to undergo robust differentiation into mature adipocytes that exhibit many hallmarks of adipose tissue physiology, including insulin-stimulated glucose uptake via GLUT4 translocation and the capacity to store large amounts of triacylglycerols (Klip, McGraw and James, 2019).

Despite its utility, differentiated 3T3-L1 adipocytes are known to display considerable heterogeneity in cellular and lipid droplet morphology (Loo *et al.*, 2009; Ahmed, Lai and Kim, 2021). Within the same culture, individual cells can range from small, multilocular adipocytes reminiscent of brown adipose tissue (BAT) to large, unilocular cells more characteristic of white adipose tissue (WAT). This morphological diversity is functionally significant because lipid droplet size and number are strongly correlated with metabolic phenotype: enlarged unilocular droplets are associated with insulin resistance, impaired glucose uptake, and increased lipolysis, whereas smaller, multilocular droplets are generally linked to higher insulin sensitivity and oxidative capacity (Konige, Wang and Sztalryd, 2014; Kim *et al.*, 2015). Adding to this complexity, differentiated 3T3-L1 adipocytes exhibit mixed phenotypic features of both WAT and BAT, prompting ongoing debate about whether the cell line is best regarded as a model of WAT, BAT, or a transitional/hybrid state (Morrison and McGee, 2015; Olson, 2018; Machado *et al.*, 2022). Given that lipid droplet morphology is intimately linked to insulin sensitivity, such heterogeneity raises important questions

about the reliability and reproducibility of population-level data generated from this model, particularly when investigating insulin resistance.

Preliminary observations from ongoing work in Gould Lab have suggested that 3T3-L1 adipocytes become progressively less morphologically heterogeneous as they age in culture. Although the primary focus of this thesis is the identification of circulating factors in serum from PWT2D that induce insulin resistance, understanding how culture affects the metabolic phenotype of 3T3-L1 adipocytes is an important complimentary question. It addresses a fundamental methodological issue: whether the differentiation state or age of these cells at the time of experimentation can influence their responsiveness to insulin or to potential humoral mediators. The work described in this chapter was therefore undertaken as a discrete investigation to characterise age-related changes in lipid droplet morphology in 3T3-L1 adipocytes and to determine whether these changes are associated with functional changes in insulin action.

5.2 Aims

The general aims of this chapter were (1) to characterise changes in lipid droplet morphology in 3T3-L1 adipocytes as they age in culture, and (2) to examine whether these morphological changes correlate with alterations in maximal insulin-mediated glucose uptake and/or the expression and phosphorylation of key proteins involved in insulin signalling.

Our specific aims were:

- To quantify the lipid droplet number, area, and volume in Day 10, Day 20, Day 30, and Day 40 3T3-L1 adipocytes

- To measure maximal insulin-mediated glucose uptake in Day 10, Day 20, Day 30, and Day 40 3T3-L1 adipocytes

- To measure maximal insulin-mediated glucose uptake at 27 °C in Day 10, and Day 40 3T3-L1 adipocytes

- To compare protein levels of insulin receptor beta subunit, AMPK, AS160, Syntaxin-16, IRAP, AKT, GLUT1, GLUT4, and Acetyl-CoA Carboxylase (ACC) between Day 40 and Day 10 3T3-L1 adipocytes

5.3 Results

5.3.1 3T3-L1 adipocyte morphology changes over time

We generated images of 3T3-L1 adipocytes at 10-day intervals after beginning the differentiation process, quantifying the number, cross-sectional area, and volume of the lipid droplets contained in these cells. As **Figure 5-1** shows, Day 10 3T3-L1 adipocytes contain multiple small lipid droplets (characteristic of brown adipose cells (Figueroa *et al.*, 2023)). Contrastingly, as they age (from Day 20 to Day 40) the number of lipid droplets decreases with the droplets becoming larger and more voluminous (which is characteristic of white adipose cells (Olson, 2018)). Thus, these data indicate that 3T3-L1 adipocytes become more WAT-like as they age. As white adipose cells and brown adipose cells have different metabolic profiles (Rosell *et al.*, 2014), including different sensitivities to insulin (Orava *et al.*, 2011), we investigated whether the phenotypic changes we saw in aged 3T3-L1s would correlate with differences in insulin-mediated glucose uptake,

and/or differences in levels of key proteins involved in insulin signalling/response pathways.

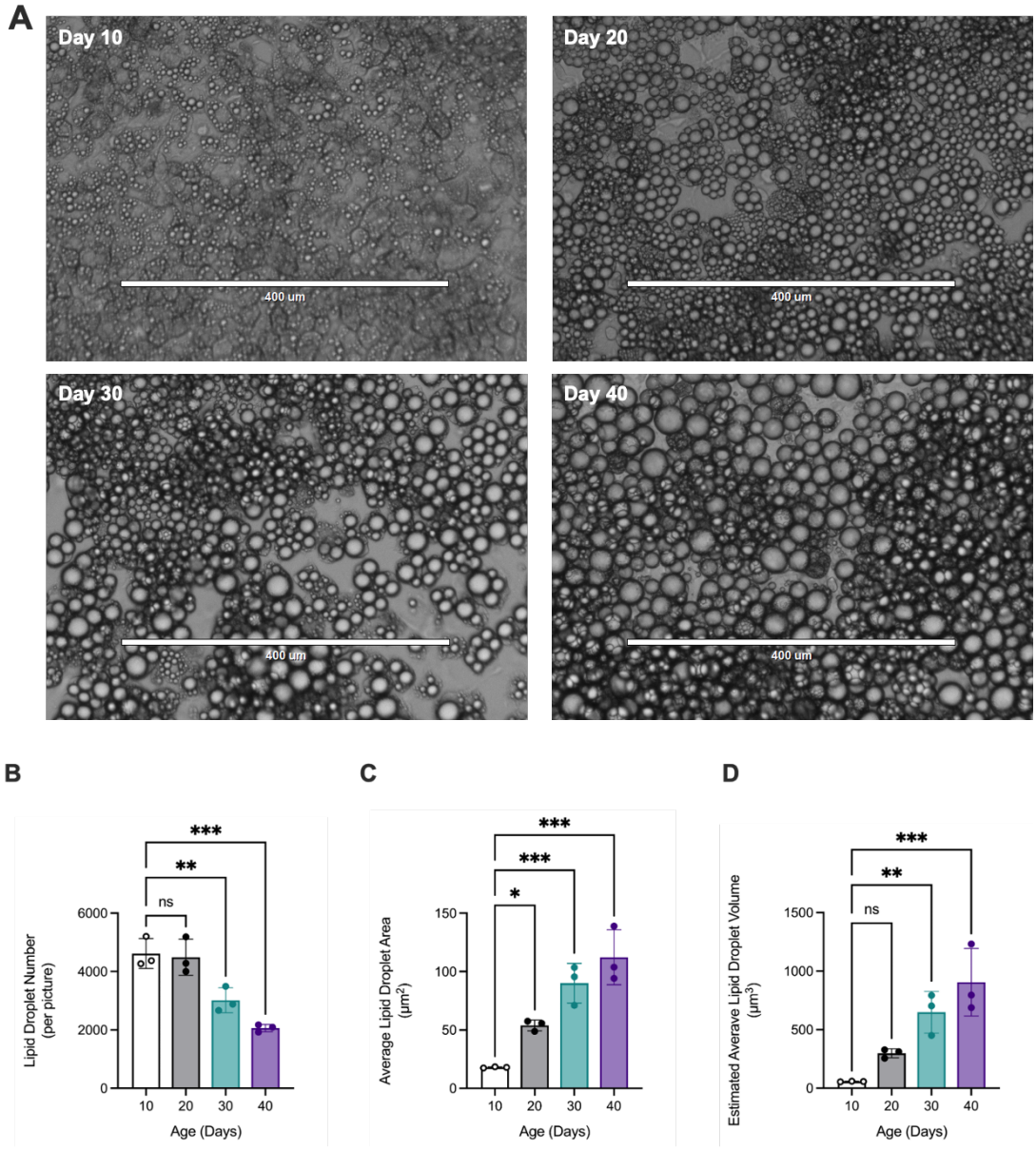

Figure 5-1: 3T3-L1 adipocyte morphology changes over time
(A) Representative images of 3T3-L1 adipocytes of the indicated ages from one biological replicate (labelled as number of days since the differentiation process was started). Scale bars of 400 µm are shown. **(B, and C)** Quantification of lipid droplet number and cross-sectional area in (μm^2) per time point from 3 biological replicates (that is, data from 3 images per time point, each containing around 1500 cells). (**D**) Estimated average volume of lipid droplets per time point, from 3 biological replicates. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

5.3.2 No difference in maximum insulin-mediated glucose uptake between new and aged 3T3-L1 adipocytes

We hypothesised that the more WAT-like morphology we saw develop as 3T3-L1 adipocytes age might correlate with differences in insulin-mediated glucose uptake. To test this idea, we measured the ability of 100 nM insulin to stimulate glucose uptake in 3T3-L1 adipocytes at 10-day intervals up to 40 days after starting differentiation. As **Figure 5-2** shows, there were no statistically different differences in basal glucose uptake and maximally stimulated insulin-mediated glucose uptake in 3T3-L1 adipocytes, at 10-, 20-, 30-, and 40-days post differentiation. Thus, although 3T3-L1 adipocytes of increasing ages displayed different morphologies, they exhibited similar maximally stimulated insulin-mediated glucose uptake. An intriguing finding here is that, judging by the decreasing size of the error bars of the responses to 100 nM insulin, the intra-experiment variability decreased as 3T3-L1 adipocytes aged. Thus, as the cells became more homogeneous as they aged, so too did their responses to maximal insulin.

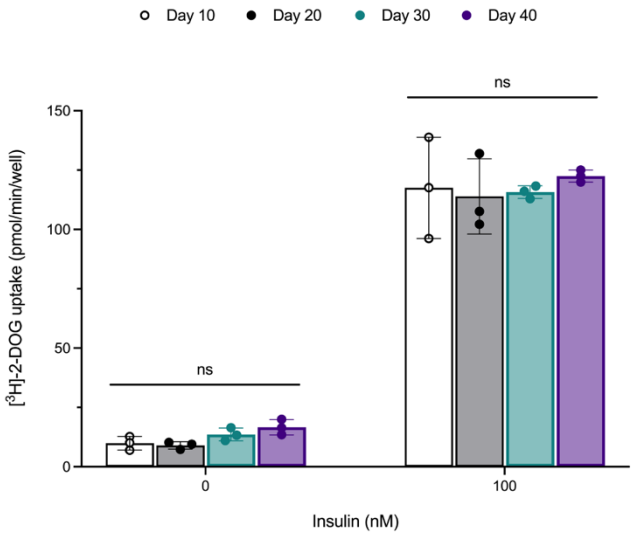

Figure 5-2: No difference in insulin-mediated glucose uptake between new and aged 3T3-L1 adipocytes
3T3-L1 adipocytes of the indicated age post differentiation were serum starved and treated with 100 nM insulin for 20 minutes. Glucose uptake was measured by the accumulation of [^{3}H]-2-deoxyglucose ([^{3}H]-2-DOG). Data from three independent experiments shown. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

5.3.3 No difference in maximum insulin-mediated glucose uptake between new and aged 3T3-L1 adipocytes at 27°C

The initial rate of insulin-mediated glucose uptake in adipocytes is extremely rapid, reaching an equilibrium value in approximately 20 s with a half-time of 4 s (Ludvigsen and Jarett, 1979). Measuring these changes over such short timescales is challenging. Previous work conducted in Gould Lab, used glucose uptake assays performed at 27°C to slow down the kinetic changes with time after addition of insulin (Gibbs, Lienhard and Gould, 1988). We hypothesised that using this method might reveal changes in the insulin responses of aged 3T3-L1s that could not detected at 37°C (**Figure 5-2**). To test this idea, we, performed a glucose uptake assay at 27°C, comparing the effects of 100 nM insulin to stimulate glucose uptake in 40-day old cells (post differentiation) against that of Day 10 cells. As **Figure 5-3 (A)** shows, there was no difference in insulin-mediated glucose uptake at 27°C between Day 40 and Day 10 cells. However, at 30 minutes the rate of uptake was still increasing (maximum insulin-mediated glucose uptake had not been reached). To make sure that we did not miss any age-related changes in glucose uptake that occurred after 30 mins, we repeated the experiment, this time extending the final time point to 60 mins. As **Figure 5-3 (B)** shows, a similar result was evident; we still could not see any difference in maximum insulin-mediated glucose uptake between aged and Day 10 adipocytes at 27°C. This is further evidence that although 3T3-L1 adipocyte morphology changes as they age, their responses to maximal insulin-mediated glucose uptake are not affected by this.

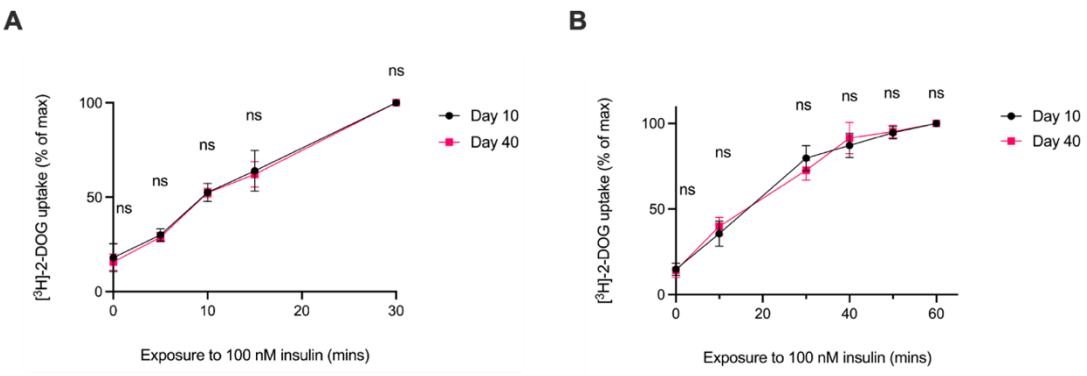

Figure 5-3: No difference in maximum insulin-mediated glucose uptake in aged 3T3-L1 adipocytes at 27°C
3T3-L1 adipocytes of the indicated age post differentiation (labelled Day 10 or Day 40) were serum starved, cooled to 27°C, and treated with 100 nM insulin for **(A)** 30 mins or **(B)** 60 mins. Glucose uptake was measured by the accumulation of [^{3}H]-2-DOG. Data from three independent experiments shown. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by two-way ANOVA and Tukey's multiple comparisons test. 'ns' represents not significant.

5.3.4 Aged 3T3-L1 adipocytes show differences in key proteins involved in insulin responses

Due to the reported differences in insulin sensitivities between WAT and BAT (Orava *et al.*, 2011; Kim *et al.*, 2015), we hypothesised that the age-related changes in 3T3-L1 adipocyte morphology seen in **Figure 5-1** may have correlated with changes in key proteins involved in insulin signalling/responses. To test this idea, we used western blotting to compare Day 40 3T3-L1 adipocyte lysates to Day 10 lysates. As **Figure 5-4 (A)** shows, levels of the insulin receptor beta subunit were significantly lower in Day 40 lysates, implying that aged cells had fewer insulin receptors than younger ones. Moreover, proteins involved in insulin-mediated glucose uptake, AMPK, AS160, Syntaxin-16, and IRAP were also significantly lower compared to controls (**Figure 5-4 (B-E)**), indicating that the glucose uptake cascade was modified in aged cells (although not to the degree that it affected insulin-mediated glucose uptake (**Figure 5-2 & Figure 5-3**). There are several potential explanations for this finding, some of which are given in the following section. Finally, levels of AKT, GLUT1, GLUT4, and Acetyl-CoA

Carboxylase (ACC), an enzyme involved in insulin-mediated fatty acid metabolism, had not changed (**Figure 5-4 (F-H)**), indicating that not all proteins are affected by the aging process in these cells. Taken together, the results of this chapter show that 3T3-L1 adipocytes become morphologically more WAT-like as they age. This coincides with lowered levels of proteins involved in insulin signalling/responses, including the insulin receptor beta subunit, AMPK, AS160, Syntaxin-16, and IRAP. However, these changes did not affect maximally stimulated insulin-mediated glucose uptake in aged cells. This suggests that there was enough redundancy in the insulin signalling system to compensate for changes in these specific protein levels. We would have liked to have looked for further changes in aged 3T3-L1s in this and other contexts, but, unfortunately, my lab time for this PhD project ran out shortly after obtaining these results.

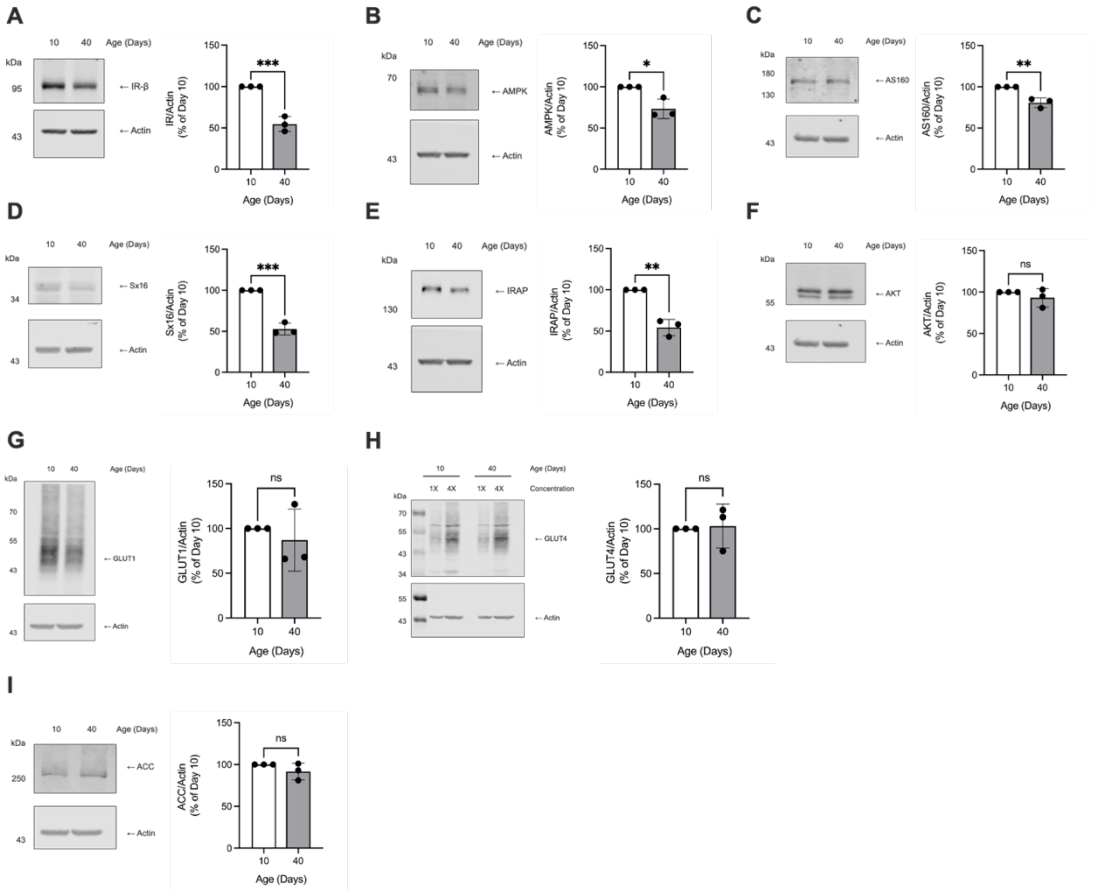

Figure 5-4: Western blots of selected proteins involved in insulin-mediated responses in aged 3T3-L1 adipocytes
3T3-L1 adipocytes were cultured for either 10- or 40-days (as labelled) after beginning differentiation. Cells were serum starved for and lysed. Lysates were probed with antibodies raised against the indicated proteins. Molecular weights shown in kilodaltons (kDa). **(A, B, C, D, E, F, G, H, and I)** Each show a picture of a representative western blot of one biological replicate and a graph showing the densitometric analysis of three biological replicates. Statistical significance (depicted by asterisks) or lack thereof (depicted by ns) was determined by unpaired t-test. *, **, ***, ****, and ns represent P≤0.05, P≤0.01, P≤0.001, P≤0.0001, and not significant, respectively.

5.4 Discussion

The aim of this chapter was to characterise age-related changes in 3T3-L1 adipocyte morphology and to determine whether these changes are associated with alterations in insulin action. As the cells aged in culture, they underwent a progressive morphological shift: the number of lipid droplets decreased while the remaining droplets became larger and more voluminous, resulting in a transition toward a more WAT-like phenotype. Despite this clear morphological remodelling, maximal insulin-stimulated uptake remained unchanged between young (Day 10) and aged (Day 40) adipocytes, even when measured at a reduced temperature of 27 °C to slow membrane trafficking. Consistent with the functional data, protein levels of key glucose transporters (GLUT4 and GLUT1) and the central signalling kinase AKT were unaltered with age. However, significant reductions were observed in several proteins involved in insulin signalling and GLUT4 trafficking, including the insulin receptor beta subunit, AMPK, AS160, Syntaxin-16 and IRAP.

These findings reveal a dissociation between morphological maturation and insulin responsiveness in 3T3-L1 adipocytes. The development of larger, unilocular lipid droplets is generally associated with insulin resistance in native adipose tissue (Konige, Wang and Sztalryd, 2014; Kim *et al.*, 2015). The fact that aged 3T3-L1 cells adopted a more WAT-like morphology without a corresponding decline in maximal glucose uptake suggests that this cell line may possess compensatory mechanisms that maintain insulin sensitivity despite hypertrophic changes. This is an important observation because

3T3-L1 adipocytes are one of the most commonly used models for studying insulin action and resistance. The data indicate that culture age can significantly alter cellular phenotype, yet this does not necessarily translate into functional insulin resistance under the conditions tested.

The reduction in insulin receptor beta subunit levels is noteworthy. Although fewer receptors might be expected to impair insulin action, maximal glucose uptake was preserved. This is consistent with the concept of spare capacity in the insulin signalling system, whereby only a fraction of receptors need to be activated to elicit a full biological response (Kahn, 1978). Similarly, the decrease in AS160, Syntaxin-16, and IRAP (all proteins involved in GLUT4 vesicle trafficking) did not result in measurable impairment of maximal insulin-stimulated glucose uptake. This suggests that the remaining levels of these proteins were still sufficient to support full GLUT4 translocation under saturating insulin concentrations. Whether sub-maximal insulin responses or more subtle aspects of GLUT4 trafficking (such as vesicle tethering or fusion kinetics) are affected remains to be determined.

The reduction in AMPK levels in aged cells is also consistent with a shift towards a more WAT-like phenotype, as AMPK is known to promote brown adipocyte characteristics while inhibiting insulin-stimulated glucose uptake in white adipocytes (Salt, Connell and Gould, 2000; Ahmad *et al.*, 2020; Göransson, Kopietz and Rider, 2023). However, the functional consequences of this reduction in the context of maximal insulin action appear limited in this model.

Several limitations should be acknowledged. Our study relied on a single immortalised cell line, and the observed changes may not fully translate to primary adipocytes or in vivo adipose tissue. With the benefit of hindsight, extending the final time point of the 27 °C glucose uptake experiment or performing insulin concentration-response and time-course experiments for phosphorylated signalling intermediates may have provided additional

insight. Furthermore, although we observed clear morphological and protein-level changes, the absence of corresponding functional impairment in maximal glucose uptake raises the question of whether more sensitive or different physiologically relevant readouts (such as sub-maximal insulin responses, lipolysis rates, or adipokine secretion) might reveal differences not captured here.

In conclusion, this chapter demonstrates that 3T3-L1 adipocytes undergo significant age-related morphological remodelling towards a more WAT-like state, accompanied by selective changes in the levels of several insulin signalling and GLUT4 trafficking proteins. However, these changes did not result in impaired maximal insulin-stimulated glucose uptake under the conditions tested. The findings highlight the importance of considering culture age when using 3T3-L1 cells as a model system and suggest that morphological maturation and insulin responsiveness can be partially dissociated in this cell line. Future work should extend these observations to primary adipocytes, examine sub-maximal insulin responses, and explore whether the age-related protein changes influence other aspects of adipocyte metabolism, such as lipolysis or secretory function. Such studies will improve our understanding of the reliability of 3T3-L1 cells and may provide further insight into the relationship between adipocyte morphology and insulin sensitivity in the context of T2D.

Future Perspectives and Final Conclusions

In this section, we will propose plans to advance the work carried out during this project based on what we have learned and what might be accomplished if suitable funding for several years of work was obtained. We will then place our results into the context of the field before stating our final conclusions.

Serum collection and confirmatory testing

Our findings suggest that the insulin resistance-promoting factor(s) in T2D serum may be most active, or only detectable, in the early stages of the disease, specifically in newly diagnosed or pre-T2D individuals, before the implementation of diet or pharmacological therapies. Recruiting from research registries of individuals managing T2D through lifestyle measures alone appears suboptimal for detecting this activity. Future studies should therefore prioritise serum collection from diabetic clinics, targeting individuals with HbA1c ≥48 mmol/mol, with samples obtained prior to the initiation of glucose-lowering medications. To account for potential sex differences, serum should ideally be collected from at least 20 males and 20 females. If access to diabetic clinics is limited, serum from recently diagnosed individuals with pre-T2D could be sourced through diabetes prevention programmes (again, collected before commencement of medication). Our preliminary data showing that serum from a small cohort of pre-T2D individuals induced insulin resistance in 3T3-L1 adipocytes supports the value of focusing on early-stage patients.

Cultured cell models remain the most appropriate and practical approach for assessing the effects of human T2D serum. In vivo administration of human serum to animals is not feasible due to the risk of severe immunological reactions. In addition to being more ethically acceptable, cell-based systems are significantly more cost-effective and logistically tractable than animal studies. Importantly, this approach has already been successfully used by Logie *et al.* (2010) and Renstrom *et al.* (2009) to demonstrate that serum

from people with T2D can induce insulin resistance. Future studies should therefore build upon or seek to replicate these findings using standardised cell-based bioassays.

If an effect of T2D serum is confirmed in future studies, the next critical step would be to identify the responsible factor(s) and elucidate their mechanism of action. Given the volume of serum likely required for fractionation and downstream analyses, a pooled serum approach would be practical. An initial strategy could involve testing pooled serum from male and female donors separately to determine whether sex differences influence the insulin resistance-promoting activity. Should pooled samples prove insufficiently sensitive, testing individual sera (as performed by Logie *et al*. (2010)) would allow detection of high-potency donors and preserve information on inter-individual variability. If activity is detected in only a subset of samples, those active sera could be pooled for fractionation studies. In cases where clinic-recruited T2D serum is unavailable, serum from recently diagnosed pre-T2D individuals could be used as an alternative, based on the preliminary activity observed in this project.

Whether or not an effect is observed in H4-II-E hepatocytes, pooled serum from male donors with T2D (or pre-T2D) should be tested in the 3T3-L1 adipocyte glucose uptake assay. If no effect is seen with serum from individuals with established T2D, this would suggest that the insulin resistance-promoting factor(s) may be primarily active in the pre-T2D stage. Conversely, if no effect is seen with pre-T2D serum, the preliminary finding from this study would require further validation or may not be reproducible. Should a clear effect be detected, the 3T3-L1 glucose uptake assay would be the preferred "workhorse" model for future studies aimed at identifying the responsible factor(s). This assay offers two major practical advantages: a short incubation period of only 24 hours (compared with three weeks for the *Pck1* assay), allowing higher experimental throughput, and the ability to

maintain cell health in the absence of serum, enabling the testing of small serum fractions without compromising viability.

Fractionation

If an effect of serum from PWPT2D is seen using the 3T3-L1 assay, then experiments to fractionate the serum could be conducted. Size exclusion columns can be used to separate bioactive molecules (Krishnamurthy and Belur, 2018; Zdaniewicz *et al.*, 2024). To this end, the serum could be filtered through such a column and pooled into fractions according to elution time. These fractions could then be tested 3T3-L1 assay to identify the fraction that maintains an insulin desensitising effect. The active fraction could then be sub fractionated by using a different column designed to separate biomolecules of smaller sizes. Alternatively, ion exchange columns which separate biomolecules based on charge could be used separately or in combination (Conze *et al.*, 2008) to isolate the smallest active fraction. The smallest active fraction could then be boiled to determine if it is a protein. If the effect on 3T3-L1s is lost after boiling, then that would indicate that this is the case. Mass spectrometry could be used to identify such a protein (Tardif *et al.*, 2022). To this end, the smallest active fraction of serum from PWT2D would be compared to an equivalent fraction of serum from healthy volunteers to identify the factor. If an active serum fraction is identified that takes longer to pass through the size exclusion column and cannot be separated further, this could suggest that the fraction contains a mixture of small metabolites. If that is the case, liquid chromatography-mass spectrometry could be used to identify the metabolite (Xiao, Zhou and Ressom, 2012) responsible for the effect on 3T3-L1s. Before identification by the means described above, the active fraction of serum could be tested in the *Pck1* assay to determine if the same fraction also affects H4-II-E cells. If this is not the case, then other fractions stored from previous steps could be tested by these means until the relevant active fraction is found and can be identified. Once a factor or factors have been identified, purified samples

could be obtained. These purified factors could be tested in both assays alongside serum samples where the active factor has been removed to establish causation.

Isolation of lipid fraction

Lipid dysregulation is a hallmark of T2D that often occurs many years preceding the diagnosis of overt disease (Adiels *et al.*, 2008; Miao *et al.*, 2021). There are 9 classes of lipid that make up 99% of the human serum lipidome (Pellegrino *et al.*, 2014). A large proportion these have been linked to the development of insulin resistance (Petersen *et al.*, 2024; Mir *et al.*, 2025), including the following 3 examples. First, Free fatty acids become elevated (Sobczak, Blindauer and Stewart, 2019) which cause insulin resistance in muscle (Roden *et al.*, 1996), liver, and adipose tissue via diacylglycerol intermediates which activate protein kinase c isoforms (Kolczynska *et al.*, 2020). Second, an increased triglyceride/high-density lipoprotein cholesterol ratio has shown to be a marker of insulin resistance as measured by insulin suppression test (McLaughlin *et al.*, 2005; James, Stockli and Birnbaum, 2018). Third, ceramides contained in low density lipoproteins are elevated in T2D which promote insulin resistance by stimulating dephosphorylation of AKT (Boon *et al.*, 2013) in muscle, liver, heart, and adipose tissue (Field, Gordillo and Scherer, 2020). In short, it is plausible that the insulin resistance inducing serum factor is a lipid. Therefore, this line of inquiry could be explored. A methanol/chloroform /methyl-tert-butyl ether extraction has been shown to recover close to 100% of the 9 classes of human serum lipids (Pellegrino *et al.*, 2014). This method could be used to extract the lipids from human serum which could then be complexed to BSA (Alsabeeh *et al.*, 2018) and tested in the 3T3-L1 assay. If an effect is seen, a liquid chromatography-mass spectrometry approach could be used to identify differences in lipid content between serum from PWT2D and control serum (Pellegrino *et al.*, 2014). Interestingly some circulating lipids promote insulin sensitivity, such as palmitoleic acid (Tricò

et al., 2020) and branched fatty acid esters of hydroxy fatty acids (Aryal *et al.*, 2021). Therefore, the insulin resistance promoting properties of serum from PWT2D could conceivably be caused by a lack of these or other such factors.

Isolation of exosomes

Exosomes are small (30-150 nm) endosomally derived phospholipid bilayer vesicles that are released by prokaryotic and eukaryotic cells (Chen *et al.*, 2024). Discovered in the late 1980s they were thought to contain cellular waste products with no function (Zhang *et al.*, 2019b). However, more recently, a paradigm shift has occurred, as research has demonstrated that exosomes are in fact important components of an inter-tissue communication system, capable of carrying biological signals from one tissue type to another (Isaac *et al.*, 2021). Exosomal cargo can consist of proteins, lipids, several types of RNA, including mRNA, microRNA, and long non-coding RNA, DNA, metabolites, and even oxidatively damaged mitochondria (Janas *et al.*, 2015; Crewe *et al.*, 2021; Lu *et al.*, 2025). These cargoes can significantly alter the behaviour of target cells, including by the promotion of insulin resistance in T2D (Wang *et al.*, 2023). In short, it is plausible that the insulin resistance inducing serum factor is exosome mediated. Therefore, this hypothesis might be tested. To this end, exosomes could be isolated from human serum by differential centrifugation, which is considered to be the gold standard method for this purpose (Gao *et al.*, 2023). To ensure reproducibility, quality control on the isolation procedure could be carried out by particle tracking analysis and western blotting for common markers, including Alix, Hsp70, and CD9, for example (McAndrews and Kalluri, 2019; Comfort *et al.*, 2021). Once isolated, exosomes could be tested in the 3T3-L1 assay against exosomes from healthy individuals, and alongside serum from PWT2D from which the exosomes have been depleted, to establish causation.

If an effect is seen, steps could be taken to elucidate what the causative exosome cargo is. Similar to above, if the active cargo is a protein, this could

be determined by boiling extracted exosomes before re-testing in the 3T3-L1 assay. If this is the case, the protein responsible might be isolated via the classical methods described above, or by proteomics using global or targeted approaches (Schey, Luther and Rose, 2015). If the active cargo is a lipid, this could be determined by extracting the lipids from the exosome fraction by the solvent method described above or by the Bligh and Dyer, (1959) method and then testing them in the 3T3-L1 assay. The responsible lipid might then be identified by shotgun lipidomics, or other mass spectrometry or nuclear magnetic resonance approaches (Donoso-Quezada, Ayala-Mar and González-Valdez, 2021). To determine if the active cargo is RNA, a kit can be used to isolate the exosome RNA which could then be tested by transfecting this RNA back into target cells (Ghafouri-Fard *et al.*, 2023). Alternatively, commercial sequencing could be used to determine if specific mRNA, microRNA, or long non-coding RNA are over or underrepresented compared to RNA isolated from control exosomes. This would provide profiles and relative abundances of RNA in the two populations. Specific RNAs that are abundant are likely to have a biological effect (Isaac *et al.*, 2021) and so might be identified by these means. Otherwise, a bioinformatical approach could be taken. For example, the expression profile of adipocyte miRNA could be downloaded from the Gene Expression Omnibus database. This could be compared against differentially expressed exosomal miRNAs as determined from sequencing. Targets for these miRNAs could be determined by bioinformatical algorithms such as miRanda, miRDB, miRWalk, and Targetscan. Then, Cytoscape software could be used to provide functional and pathway analyses, including gene ontology analysis, and Kyoto Encyclopaedia of Genomes pathway enrichment (Zhao *et al.*, 2018). Ultimately, then, these or other such methods could be used to identify specific serum RNAs which promote insulin resistance, the genes that are involved in this, and the biological processes, cellular locations, and molecular functions that are altered.

What pathways and cell types could serum from PWT2D affect?

Increased fasting gluconeogenesis has been measured in PWT2D (Magnusson *et al.*, 1992). Yet, there is evidence suggesting that such increased fasting gluconeogenesis is not due to increased transcriptional expression of *Pck1* (Samuel *et al.*, 2009). If a serum from PWT2D mediated effect is detected with the *Pck1* assay, it is important to determine if this effect resulted in an increase in gluconeogenesis. This might be accomplished with a Promega Glucose-Glo assay kit, for example. Other commercially available kits could be used to determine the effects of serum from PWT2D on glycerol release and lipolysis rates in 3T3-L1s. This might show that serum from PWT2D affects more than one pathway in 3T3-L1s. Muscle, liver, and fat are sometimes referred to as the big three, in terms of the development of insulin resistance in T2D (Schmidt and Hickey, 2009). If effects are seen in liver and adipocytes, it would be good to potentially complete this trifecta. This might be done by conducting glucose uptake assays in C2C12 myotubes, treated with serum from PWT2D, for example (Yun *et al.*, 2009). Human primary hepatocytes can be cultured for at least 24 days (Klaas *et al.*, 2021). Therefore, the effects of serum from PWT2D could be determined by using our assays in primary hepatocytes (and primary adipocytes (Renstrom *et al.*, 2009)) to provide stronger evidence that these findings are relevant to human disease.

RNA sequencing

If effects are seen in primary human cells after treatment with serum from PWT2D, then RNA sequencing could be used to discern what changes in gene expression have occurred to underpin these effects (Li *et al.*, 2021). Such sequencing could be carried out after insulin treatments to assess the transcriptional remodelling by insulin in these models of insulin resistance compared to controls (Batista *et al.*, 2019). A study using similar means uncovered changes in gene networks related to mitochondrial function,

electron transport chain, TCA cycle activity, transcriptional regulation, and transport of organic anions and cationic amino acids, for example (Batista *et al.*, 2019). Another such study developed an integrative roadmap for identifying lncRNAs in primary hepatocytes under pathophysiological conditions (Yang *et al.*, 2016). This roadmap could be followed to identify potential lncRNAs upregulated by treatments with serum from PWT2D. Finally, a related study illustrates how RNA sequencing can be used to identify miRNAs upregulated in insulin resistant liver (Zhao *et al.*, 2019). Again, these methods could be followed to identify potential miRNAs upregulated by treatments with serum from PWT2D. Any genes, miRNAs, or lncRNAs found to be upregulated could be validated with quantitative real-time PCR (Zhao *et al.*, 2019). In addition, the effects of any upregulated RNA species could be confirmed via RNAi knockdown (Batista *et al.*, 2019). Finally, the same or similar bioinformatical approaches to those described in the exosome RNA section could be utilised to identify the specific biological pathways that have been altered. Changes in the expression of genes controlling expected pathways such as glucose and lipid utilisation, or other less known pathways such as chromatin remodelling might be discovered by these methods (Batista *et al.*, 2019).

Summary

The work outlined above could determine whether serum from PWT2D promotes insulin resistance in cultured cells, and if that is the case, it could generate new insights into the mechanisms involved in the development insulin resistance by identifying the causative factor and its mode of action. Longer term, this could enable the development of new treatments and perhaps lead to the development of new tools to stratify PWT2D (and those at risk of developing T2D) to enable more precisely tailored treatments.

Placing our results into the context of the field

One of the long-term aims of this project was to identify the circulating factor(s) in the blood of people with type 2 diabetes (T2D) and to elucidate their mode of action, with the ultimate goal of developing them as biomarkers for patient stratification, early risk identification, or as targets for new or precision therapies. Despite the initial promise of Logie et al. (2010), this approach has not yet yielded a robust and reproducible circulating mediator suitable for clinical translation. The present findings, which showed no detectable insulin resistance-inducing activity in serum from individuals with established, lifestyle-managed T2D, suggest that the potency of such humoral factors is highly dependent on disease stage. Consequently, at the current time, serum from patients with longer-standing T2D does not appear to offer a reliable route for biomarker discovery or patient stratification using the assays employed in this study. The following section discusses how others in the field are attempting to achieve similar goals through alternative biomarker strategies and evaluates their relative success, before considering opportunities to optimise currently available treatments for T2D.

The Generation Scotland biobank contains genome-wide whole genome and DNA methylation (DNAm) data linked to the electronic health records of 14,613 PWT2D and 626 people who developed T2D during the 15-year follow up period since the biobank's inception (Smith *et al.*, 2013; Cheng *et al.*, 2023). Cheng *et al.* (2023) used this resource to develop and validate an epigenetic score system to predict incidence of T2D within 10 years. They found that the best model using traditional risk factors (age, sex, BMI, hypertension and family history) produced an Area Under the Curve (AUC) value of 0.839 compared to 0.872 when DNAm was also considered. Thus, this technique appears to provide a significant but relatively modest improvement in the prediction of 10-year T2D incidence risk. However, implementing the sequencing methods required for this and other

sequencing-based approaches to enable risk prediction and stratification are currently hampered by technical complexities, high cost, and a lack of standardised clinical practice guidelines (Haghshenas *et al.*, 2020). In addition, the ability of multiple genetic loci to accurately predict onset of T2D has been described as modest, at best (Lugner *et al.*, 2024). Taken together, this suggests there are several obstacles to be overcome before this or other such other sequencing-based approaches could be adopted by the clinic.

Bragg *et al.* (2022) used nuclear magnetic resonance (NMR) metabolomic profiling to analyse the plasma samples of 50,519 UK Biobank participants, 1211 of whom developed T2D during the 11-year follow up period. They found that adding the data of 143 individual circulating metabolic biomarkers (including lipids, lipoproteins, fatty acids, amino acids, ketone bodies and other low molecular weight metabolic biomarkers) modestly improved the established model of T2D risk prediction (based on traditional risk factors) as assessed by the c-statistic from 0.802 to 0.830. Even more recently, and along similar lines, Xie *et al.* (2025) used NMR profiling to analyse the plasma samples of 86,232 UK Biobank participants and 4383 participants from a German biobank, 3537, and 495 of whom (respectively) developed T2D during the 10-year follow up period. They analysed 249 circulating metabolites, eventually incorporating 11 metabolomic biomarkers (including glycolysis related metabolites, ketone bodies, amino acids, and lipids) into a model to predict 10-year risk of T2D. This increased the c-statistic of the Cambridge Diabetes Risk Score (a model of T2D risk prediction, based on traditional risk factors) from 0.815 to 0.834. Another significant (but modest) improvement. Taken together, these studies indicate that metabolomics approaches could be used to achieve improvements in T2D risk prediction. However, clinical challenges such as increased costs, the need for specialised equipment, complex data analysis, biological variability, and standardisation of methods to ensure reproducibility must be overcome for this to take place (Vo and Trinh, 2024).

A potentially cheaper route to improving the prediction of T2D risk that uses equipment already incorporated into clinical practice is the Artificial Intelligence (AI) electrocardiogram Risk Estimation for Diabetes Mellitus (AIRE-DM) (Pastika *et al.*, 2024). AIRE-DM uses AI software to predict T2D risk based on the ECG readings from routine heart scans. The system was trained on 1,163,401 ECG readings from Beth Israel Deaconess Medical Center and validated using UK Biobank data from 65,606 individuals. It has been shown to identify prevalent T2D with an AUC of 0.712 and predict incipient T2D up to 13 years in advance with a c-statistic of 0.666 (Pastika *et al.*, 2024). Importantly, AIRE-DM can be used to improve the accuracy of T2D risk prediction based on traditional risk factors and is scheduled to be trialled in two London Hospitals this year (2025). If AIRE-DM is successful in these trials, it could be rapidly rolled out across the NHS to identify people at risk of developing T2D so that preventative measures could be offered to them. Furthermore, AIRE-DM demonstrates the potential for AI-based tools to be applied to large datasets to generate new models of disease prediction. Consequently, steps are being taken to apply similar machine-learning approaches to large T2D multi-omic data sets to this end (Carrasco-Zanini *et al.*, 2024; Rönn *et al.*, 2024).

Continuing the topic of machine-learning, such an approach was recently used to identify the top ten predictors of the development of T2D from UK biobank data (Lugner *et al.*, 2024). When data from 448,277 participants, 12,148 of whom developed T2D within 10 years, was analysed in this way, HbA1c emerged as the foremost predictor, followed by BMI, waist circumference, blood glucose, family history of diabetes, gamma-glutamyl transferase, waist-hip ratio, HDL cholesterol, age, and urate. Impressively, the group's XGboost classification model (which incorporated the 10 variables mentioned) to predict 10-year risk of T2D had an AUC of 0.88. Because existing biomarkers like these can be used to accurately predict who might develop T2D, it is tempting to think that we do not need to develop new biomarkers. However, these traditional markers have weaknesses. For

example, HbA1c and blood glucose primarily identify hyperglycaemia after it develops, missing the preceding period where beta cell dysfunction and peripheral insulin resistance are developing (Chen *et al.*, 2017). These defects are detectable by hyperinsulinemic-euglycemic clamp, but this is a complex and costly technique, generally not suitable for clinical practice. In addition, T2D is a heterogeneous disease that can lead to distinct complications like cardiomyopathy and retinopathy. Traditional markers have been shown to have limited sensitivity for predicting such outcomes (Ahmed *et al.*, 2025). Therefore, it is hoped that the detection and validation of new biomarkers will enable better subgroup classifications, disease prediction, and complication forecasting. As described above, there is potential for several novel biomarkers to be used (perhaps in combination) to improve the power of traditional ones once several key obstacles can be overcome.

Another route to developing precision medical approaches is to use the currently available drugs in a more optimised way. To this end, Dennis *et al.* (2025) developed and validated a model to predict the glycaemic effectiveness (12-month HbA1c) of 5 classes of drugs (dipeptidyl peptidase-4 inhibitors, glucagon-like peptide-1 receptor agonists (GLP-1 RAs), sodium-glucose co-transporter-2 (SGLT2) inhibitors, sulfonylureas, and thiazolidinediones after initial treatment with metformin. The model used nine traditional biomarkers (age, duration of diabetes, sex, and baseline HbA1c, BMI, estimated glomerular filtration rate, HDL cholesterol, total cholesterol, and alanine aminotransferase) as predictive factors. It was created using observational data from over 100,000 patients with T2D in England and validated using clinical trial data. Importantly, individuals that were on the model-predicted model therapy were less likely to need additional glucose-lowering therapies and less likely to develop complications. In addition, and somewhat disappointingly, the model predicted that since 2019 only 18% of patients with T2D in England were given the optimum drug for glycaemic effectiveness. Thus, implementing this

model could quickly result in beneficial outcomes. To this end, according to Diabetes UK, Dennis *et al.* (2025)'s model is currently being tested in clinical practices in Scotland with 22,500 PWT2D. Results from this will guide its potential roll out to the rest of the UK and globally.

Dennis et al. (2025) developed a model to predict the most effective second-line drug after metformin, which remains the most common first-line pharmacological therapy for T2D in the UK following lifestyle intervention. However, metformin itself may not be the optimal initial choice for all patients. For example, glucagon-like peptide-1 receptor agonists (GLP-1 RAs) address six of the eight core pathophysiological defects in T2D — including impaired insulin secretion, increased hepatic glucose production, reduced muscle glucose uptake, hyperglucagonaemia, incretin deficiency, and central neurotransmitter dysfunction — whereas metformin primarily suppresses hepatic glucose production (Abdul-Ghani *et al.*, 2025). Clinical trial data also indicate that GLP-1 RAs provide superior cardiovascular risk reduction compared with metformin. Furthermore, emerging evidence suggests that initial triple therapy can achieve better glycaemic control, slower progression of atherosclerosis, and greater reductions in hepatic fat content and fibrosis than conventional sequential add-on therapy starting with metformin (Abdul-Ghani *et al.*, 2025).

Importantly, GLP-1 RAs are most effective when used in combination with structured lifestyle intervention. Major guidelines (ADA/EASD, WHO, and The Obesity Society) now recommend that GLP-1-based therapies should be prescribed alongside intensive nutrition therapy, physical activity (including resistance training), and behavioural support. This combined approach not only enhances weight loss and glycaemic control but also helps preserve lean muscle mass, improves long-term adherence, and supports sustained benefits even after medication discontinuation (Lundgren *et al.*, 2021). With these considerations in mind, refining or extending Dennis et al.'s (2025) predictive model to better personalise first-line treatment (whether as

monotherapy or early combination therapy, alongside an optimised lifestyle intervention) could enable a larger proportion of people with T2D who are unable to achieve remission through lifestyle measures alone to receive the most effective therapy earlier in the disease course.

Summary

Several novel biomarkers are currently being developed and validated to improve risk prediction of developing T2D and to better stratify those who already have the disease for more effective treatments. In addition, steps are underway to better personalise currently available therapies to better treat PWT2D.

Final Conclusions

This project set out to replicate the key finding of Logie *et al*. (2010) (that serum from PWT2D can directly induce insulin resistance in cultured cells) as a platform for identifying the responsible circulating factor(s). Although we successfully validated sensitive functional assays in both hepatoma and adipocyte models, pooled serum from individuals with longer-standing, lifestyle managed T2D did not induce detectable insulin resistance. In contrast, preliminary experiments using serum from a small cohort of people with pre-T2D showed a selective impairment in insulin-stimulate glucose uptake in adipocytes, without corresponding changes in proximal signalling (AKT or AS160 phosphorylation). These results suggest that the insulin resistance-promoting activity of serum from PWT2D may be transient and most evident in the early stages of disease, prior to long-term management.

In a parallel investigation, we characterised age-related changes in 3T3-L1 adipocytes. As the cells aged, they adopted a more white adipose tissue-like morphology (fewer but larger lipid droplets), accompanied by reductions in several proteins involved in insulin signalling and GLUT4 trafficking. However, maximal insulin-stimulated glucose uptake remained unchanged,

indicating a degree of functional resilience despite morphological and proteomic remodelling.

Taken together, this work highlights both the promise and the challenges of using human serum to study the humoral mediators of insulin resistance. It highlights the importance of donor disease stage and careful methodological standardisation. While we did not replicate the original Logie *et al*. (2010) finding with our cohorts, the preliminary effect observed with serum from people with pre-T2D, combined with the robust assay platforms developed, provides a foundation for future, more targeted investigations. Identifying the specific circulating factor(s) responsible (whether proteins, lipids, RNAs or exosome-mediated) could ultimately yield new biomarkers for early detection or stratification of T2D and open avenues for novel therapeutic strategies aimed at interupting the propagation of insulin resistance.

Appendix

IRAS form

IRAS Form Reference: IRAS Version 5.21

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please complete the questions in order. If you change the response to a question, please select 'Save' and review all the questions as your change may have affected subsequent questions.

Please enter a short title for this project (maximum 70 characters)
Diabetes BFX Sampling study

1. Is your project research?

(•) Yes ( ) No

2. Select one category from the list below:

( ) Clinical trial of an investigational medicinal product
( ) Clinical investigation or other study of a medical device
( ) Combined trial of an investigational medicinal product and an investigational medical device
( ) Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice
(•) Basic science study involving procedures with human participants
( ) Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
( ) Study involving qualitative methods only
( ) Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)
( ) Study limited to working with data (specific project only)
( ) Research tissue bank
( ) Research database

If your work does not fit any of these categories, select the option below:

( ) Other study

2a. Will the study involve the use of any medical device without a UKCA/CE UKNI/CE Mark, or a UKCA/CE UKNI/CE marked device which has been modified or will be used outside its intended purposes?

( ) Yes (•) No

2b. Please answer the following question(s):

a) Does the study involve the use of any ionising radiation?	( ) Yes	(•) No
b) Will you be taking new human tissue samples (or other human biological samples)?	(•) Yes	( ) No
c) Will you be using existing human tissue samples (or other human biological samples)?	( ) Yes	(•) No

d) Will the study involve any other clinical procedures with participants (e.g. MRI, ultrasound, physical examination)? ○ Yes ◉ No

3. In which countries of the UK will the research sites be located? *(Tick all that apply)*

- ☐ England
- ☑ Scotland
- ☐ Wales
- ☐ Northern Ireland

3a. In which country of the UK will the lead NHS R&D office be located:

- ○ England
- ◉ Scotland
- ○ Wales
- ○ Northern Ireland
- ○ This study does not involve the NHS

4. Which applications do you require?

- ☑ IRAS Form
- ☐ Confidentiality Advisory Group (CAG)
- ☐ Her Majesty's Prison and Probation Service (HMPPS)

Most research projects require review by a REC within the UK Health Departments' Research Ethics Service. Is your study exempt from REC review?

○ Yes ◉ No

5. Will any research sites in this study be NHS organisations?

◉ Yes ○ No

6. Do you plan to include any participants who are children?

○ Yes ◉ No

7. Do you plan at any stage of the project to undertake intrusive research involving adults lacking capacity to consent for themselves?

○ Yes ◉ No

Answer Yes if you plan to recruit living participants aged 16 or over who lack capacity, or to retain them in the study following loss of capacity. Intrusive research means any research with the living requiring consent in law. This includes use of identifiable tissue samples or personal information, except where application is being made to the Confidentiality Advisory Group to set aside the common law duty of confidentiality in England and Wales. Please consult the guidance notes for further information on the legal frameworks for research involving adults lacking capacity in the UK.

8. Do you plan to include any participants who are prisoners or young offenders in the custody of HM Prison Service or who are offenders supervised by the probation service in England or Wales?

○ Yes ◉ No

9. Is the study or any part of it being undertaken as an educational project?

◉ Yes ○ No

Please describe briefly the involvement of the student(s):
Laboratory research on blood samples generated by the clinical research.

9a. Is the project being undertaken in part fulfilment of a PhD or other doctorate?

◉ Yes ○ No

10. Will this research be financially supported by the United States Department of Health and Human Services or any of its divisions, agencies or programs?

○ Yes ◉ No

Integrated Research Application System
Application Form for Basic science study involving procedures with human participants

IRAS Form (project information)

Please refer to the E-Submission and Checklist tabs for instructions on submitting this application.

The Chief Investigator should complete this form. Guidance on the questions is available wherever you see this symbol displayed. We recommend reading the guidance first. The complete guidance and a glossary are available by selecting Help.

Please define any terms or acronyms that might not be familar to lay reviewers of the application.

Short title and version number: (maximum 70 characters - this will be inserted as header on all forms)
Diabetes BFX Sampling study

Please complete these details after you have booked the REC application for review.

REC Name:

REC Reference Number: **Submission date:**

PART A: Core study information

1. ADMINISTRATIVE DETAILS

A1. Full title of the research:

Diabetes Blood Factor X Sampling Study

A2-1. Educational projects

Name and contact details of student(s):

Student 1

Title	Forename/Initials	Surname
Mr	Roderick	Milligan

Address	C/O Prof Sutherland, Department of Cellular Medicine
	Ninewells Hospital and Medical School
	Dundee
Post Code	DD1 9SY
E-mail	
Telephone	
Fax	

Give details of the educational course or degree for which this research is being undertaken:

Name and level of course/ degree:
PhD

Name of educational establishment:
University of Strathclyde

Name and contact details of academic supervisor(s):

Academic supervisor 1

	Title	Forename/Initials	Surname
	Prof	Calum	Sutherland
Address	Department of Cellular Medicine		
	Ninewells Hospital and Medical School		
	Dundee		
Post Code	DD1 9SY		
E-mail	c.d.sutherland@dundee.ac.uk		
Telephone	01382383098		
Fax			

Academic supervisor 2

	Title	Forename/Initials	Surname
	Prof	Gwyn	Gould
Address	Strathclyde Institute of Pharmacy and Biomedical Sciences		
	University of Strathclyde		
	161 Cathedral Street, Glasgow		
Post Code	G4 0RE		
E-mail	gwyn.gould@strath.ac.uk		
Telephone	01415484805		
Fax			

Please state which academic supervisor(s) has responsibility for which student(s):
Please click "Save now" before completing this table. This will ensure that all of the student and academic supervisor details are shown correctly.

Student(s)	Academic supervisor(s)
Student 1 Mr Roderick Milligan	☑ Prof Calum Sutherland ☑ Prof Gwyn Gould

A copy of a current CV for the student and the academic supervisor (maximum 2 pages of A4) must be submitted with the application.

A2-2. Who will act as Chief Investigator for this study?

- ○ Student
- ◉ Academic supervisor
- ○ Other

A3-1. Chief Investigator:

	Title	Forename/Initials	Surname
	Prof	Calum	Sutherland
Post	Professor		
Qualifications	PhD		
ORCID ID	0000 0003 4398 7434		
Employer	University of Dundee		
Work Address	Department of Cellular Medicine		
	Ninewells Hospital and Medical School		
	Dundee		
Post Code	DD1 9SY		
Work E-mail	c.d.sutherland@dundee.ac.uk		
* Personal E-mail			
Work Telephone	01382383098		
* Personal Telephone/Mobile			
Fax			

** This information is optional. It will not be placed in the public domain or disclosed to any other third party without prior consent.*
A copy of a current CV (maximum 2 pages of A4) for the Chief Investigator must be submitted with the application.

A4. Who is the contact on behalf of the sponsor for all correspondence relating to applications for this project?
This contact will receive copies of all correspondence from REC and HRA/R&D reviewers that is sent to the CI.

	Title	Forename/Initials	Surname
	Dr	Patricia	Burns
Address	TASC, R&D Department, Ninewells Hospital		
	Dundee		
Post Code	DD1 9SY		
E-mail	TASCgovernance@dundee.ac.uk		
Telephone	01382383297		
Fax			

A5-1. Research reference numbers. *Please give any relevant references for your study:*

Applicant's/organisation's own reference number, e.g. R & D (if available):	
Sponsor's/protocol number:	2.013.21
Protocol Version:	1.0
Protocol Date:	24/09/2021
Funder's reference number (enter the reference number or state not applicable):	20/0006218
Project website:	

Registry reference number(s):
The UK Policy Framework for Health and Social Care Research sets out the principle of making information about research publicly available. Furthermore: Article 19 of the World Medical Association Declaration of Helsinki adopted in 2008 states that "every clinical trial must be registered on a publicly accessible database before recruitment of the first subject"; and the International Committee of Medical Journal Editors (ICMJE) will consider a clinical trial for publication only if it has been registered in an appropriate registry. Please see guidance for more information.

International Standard Randomised Controlled Trial Number (ISRCTN):

ClinicalTrials.gov Identifier (NCT number):

Additional reference number(s):

Ref.Number Description	Reference Number

A5-2. Is this application linked to a previous study or another current application?

○ Yes ◉ No

Please give brief details and reference numbers.
This study follows on from previous work but is not linked or part of a programme.

2. OVERVIEW OF THE RESEARCH

To provide all the information required by review bodies and research information systems, we ask a number of specific questions. This section invites you to give an overview using language comprehensible to lay reviewers and members of the public. Please read the guidance notes for advice on this section.

A6-1. Summary of the study. *Please provide a brief summary of the research (maximum 300 words) using language easily understood by lay reviewers and members of the public. Where the research is reviewed by a REC within the UK Health Departments' Research Ethics Service, this summary will be published on the Health Research Authority (HRA) website following the ethical review. Please refer to the question specific guidance for this question.*

An early feature of Type 2 Diabetes (T2D) is a progressive reduction in whole-body insulin sensitivity (insulin resistance). This appears even before the development of T2D, which occurs when insulin secretion no longer overcomes the underlying insulin resistance, resulting in hyperglycaemia. There are many potential molecular mechanisms leading to whole body insulin resistance.
This study is designed to collect blood samples from appropriate groups of volunteers. These samples will permit lab based (funded) experimental studies to investigate the molecular basis of serum-mediated generation of insulin resistance via the following strategies:
• What is the nature of the blood borne factor(s)?
• Is the observed insulin resistance tissue-specific?
• Does the insulin resistance generation in culture correlate with any clinical features of the individual donors?

A6-2. Summary of main issues. *Please summarise the main ethical, legal, or management issues arising from your study and say how you have addressed them.*

Not all studies raise significant issues. Some studies may have straightforward ethical or other issues that can be identified and managed routinely. Others may present significant issues requiring further consideration by a REC, HRA, or other review body (as appropriate to the issue). Studies that present a minimal risk to participants may raise complex organisational or legal issues. You should try to consider all the types of issues that the different reviewers may need to consider.

This is a research study to obtain blood samples for further laboratory research. The use of the samples and the destinations of the samples are clearly explained. Two collaborating research labs in Scotland are working on the project, with the PhD student travelling between the two laboratories to make use of the specialist equipment and expertise present in each location.
Aside from sample and data use there are no ethical issues since this is a non-interventional study.

3. PURPOSE AND DESIGN OF THE RESEARCH

A7. Select the appropriate methodology description for this research. *Please tick all that apply*:

☐ Case series/ case note review
☑ Case control
☐ Cohort observation

- ☐ Controlled trial without randomisation
- ☐ Cross-sectional study
- ☐ Database analysis
- ☐ Epidemiology
- ☐ Feasibility/ pilot study
- ☑ Laboratory study
- ☐ Metanalysis
- ☐ Qualitative research
- ☐ Questionnaire, interview or observation study
- ☐ Randomised controlled trial
- ☐ Other (please specify)

A10. What is the principal research question/objective? *Please put this in language comprehensible to a lay person.*

1) To improve our understanding of the identity of the blood borne factor that causes insulin resistance in patients with T2D and Pre-diabetes (visit 1 and 2 samples).

2) To generate appropriate assays for adipose tissue and skeletal muscle and then establish whether the serum from T2D volunteers causes insulin resistance in a tissue-selective manner (visit 1 samples).

3) To investigate whether the resistance generation property of the blood associates with any clinical feature of T2D or pre-diabetes (visit 1 and 2 samples).

4) To investigate whether lifestyle intervention in the Tayside diabetes prevention programme significantly alters the resistance generation property of the donated blood (visit 1 and 2 samples).

A11. What are the secondary research questions/objectives if applicable? *Please put this in language comprehensible to a lay person.*

To investigate the reproducibility of the cell based assay systems by comparing samples from two visits of the individual T2D volunteers and controls (visit 1 and 2 samples).

A12. What is the scientific justification for the research? *Please put this in language comprehensible to a lay person.*

This study follows on from a pilot study which looked at the effects of serum from patients with diabetes on cell cultures and showed that an insulin resistance factor is present in the blood.

This study is designed to collect blood samples from appropriate groups of volunteers. These samples will permit lab based (funded) experimental studies to investigate the molecular basis of our previously observed serum-mediated generation of insulin resistance via the following strategies:
• What is the nature of the blood borne factor(s)?
• Is the observed insulin resistance (Fig.1) tissue-specific?
• Does the insulin resistance generation in culture correlate with any clinical features of the individual donors?

A13. Please summarise your design and methodology. *It should be clear exactly what will happen to the research participant, how many times and in what order. Please complete this section in language comprehensible to the lay person. Do not simply reproduce or refer to the protocol. Further guidance is available in the guidance notes.*

This non-interventional study will recruit 3 cohorts of people - healthy volunteers, those with pre-diabetes and those with established type 2 diabetes. The participants will provide blood for laboratory analysis on 2 occasions approximately 10 months apart. Comparisons of sample activity in cell based assays will be made between timepoints and between cohorts.

A14-1. In which aspects of the research process have you actively involved, or will you involve, patients, service users, and/or their carers, or members of the public?

- [x] Design of the research
- [ ] Management of the research
- [x] Undertaking the research
- [ ] Analysis of results
- [ ] Dissemination of findings
- [ ] None of the above

Give details of involvement, or if none please justify the absence of involvement.
As part of the funding application preparation which took place in June 2020, a Diabetes PPI group operated by the NHS Research Scotland Diabetes Network reviewed the funding application and commented on the study.

One patient wrote:
How easy is it to understand?
1)All terminology and language is easy to understand and straightforward. The aims and purpose of the study is clear.

Specific comments on the "do's and don'ts" for asking people with type 2 diabetes to donate fasted blood:
2) I think anyone with type 2 will have had a fasting blood test and be aware of the scenario. The offer of a drink and snack will offset any issue with the possibility of feeling lightheaded. The only issue I can see is with people getting to the CRC at that time in the morning having not had breakfast, will they be provided with transport? Will they have had their meds and there be a possibility of hypo?

General thoughts on the importance of doing prevention/early detection work, and developing a test that could help us decide on best treatment (including prevention) option for people who may have different causes of type 2 diabetes. Although this is theoretical at the moment we believe that the reason people experience their diabetes very differently is due to having different reasons for developing type 2 diabetes. We want to prove this by developing 'biomarkers'. These could then help us divide type 2 diabetes by specific causes, allowing us to target the cause with treatment rather than treating everyone the same.
3) This looks like a really exciting, interesting study. It has the potential to find many people who don't know they have type 2, get them early treatment which will reduce complications.

4. RISKS AND ETHICAL ISSUES

RESEARCH PARTICIPANTS

A15. What is the sample group or cohort to be studied in this research?

Select all that apply:

- [ ] Blood
- [ ] Cancer
- [ ] Cardiovascular
- [ ] Congenital Disorders
- [ ] Dementias and Neurodegenerative Diseases
- [x] Diabetes
- [ ] Ear
- [ ] Eye
- [x] Generic Health Relevance
- [ ] Infection
- [ ] Inflammatory and Immune System
- [ ] Injuries and Accidents
- [ ] Mental Health

- ☐ Metabolic and Endocrine
- ☐ Musculoskeletal
- ☐ Neurological
- ☐ Oral and Gastrointestinal
- ☐ Paediatrics
- ☐ Renal and Urogenital
- ☐ Reproductive Health and Childbirth
- ☐ Respiratory
- ☐ Skin
- ☐ Stroke

Gender:	Male and female participants
Lower age limit: 18	Years
Upper age limit:	Years

A17-1. Please list the principal inclusion criteria (list the most important, max 5000 characters).

• Person with Type 2 Diabetes Diet controlled (not currently using diabetes medications and have not used diabetes medications for the past 3 months)
• Aged 18 or over
• Able to read and write in English

Cases (Pre-Diabetes):
• Person with Pre-Diabetes (HbA1c between 42-49 mmol/mol) being enrolled onto the Tayside Diabetes Prevention Programme
• Aged 18 or over
• Able to read and write in English

Controls:
• Person without diabetes or Pre-diabetes (<42 mmol/mol HbA1c)
• Age and sex matched to cases
• Able to read and write in English

A17-2. Please list the principal exclusion criteria (list the most important, max 5000 characters).

• Participating in the clinical phase of another interventional study or have done so within the last 30 days.

RESEARCH PROCEDURES, RISKS AND BENEFITS

A18. Give details of all non-clinical intervention(s) or procedure(s) that will be received by participants as part of the research protocol. *These include seeking consent, interviews, non-clinical observations and use of questionnaires.*

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days)
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4

Informed consent	1	0	30 mins	A member of the research team in the clinical research area
Demographic information collected	1	0	10 mins	A member of the research team in the clinical research area

A19. Give details of any clinical intervention(s) or procedure(s) to be received by participants as part of the research protocol. *These include uses of medicinal products or devices, other medical treatments or assessments, mental health interventions, imaging investigations and taking samples of human biological material. Include procedures which might be received as routine clinical care outside of the research.*

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days).
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4
Height measurement	2	0	5 mins	A member of the research team in the clinical research area
Weight measurement	2	0	5 mins	A member of the research team in the clinical research area
Bioimpedence scales	2	0	5 mins	A member of the research team in the clinical research area
Blood sampling collection for research	2	0	15 mins	A member of the research team in the clinical research area
Cases: HbA1c if required for routine case	2	2	2 mins	A member of the research team in the clinical research area
Controls: HbA1c to confirm no diabetes	2	0	2 mins	A member of the research team in the clinical research area

A21. How long do you expect each participant to be in the study in total?

Each participant will attend on 2 occasions for about 50 minutes/visit.
There are 2 visits spaced about 10 months apart. There is flexibility in the time window to support participant convenience.

A22. What are the potential risks and burdens for research participants and how will you minimise them?

For all studies, describe any potential adverse effects, pain, discomfort, distress, intrusion, inconvenience or changes to lifestyle. Only describe risks or burdens that could occur as a result of participation in the research. Say what steps would be taken to minimise risks and burdens as far as possible.

Participants may experience some risks from the blood sampling procedure - these include bruising, infection at the needle site, inflammation of the area around the needle entry, and in rare instances individuals can experience loss of consciousness. This is unlikely however, as patients with diabetes deal with finger prick sampling on a routine basis and generally cope well with needles. To support the participant, a research staff member will stop the procedure if the participant faints or experiences distress.

Inconvenience will be minimised by arranging study visit appointments at a date and time convenient to the participant.

Risks associated with covid will be minimised by use of appropriate PPE for research staff and participants and by operating local procedures for covid checks the day in advance of a planned study visit. We anticipate that the majority of participants will have received a covid vaccination before this study commences.

A24. What is the potential for benefit to research participants?

There is no likelihood of benefit to the research participants.

For those attending the study visit, if routine care clinical tests for HbA1c have not been done in primary care these will be done as part of the study. This could help to identify patients who need to see their GP for diabetes care and these patients will then be advised to see their GP.

RECRUITMENT AND INFORMED CONSENT

In this section we ask you to describe the recruitment procedures for the study. Please give separate details for different study groups where appropriate.

A27-1. How will potential participants, records or samples be identified? Who will carry this out and what resources will be used?*For example, identification may involve a disease register, computerised search of GP records, or review of medical records. Indicate whether this will be done by the direct healthcare team or by researchers acting under arrangements with the responsible care organisation(s).*

Participants will be recruited
a) From the NHS Research Scotland (NRS) Diabetes Research Network permission to contact database of people who have expressed a willingness to be involved in research. This register has a large group of people with diabetes (and some with pre-diabetes/without frank diabetes) who have given assent to be approached directly via telephone, email or letter using templates approved under the approval for that project.
b) Patients will be approached by their care team in diabetes clinics or for pre-diabetes cohorts they will be approached by their care team in weight management services/diabetes prevention programme.
c) Pre-diabetes patients within the diabetes prevention programme may be approached in writing using a "clinic letter".
d) Targeted advertising eg. via posters placed in diabetes clinics and eye screening clinics (for diabetes patients) and in workplace corridors and on notice boards (for controls).
e) Social media posts will also be used to engage individuals in the local community.

A27-2. Will the identification of potential participants involve reviewing or screening the identifiable personal information of patients, service users or any other person?

(●) Yes ( ) No

Please give details below:
Patients who are part of the Diabetes Research Register system have already given permission to be approached for research. The screening process will use the flexible query system of SCI Diabetes to identify patients who are part of the research register system and who meet the age criteria for the study. This process will follow the SOPs in place for the Diabetes Research Register project (IRAS 246515) using templates approved within that project.

A27-3. Describe what measures will be taken to ensure there is no breach of any duty of confidentiality owed to patients, service users or any other person in the process of identifying potential participants.*Indicate what steps have been or will be taken to inform patients and service users of the potential use of their records for this purpose. Describe the arrangements to ensure that the wishes of patients and service users regarding access to their records are respected. Please consult the guidance notes on this topic.*

Participants in the Diabetes Research Register system have already given permission by email or completed a sign up leaflet to be approached for research projects for which they are suitable/eligible.
The clinical research team will maintain all contact information within NHS systems.

A27-4. Will researchers or individuals other than the direct care team have access to identifiable personal information of any potential participants?

(●) Yes ( ) No

A27-5. Has prior consent been obtained or will it be obtained for access to identifiable personal information?

◉ Yes ○ No

If Yes, please give details below.

Potential participants may already be part of the diabetes research register and have given permission to be contacted.
The participants who will visit the clinical research area for the study will provide written consent for identifiable information to be kept as explained in PIS.

A28. Will any participants be recruited by publicity through posters, leaflets, adverts or websites?

◉ Yes ○ No

If Yes, please give details of how and where publicity will be conducted, and enclose copy of all advertising material (with version numbers and dates).

A poster will be used in secondary care diabetes clinics and eye screening areas.
Posters to attract control participants will be used in workplace corridors and notice boards. Social media posts will also be used to provide initial information about the study.

A29. How and by whom will potential participants first be approached?

Potential participants will be approached by Research Network staff in line with the SOPs used for the Diabetes Research Register project.
Potential participants may also be approached in clinical areas by a member of the care team.

Once an individual expresses an interest in the study, the clinical research team will follow up to ensure a PIS (control or case as applicable) is provided.

A30-1. Will you obtain informed consent from or on behalf of research participants?

◉ Yes ○ No

If you will be obtaining consent from adult participants, please give details of who will take consent and how it will be done, with details of any steps to provide information (a written information sheet, videos, or interactive material). Arrangements for adults unable to consent for themselves should be described separately in Part B Section 6, and for children in Part B Section 7.

If you plan to seek informed consent from vulnerable groups, say how you will ensure that consent is voluntary and fully informed.

Participants in the study will complete a written informed consent form prior to any study procedures taking place.

If you are not obtaining consent, please explain why not.

Please enclose a copy of the information sheet(s) and consent form(s).

A30-2. Will you record informed consent (or advice from consultees) in writing?

◉ Yes ○ No

A31. How long will you allow potential participants to decide whether or not to take part?

Participants can take as long as they wish to decide whether to take part. Those who express an interest will be followed up and provided with a Participant Information Sheet (PIS) appropriate to them.

A visit will be booked at least 24 hours after this has been sent. Cases with diabetes will need to be recruited ahead of controls in order for age and sex matching to be effective.

A32. Will you recruit any participants who are involved in current research or have recently been involved in any

research prior to recruitment?

- (●) Yes
- ( ) No
- ( ) Not Known

If Yes, please give details and justify their inclusion. If Not Known, what steps will you take to find out?

For the participants who take part in the study we have specified that they should not already be part of an interventional study (exclusion criteria). Potential participants can choose to delay their study visit until after any ongoing interventional study has been completed.
Participants who have recently donated blood should also be delayed to enable sufficent recovery time.

A33-1. What arrangements have been made for persons who might not adequately understand verbal explanations or written information given in English, or who have special communication needs?*(e.g. translation, use of interpreters)*

We will be able to support participants who have reading problems, visual impairment or hearing impairment.
We are not planning to make translations of the PIS available in different languages due to the small number of participants that will be sought for this study, but local NHS translation service could be used if needed.

A35. What steps would you take if a participant, who has given informed consent, loses capacity to consent during the study? *Tick one option only.*

- ( ) The participant and all identifiable data or tissue collected would be withdrawn from the study. Data or tissue which is not identifiable to the research team may be retained.
- ( ) The participant would be withdrawn from the study. Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected or any other research procedures carried out on or in relation to the participant.
- ( ) The participant would continue to be included in the study.
- ( ) Not applicable – informed consent will not be sought from any participants in this research.
- (●) Not applicable – it is not practicable for the research team to monitor capacity and continued capacity will be assumed.

Further details:

For the study visit participants will be assessed for capacity prior to written informed consent. As the study visit is up to 1 hour on the same day it is unlikely that capacity will be lost during this timeframe.
Participants will be asked to carry out a second study visit approx. 10 months after the first visit. The research team will check patient status in health records (where possible) ahead of contacting the participant for visit 2 and will assess capacity and capability at the visit 2 point and confirm ongoing consent prior to the visit 2 study procedures.
If it is deemed that the participant is incapacitated then the second study visit will not go ahead (participant will be withdrawn). Likewise if the participant's capacity changes between their self-nomination for the research and the first study visit or the patient is deemed to have impaired capacity such that they could not provide adequate consent, the research team staff will not progress with the informed consent process.

CONFIDENTIALITY

In this section, personal data means any data relating to a participant who could potentially be identified. It includes pseudonymised data capable of being linked to a participant through a unique code number.

Storage and use of personal data during the study

A36. Will you be undertaking any of the following activities at any stage (including in the identification of potential participants)?*(Tick as appropriate)*

- [x] Access to medical records by those outside the direct healthcare team

- [ ] Access to social care records by those outside the direct social care team
- [x] Electronic transfer by magnetic or optical media, email or computer networks
- [ ] Sharing of personal data with other organisations
- [ ] Export of personal data outside the EEA
- [x] Use of personal addresses, postcodes, faxes, emails or telephone numbers
- [ ] Publication of direct quotations from respondents
- [ ] Publication of data that might allow identification of individuals
- [ ] Use of audio/visual recording devices
- [x] Storage of personal data on any of the following:
  - [ ] Manual files (includes paper or film)
  - [x] NHS computers
  - [ ] Social Care Service computers
  - [ ] Home or other personal computers
  - [x] University computers
  - [ ] Private company computers
  - [x] Laptop computers

Further details:
Personal addresses, postcodes, emails and telephone numbers will be used to contact participants to arrange study visits.
Personal data will be limited to NHS computers where possible.
The clinical research team use University laptops to support working from home and may need to store contact details on these devices but will limit this where possible.
All staff have received mandatory training on GDPR.

A37. Please describe the physical security arrangements for storage of personal data during the study?

During the study, most study data will be collected and stored on the paper CRF prior to transfer to the laboratory research team who will transfer the subject ID linked dataset to an electronic format hosted on a secure University of Dundee server (details of CRF visit, study ID, follow up health data). This data will include a limited panel of demographic information (age, ethnicity, sex, diabetes type, diabetes duration).
Exceptions (stored on NHS computers and/or University laptops within the responsibility of clinical research team members):
1. Screening log for invitation to study – this will include patient identifiers restricted to CHI, name, telephone number, email address, as well as study process details (date contacted, consent to be contacted).
2. Linked file of CHI and study ID for study only. After informed consent has been given a study ID is assigned. Importantly the CHI is not stored but will be kept on an NHS computer with the linked study ID.
This is to allow linkage to routine clinical information (with consent) and eventual addition of this pseudonymised information to the final study dataset.
3. Signed informed consent forms for the study will be held in local Investigator site files and will not be uploaded to the data management platform held at The University of Dundee. Only date of consent and time of consent will be captured in the case report form/data management system.
At the end of the study a final study dataset will be downloaded to files for longer term storage in The University of Dundee secure data storage system along with lab data (stored with study ID and no personal identifiers), and pseudonymised data from linkage to NHS data sets (with consent). Other information including email and telephone will not be stored in this final stored dataset.

A38. How will you ensure the confidentiality of personal data?*Please provide a general statement of the policy and procedures for ensuring confidentiality, e.g. anonymisation or pseudonymisation of data.*

As above a minimal amount of personal data is stored and only in secure conditions.
Personal identifiers such as CHI are stored in screening logs and linked file of CHI and study ID and this information held separately to the study files on NHS computers.

All other information will be held with study ID as the participant identifier.

A40. Who will have access to participants' personal data during the study? *Where access is by individuals outside the direct care team, please justify and say whether consent will be sought.*

Information from the Diabetes Research Register - permission given already by the individuals signed up to the register.
The clinical research team will be sent lists of eligible participants in line with the Diabetes Research Register SOPs, along with instructions on safe handling of this data. Information containing patient identifiers is only sent to NHS computers.
The clinical research team will store study participant details on NHS computers and/or site files to facilitate contacting participants for the study visits. This will be stored in secure research facility areas including the Diabetes Support Unit at Ninewells Hospital which is card entry. Office doors are locked overnight.

Storage and use of data after the end of the study

A41. Where will the data generated by the study be analysed and by whom?

The anonymised data generated by the study will be analysed in two research laboratories, the lab of Prof Calum Sutherland, University of Dundee, and the lab of Prof Gwyn Gould, University of Strathclyde. Data generated from the assays specific to each location will be analysed to establish assay specific effects of sera from each group. Subsequently all data will be transferred securely to Prof Calum Sutherland as PI for comparative analysis of the group data between assays. Finally, Prof Sutherland is responsible for linking all assay data with anonymised clinical information for more individualised analysis and future publication.

A42. Who will have control of and act as the custodian for the data generated by the study?

	Title	Forename/Initials	Surname
	Prof	Calum	Sutherland
Post	Professor		
Qualifications	PhD		
Work Address	Department of Cellular Medicine		
	Ninewells Hospital and Medical School		
	Dundee		
Post Code	DD1 9SY		
Work Email	c.d.sutherland@dundee.ac.uk		
Work Telephone	01382383098		
Fax			

A43. How long will personal data be stored or accessed after the study has ended?

- ○ Less than 3 months
- ○ 3 – 6 months
- ○ 6 – 12 months
- ○ 12 months – 3 years
- ◉ Over 3 years

If longer than 12 months, please justify:
The participants will be followed up annually via their health records.

A44. For how long will you store research data generated by the study?

Years: 10
Months: 0

A45. Please give details of the long term arrangements for storage of research data after the study has ended. *Say where data will be stored, who will have access and the arrangements to ensure security.*

Anonymised data will be stored on the University of Dundee secure server for the longer term. It will be password protected and accessible by the PI only.

INCENTIVES AND PAYMENTS

A46. Will research participants receive any payments, reimbursement of expenses or any other benefits or incentives for taking part in this research?

(•) Yes ( ) No

If Yes, please give details. For monetary payments, indicate how much and on what basis this has been determined.
Travel expenses of a reasonable nature will be reimbursed.
This is stated in the PIS.
All participants will receive a breakfast.

A47. Will individual researchers receive any personal payment over and above normal salary, or any other benefits or incentives, for taking part in this research?

( ) Yes (•) No

A48. Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g. financial, share holding, personal relationship etc.) in the organisations sponsoring or funding the research that may give rise to a possible conflict of interest?

( ) Yes (•) No

NOTIFICATION OF OTHER PROFESSIONALS

A49-1. Will you inform the participants' General Practitioners (and/or any other health or care professional responsible for their care) that they are taking part in the study?

(•) Yes ( ) No

If Yes, please enclose a copy of the information sheet/letter for the GP/health professional with a version number and date.

A49-2. Will you seek permission from the research participants to inform their GP or other health/ care professional?

(•) Yes ( ) No

It should be made clear in the participant's information sheet if the GP/health professional will be informed.

PUBLICATION AND DISSEMINATION

A50. Will the research be registered on a public database?

The UK Policy Framework for Health and Social Care Research sets out the principle of making information about research publicly available. Furthermore: Article 19 of the World Medical Association Declaration of Helsinki adopted

in 2008 states that "every clinical trial must be registered on a publicly accessible database before recruitment of the first subject"; and the International Committee of Medical Journal Editors (ICMJE) will consider a clinical trial for publication only if it has been registered in an appropriate registry. Please see guidance for more information.

○ Yes ◉ No

Please give details, or justify if not registering the research.
This is not a clinical trial.

Please ensure that you have entered registry reference number(s) in question A5-1.

A51. How do you intend to report and disseminate the results of the study? *Tick as appropriate:*

- ☑ Peer reviewed scientific journals
- ☐ Internal report
- ☑ Conference presentation
- ☐ Publication on website
- ☐ Other publication
- ☐ Submission to regulatory authorities
- ☐ Access to raw data and right to publish freely by all investigators in study or by Independent Steering Committee on behalf of all investigators
- ☐ No plans to report or disseminate the results
- ☐ Other (please specify)

A52. If you will be using identifiable personal data, how will you ensure that anonymity will be maintained when publishing the results?

The dataset will not contain identifiable personal data.

A53. How and when will you inform participants of the study results?

If there will be no arrangements in place to inform participants please justify this.
Participants in the study will be told their individual results during the visit and the results will be added to SCI Diabetes electronic health record for diabetes patients.
We do not plan to inform the study participants of the compiled data for the study but will use Twitter to advertise any publication that results from the data analysis.

5. Scientific and Statistical Review

A54. How has the scientific quality of the research been assessed? *Tick as appropriate:*

- ☑ Independent external review
- ☐ Review within a company
- ☑ Review within a multi−centre research group
- ☑ Review within the Chief Investigator's institution or host organisation
- ☑ Review within the research team
- ☐ Review by educational supervisor
- ☑ Other

Justify and describe the review process and outcome. If the review has been undertaken but not seen by the researcher, give details of the body which has undertaken the review:
The project was externally reviewed as part of the assessment process for Diabetes UK funding.

The multi-centre research team have also held meetings to discuss the protocol, the best approach to carry out the research and other details of the project post funding award.

The NHS Research Scotland Diabetes Network are leading the organisation of the clinical research aspect of the project.

For all studies except non-doctoral student research, please enclose a copy of any available scientific critique reports, together with any related correspondence.

For non-doctoral student research, please enclose a copy of the assessment from your educational supervisor/ institution.

A56. How have the statistical aspects of the research been reviewed? *Tick as appropriate:*

- [x] Review by independent statistician commissioned by funder or sponsor
- [ ] Other review by independent statistician
- [ ] Review by company statistician
- [ ] Review by a statistician within the Chief Investigator's institution
- [ ] Review by a statistician within the research team or multi-centre group
- [ ] Review by educational supervisor
- [ ] Other review by individual with relevant statistical expertise
- [ ] No review necessary as only frequencies and associations will be assessed – details of statistical input not required

In all cases please give details below of the individual responsible for reviewing the statistical aspects. If advice has been provided in confidence, give details of the department and institution concerned.

	Title Forename/Initials Surname
Department	VIA DIABETES UK GRANT FUNDING REVIEW PROCESS
Institution	
Work Address	
Post Code	
Telephone	
Fax	
Mobile	
E-mail	

Please enclose a copy of any available comments or reports from a statistician.

A57. What is the primary outcome measure for the study?

The main analysis will be a variety of insulin responses in cell based assays from cells grown in sera donated by volunteers. This includes gene expression, glucose transport and glucose oxidation assays in response to a range of insulin concentrations and different time points. EC50 measures for insulin as well as slope of dose response curves will be compared between pooled sera from each group of donated sera, but also across the individual patient samples and between visit 1 and visit 2 within each group. Correlations to clinical data will be attempted where significant variations occur between the groups, visits or individual cases.

A58. What are the secondary outcome measures? *(if any)*

Comparing the responses of serum samples from two visits.

A59. What is the sample size for the research? *How many participants/samples/data records do you plan to study in total? If there is more than one group, please give further details below.*

Total UK sample size:	60
Total international sample size (including UK):	60
Total in European Economic Area:	60

Further details:
No recruitment outside of the UK.

A60. How was the sample size decided upon? *If a formal sample size calculation was used, indicate how this was done, giving sufficient information to justify and reproduce the calculation.*

This is based on our previous work which demonstrated this number of Type 2 Diabetes volunteers generated >2 fold response with a p-value of 0.05.

A61. Will participants be allocated to groups at random?

○ Yes ◉ No

A62. Please describe the methods of analysis (statistical or other appropriate methods, e.g. for qualitative research) by which the data will be evaluated to meet the study objectives.

The data generated from this study will be in the form of a variety of 'interference scores (IS)' - for each specific assay of insulin action being employed- for every individual sera sample. The ISs give an indication of difference from the mean effect of the control sera on insulin sensitivity curves of each different assay, for every sera sample (ie the higher the IS score we hypothesise a higher level of insulin resistance causing factor X). Initially we will compare these ISs by disease diagnosis- control vs diabetes, control vs pre-diabetes and diabetes vs pre-diabetes and investigate significant differences between groups by ANOVA. This will be performed on ISs generated in each 'insulin sensitivity' assay, allowing assessment of generic or targeted interference on insulin action. More detailed analysis will be performed on the full individual sera IS data set by association with continuous parameters including HbA1C, BMI and BP (independent of disease diagnosis). In addition we will assess the concentrations of glucose, insulin and leptin in sera with the most extreme ISs (high and low) to examine the potential association of these factors. Finally, we will isolate specific molecules from these extreme IS scoring sera samples (in particular microvesicles) to investigate any differences in their composition and to test whether these molecules have the ability to generate similar ISs as the parent sera samples in each of the assays.

6. MANAGEMENT OF THE RESEARCH

A63. Other key investigators/collaborators. *Please include all grant co−applicants, protocol co−authors and other key members of the Chief Investigator's team, including non-doctoral student researchers.*

	Title Forename/Initials Surname
	Prof Gwyn Gould
Post	Professor
Qualifications	PhD
Employer	University of Strathclyde
Work Address	Strathclyde Institute of Pharmacy and Biomedical Sciences
	161 Cathedral Street
	Glasgow
Post Code	G4 0RE
Telephone	01415484805

Fax
Mobile
Work Email gwyn.gould@strath.ac.uk

A64. Details of research sponsor(s)

A64-1. Sponsor

Lead Sponsor

Status: ○ NHS or HSC care organisation
● Academic
○ Pharmaceutical industry
○ Medical device industry
○ Local Authority
○ Other social care provider (including voluntary sector or private organisation)
○ Other

Commercial status: Non-Commercial

If Other, please specify:

Contact person

Name of organisation University of Dundee
Given name Patricia
Family name Burns
Address TASC, R&D Department, Ninewells Hospital
Town/city Dundee
Post code DD1 9SY
Country
Telephone 01382383297
Fax
E-mail TASCgovernance@dundee.ac.uk

Legal representative for clinical investigation of medical device (studies involving Northern Ireland only)
Clinical Investigations of Medical Devices that take place in Northern Ireland must have a legal representative of the sponsor that is based in Northern Ireland or the EU

Contact person

Name of organisation
Given name
Family name
Address
Town/city
Post code

Country
Telephone
Fax
E-mail

A65. Has external funding for the research been secured?

Please tick at least one check box.

- [x] Funding secured from one or more funders
- [ ] External funding application to one or more funders in progress
- [ ] No application for external funding will be made

What type of research project is this?

- ( ) Standalone project
- ( ) Project that is part of a programme grant
- ( ) Project that is part of a Centre grant
- (•) Project that is part of a fellowship/ personal award/ research training award
- ( ) Other

Other – please state:

Please give details of funding applications.

Organisation	Diabetes UK
Address	Wells Lawrence House Church Lane London
Post Code	E1 1FH
Telephone	03451232399
Fax	
Mobile	
Email	info@diabetes.org.uk

Funding Application Status: (•) Secured ( ) In progress

Amount: 97,221.00

Duration
Years: 3
Months: 0

If applicable, please specify the programme/ funding stream:

What is the funding stream/ programme for this research project?
PhD studentship

A66. Has responsibility for any specific research activities or procedures been delegated to a subcontractor (other

than a co-sponsor listed in A64-1) ? *Please give details of subcontractors if applicable.*

○ Yes ◉ No

A67. Has this or a similar application been previously rejected by a Research Ethics Committee in the UK or another country?

○ Yes ◉ No

Please provide a copy of the unfavourable opinion letter(s). You should explain in your answer to question A6-2 how the reasons for the unfavourable opinion have been addressed in this application.

A68-1. Give details of the lead NHS R&D contact for this research:

	Title	Forename/Initials	Surname
	Mrs	Elizabeth	Coote
Organisation	NHS Tayside		
Address	TASC R&D Offices		
	Ninewells Hospital and Medical School		
	Dundee		
Post Code	DD1 9SY		
Work Email	liz.coote@nhs.scot		
Telephone	01382383876		
Fax			
Mobile			

Details can be obtained from the NHS R&D Forum website: http://www.rdforum.nhs.uk

http://www.rdforum.nhs.uk

A69-1. How long do you expect the study to last in the UK?

Planned start date: 01/10/2021
Planned end date: 01/10/2024
Total duration:
Years: 3 Months: 0 Days: 1

A70. Definition of the end of trial, and justification in the case where it is not the last visit of the last subject undergoing the trial (1)

The end of study is defined as reaching the number of samples from completed cases and controls that the CI or delegate deems adequate for analysis.

A71-1. Is this study?

◉ Single centre
○ Multicentre

A71-2. Where will the research take place? *(Tick as appropriate)*

☐ England

- [x] Scotland
- [ ] Wales
- [ ] Northern Ireland
- [ ] Other countries in European Economic Area

Total UK sites in study 1

Does this trial involve countries outside the EU?
○ Yes ◉ No

A72. Which organisations in the UK will host the research? *Please indicate the type of organisation by ticking the box and give approximate numbers if known:*

☐ NHS organisations in England	
☐ NHS organisations in Wales	
☑ NHS organisations in Scotland	1
☐ HSC organisations in Northern Ireland	
☐ GP practices in England	
☐ GP practices in Wales	
☐ GP practices in Scotland	
☐ GP practices in Northern Ireland	
☐ Joint health and social care agencies (eg community mental health teams)	
☐ Local authorities	
☐ Phase 1 trial units	
☐ Prison establishments	
☐ Probation areas	
☐ Independent (private or voluntary sector) organisations	
☐ Educational establishments	
☐ Independent research units	
☐ Other (give details)	
Total UK sites in study:	1

A73-1. Will potential participants be identified through any organisations other than the research sites listed above?

○ Yes ◉ No

A76. Insurance/ indemnity to meet potential legal liabilities

***Note:** in this question to NHS indemnity schemes include equivalent schemes provided by Health and Social Care (HSC) in Northern Ireland*

A76-1. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the sponsor(s) for harm to participants arising from the management of the research? *Please tick box(es) as applicable.*

Note: Where a NHS organisation has agreed to act as sponsor or co-sponsor, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For all other sponsors, please describe the arrangements and provide evidence.

☐ NHS indemnity scheme will apply (NHS sponsors only)

☑ Other insurance or indemnity arrangements will apply (give details below)

Insurance – The University of Dundee will obtain and hold a policy of Public Liability Insurance for legal liabilities arising from the study.
Tayside Health Board will maintain its membership of the Clinical Negligence and Other Risks Insurance Scheme ("CNORIS") which covers the legal liability of Tayside in relation to the study.
Where the study involves University of Dundee staff undertaking clinical research on NHS patients, such staff will hold honorary contracts with Tayside Health Board which means they will have cover under Tayside's membership of the CNORIS scheme.
Indemnity – The Co-Sponsors do not provide study participants with indemnity in relation to participation in the Study but have insurance for legal liability as described above.

Please enclose a copy of relevant documents.

A76-2. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of the sponsor(s) or employer(s) for harm to participants arising from the design of the research? *Please tick box(es) as applicable.*

Note: Where researchers with substantive NHS employment contracts have designed the research, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For other protocol authors (e.g. company employees, university members), please describe the arrangements and provide evidence.

☐ NHS indemnity scheme will apply (protocol authors with NHS contracts only)

☑ Other insurance or indemnity arrangements will apply (give details below)

Please enclose a copy of relevant documents.

A76-3. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of investigators/collaborators arising from harm to participants in the conduct of the research?

Note: Where the participants are NHS patients, indemnity is provided through the NHS schemes or through professional indemnity. Indicate if this applies to the whole study (there is no need to provide documentary evidence). Where non-NHS sites are to be included in the research, including private practices, please describe the arrangements which will be made at these sites and provide evidence.

☐ NHS indemnity scheme or professional indemnity will apply (participants recruited at NHS sites only)

☑ Research includes non-NHS sites (give details of insurance/ indemnity arrangements for these sites below)

Please enclose a copy of relevant documents.

A78. Could the research lead to the development of a new product/process or the generation of intellectual property?

◉ Yes ○ No ○ Not sure

Part B: Section 5 – Use of newly obtained human tissue(or other human biological materials) for research purposes

1. What types of human tissue or other biological material will be included in the study?

Blood samples

2. Who will collect the samples?

Diabetes clinical research team members

3. Who will the samples be removed from?

- [x] Living donors
- [ ] The deceased

4. Will informed consent be obtained from living donors for use of the samples? *Please tick as appropriate*

In this research?

(•) Yes ( ) No

In future research?

(•) Yes ( ) No ( ) Not applicable

6. Will any tissues or cells be used for human application or to carry out testing for human application in this research?

( ) Yes (•) No

8. Will the samples be stored: *[Tick as appropriate]*

In fully anonymised form? *(link to donor broken)*

( ) Yes (•) No

In linked anonymised form? *(linked to stored tissue but donor not identifiable to researchers)*

(•) Yes ( ) No

If Yes, say who will have access to the code and personal information about the donor.

The blood samples will be labelled with the subject ID.

In a form in which the donor could be identifiable to researchers?

( ) Yes (•) No

9. What types of test or analysis will be carried out on the samples?

To be completed in University research labs. A variety of insulin responses in cell based assays from cells grown in sera donated by volunteers will be completed. This includes gene expression, glucose transport and glucose oxidation assays in response to a range of insulin concentrations and different time points. EC50 measures for insulin as well as slope of dose response curves will be done.

Routine clinical measures (HbA1c). To be analysed in local accredited NHS Biochemistry labs.

10. Will the research involve the analysis or use of human DNA in the samples?

( ) Yes (•) No

11. Is it possible that the research could produce findings of clinical significance for donors or their relatives?

○ Yes ◉ No

12. If so, will arrangements be made to notify the individuals concerned?

○ Yes ○ No ◉ Not applicable

13. Give details of where the samples will be stored, who will have access and the custodial arrangements.

Blood samples for University laboratory measurement will be transferred same day to labs at The University of Dundee for processing. The lab PI is Prof C Sutherland. The lab PI will take responsibility for the samples and will store the samples in -80oC freezers, access to which are restricted by building entry pass requirements, and room access requirements. Freezers are all routinely monitored and alarmed for failure in and out of office hours, with backup freezer space available.
Samples will be transferred to University of Strathclyde via secure courier. Chain of custody will transfer to Prof Gould.

14. What will happen to the samples at the end of the research? *Please tick all that apply and give further details.*

☐ Transfer to research tissue bank

(If the bank is in England, Wales or Northern Ireland the institution will require a licence from the Human Tissue Authority to store relevant material for possible further research.)

☑ Storage by research team pending ethical approval for use in another project

(Unless the researcher's institution holds a storage licence from the Human Tissue Authority, or the tissue is stored in Scotland, or it is not relevant material, a further application for ethical review should be submitted before the end of this project.)

☐ Storage by research team as part of a new research tissue bank

(The institution will require a licence from the Human Tissue Authority if the bank will be storing relevant material in England, Wales or Northern Ireland. A separate application for ethical review of the tissue bank may also be submitted.)

☐ Storage by research team of biological material which is not "relevant material" for the purposes of the Human Tissue Act

☐ Disposal in accordance with the Human Tissue Authority's Code of Practice

☐ Other

☐ Not yet known

Please give further details of the proposed arrangements:

There may be a need to use samples in the future as information about Blood Factor X comes to light.
For remaining/unused samples (whole blood and fractionated components) these will undergo documented destruction 10 years after end of trial study declaration.

PART C: Overview of research sites

Please enter details of the host organisations (Local Authority, NHS or other) in the UK that will be responsible for the research sites. *For further information please refer to guidance.*

Investigator identifier	Research site	Investigator Name
IN1	(•) NHS/HSC Site ( ) Non-NHS/HSC Site	Forename: Calum Middle name: Family name: Sutherland Email: c.d.sutherland@dundee.ac.uk Qualification (MD...): PhD Country: United Kingdom

Organisation name: NHS Tayside
Address: Kings Croos, Clepington Road, Dundee Scotland
Post Code: DD3 8EA
Country: SCOTLAND

PART D: Declarations

D1. Declaration by Chief Investigator

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.
2. I undertake to fulfil the responsibilities of the chief investigator for this study as set out in the UK Policy Framework for Health and Social Care Research.
3. I undertake to abide by the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research.
4. If the research is approved I undertake to adhere to the study protocol, the terms of the full application as approved and any conditions set out by review bodies in giving approval.
5. I undertake to notify review bodies of substantial amendments to the protocol or the terms of the approved application, and to seek a favourable opinion from the main REC before implementing the amendment.
6. I undertake to submit annual progress reports setting out the progress of the research, as required by review bodies.
7. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer. I understand that I am not permitted to disclose identifiable data to third parties unless the disclosure has the consent of the data subject or, in the case of patient data in England and Wales, the disclosure is covered by the terms of an approval under Section 251 of the NHS Act 2006.
8. I understand that research records/data may be subject to inspection by review bodies for audit purposes if required.
9. I understand that any personal data in this application will be held by review bodies and their operational managers and that this will be managed according to the principles established in the Data Protection Act 2018.
10. I understand that the information contained in this application, any supporting documentation and all correspondence with review bodies or their operational managers relating to the application:
    - Will be held by the REC (where applicable) until at least 3 years after the end of the study; and by NHS R&D offices (where the research requires NHS management permission) in accordance with the NHS Code of Practice on Records Management.
    - May be disclosed to the operational managers of review bodies, or the appointing authority for the REC (where applicable), in order to check that the application has been processed correctly or to investigate any complaint.
    - May be seen by auditors appointed to undertake accreditation of RECs (where applicable).
    - Will be subject to the provisions of the Freedom of Information Acts and may be disclosed in response to requests made under the Acts except where statutory exemptions apply.
    - May be sent by email to REC members.
11. I understand that information relating to this research, including the contact details on this application, may be held on national research information systems, and that this will be managed according to the principles established in the Data Protection Act 2018.
12. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, I understand that the summary of this study will be published on the website of the Health Research Authority (HRA) together with the contact point for enquiries named below. Publication will take place no earlier than 3 months after the issue of the ethics committee's final opinion or the withdrawal of the application.

Contact point for publication *(Not applicable for R&D Forms)*

HRA would like to include a contact point with the published summary of the study for those wishing to seek further

information. We would be grateful if you would indicate one of the contact points below.

- ◯ Chief Investigator
- ◯ Sponsor
- ◯ Study co-ordinator
- ◯ Student
- ◯ Other – please give details
- ◯ None

Access to application for training purposes *(Not applicable for R&D Forms)*

Optional – please tick as appropriate:

☐ I would be content for members of other RECs to have access to the information in the application in confidence for training purposes. All personal identifiers and references to sponsors, funders and research units would be removed.

Signature: .....

Print Name:

Date: *(dd/mm/yyyy)*

D2. Declaration by the sponsor's representative

If there is more than one sponsor, this declaration should be signed on behalf of the co-sponsors by a representative of the lead sponsor named at A64-1.

I confirm that:

1. This research proposal has been discussed with the Chief Investigator and agreement in principle to sponsor the research is in place.
2. An appropriate process of scientific critique has demonstrated that this research proposal is worthwhile and of high scientific quality.
3. Any necessary indemnity or insurance arrangements, as described in question A76, will be in place before this research starts. Insurance or indemnity policies will be renewed for the duration of the study where necessary.
4. Arrangements will be in place before the study starts for the research team to access resources and support to deliver the research as proposed.
5. Arrangements to allocate responsibilities for the management, monitoring and reporting of the research will be in place before the research starts.
6. The responsibilities of sponsors set out in the UK Policy Framework for Health and Social Care Research will be fulfilled in relation to this research.

   Please note: *The declarations below do not form part of the application for approval above. They will not be considered by the Research Ethics Committee.*
7. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, I understand that the summary of this study will be published on the website of the National Research Ethics Service (NRES), together with the contact point for enquiries named in this application. Publication will take place no earlier than 3 months after issue of the ethics committee's final opinion or the withdrawal of the application.
8. Specifically, for submissions to the Research Ethics Committees (RECs) I declare that any and all clinical trials approved by the HRA since 30th September 2013 (as defined on IRAS categories as clinical trials of medicines, devices, combination of medicines and devices or other clinical trials) have been registered on a publically accessible register in compliance with the HRA registration requirements for the UK, or that any deferral granted by the HRA still applies.

Signature: .....

Print Name:

Post:

Organisation:

Date: *(dd/mm/yyyy)*

D3. Declaration for student projects by academic supervisor(s)

1. I have read and approved both the research proposal and this application. I am satisfied that the scientific content of the research is satisfactory for an educational qualification at this level.

2. I undertake to fulfil the responsibilities of the supervisor for this study as set out in the UK Policy Framework for Health and Social Care Research.

3. I take responsibility for ensuring that this study is conducted in accordance with the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research, in conjunction with clinical supervisors as appropriate.

4. I take responsibility for ensuring that the applicant is up to date and complies with the requirements of the law and relevant guidelines relating to security and confidentiality of patient and other personal data, in conjunction with clinical supervisors as appropriate.

Academic supervisor 1

Signature: .....

Print Name:

Post:

Organisation:

Date: *(dd/mm/yyyy)*

Academic supervisor 2

Signature: .....

Print Name:

Post:

Organisation:

Date: *(dd/mm/yyyy)*

Figure A-0-1: IRAS form (32 pages)

Ethical approval

NHS
Health Research Authority

London - West London & GTAC Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0207 104 8143

29 October 2021

Prof Calum Sutherland
Department of Cellular Medicine
Ninewells Hospital and Medical School
Dundee
DD1 9SY

Dear Prof Sutherland

Study title:	**Diabetes Blood Factor X Sampling Study**
REC reference:	**21/PR/1385**
Protocol number:	**2.013.21**
IRAS project ID:	**305555**

Thank you for your letter of 27 October 2021, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved on behalf of the PR sub-committee.

Confirmation of ethical opinion

On behalf of the Research Ethics Committee (REC), I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Good practice principles and responsibilities

The UK Policy Framework for Health and Social Care Research sets out principles of good practice in the management and conduct of health and social care research. It also outlines the responsibilities of individuals and organisations, including those related to the four elements of

research transparency:

1. registering research studies
2. reporting results
3. informing participants
4. sharing study data and tissue

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Confirmation of Capacity and Capability (in England, Northern Ireland and Wales) or NHS management permission (in Scotland) should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA and HCRW Approval (England and Wales)/ NHS permission for research is available in the Integrated Research Application System.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All research should be registered in a publicly accessible database and we expect all researchers, research sponsors and others to meet this fundamental best practice standard.

It is a condition of the REC favourable opinion that **all clinical trials are registered** on a publicly accessible database within six weeks of recruiting the first research participant. For this purpose, 'clinical trials' are defined as the first four project categories in IRAS project filter question 2. Failure to register is a breach of these approval conditions, unless a deferral has been agreed by or on behalf of the Research Ethics Committee (see here for more information on requesting a deferral:
https://www.hra.nhs.uk/planning-and-improving-research/research-planning/research-registration-research-project-identifiers/

If you have not already included registration details in your IRAS application form, you should notify the REC of the registration details as soon as possible.

Publication of Your Research Summary

We will publish your research summary for the above study on the research summaries section of our website, together with your contact details, no earlier than three months from the date of this favourable opinion letter.

Should you wish to provide a substitute contact point, make a request to defer, or require further information, please visit:
https://www.hra.nhs.uk/planning-and-improving-research/application-summaries/research-summaries/

N.B. If your study is related to COVID-19 we will aim to publish your research summary within 3 days rather than three months.

During this public health emergency, it is vital that everyone can promptly identify all relevant research related to COVID-19 that is taking place globally. If you haven't already done so, please register your study on a public registry as soon as possible and provide the REC with the registration detail, which will be posted alongside other information relating to your project. We are also asking sponsors not to request deferral of publication of research summary for any projects relating to COVID-19. In addition, to facilitate finding and extracting studies related to COVID-19 from public databases, please enter the WHO official acronym for the coronavirus disease (COVID-19) in the full title of your study. Approved COVID-19 studies can be found at:
https://www.hra.nhs.uk/covid-19-research/approved-covid-19-research/

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

After ethical review: Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study, including early termination of the study
- Final report
- Reporting results

The latest guidance on these topics can be found at
https://www.hra.nhs.uk/approvals-amendments/managing-your-approval/.

Ethical review of research sites

The favourable opinion applies to all NHS/HSC sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" above).

Approved documents

The documents reviewed and approved by the Committee are:

Document	*Version*	*Date*
Copies of materials calling attention of potential participants to the research [Public Engagement advertisement]	1	30 August 2021
Copies of materials calling attention of potential participants to the research [Poster CASES]	1	30 August 2021
Copies of materials calling attention of potential participants to the research [Poster CONTROLS]	1	30 August 2021
Covering letter on headed paper [Cover letter to REC]		27 September 2021
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance letter]		29 June 2021
GP/consultant information sheets or letters [Letter to GP]	2	26 October 2021
IRAS Application Form [IRAS_Form_28092021]		28 September 2021
IRAS Application Form XML file [IRAS_Form_28092021]		28 September 2021
Letter from funder [Funding award letter]		20 January 2021
Letter from sponsor [Sponsorship Letter]		27 September 2021
Letters of invitation to participant [Clinic letter of invitation]	1	30 August 2021
Other [Cover letter to REC (Response)]	1	26 October 2021
Participant consent form [Consent form]	2	26 October 2021
Participant information sheet (PIS) [PIS Controls]	2	26 October 2021
Participant information sheet (PIS) [PIS Diabetes Participants]	2	26 October 2021
Participant information sheet (PIS) [PIS Pre-Diabetes Participants]	2	26 October 2021
Research protocol or project proposal [Protocol]	1	24 September 2021
Summary CV for Chief Investigator (CI) [CV for CI]		27 September 2021
Summary CV for student [CV student Milligan]		27 September 2021
Summary CV for supervisor (student research) [CV supervisor Sutherland]		27 September 2021
Summary CV for supervisor (student research) [CV supervisor Gould]		28 September 2021
Summary, synopsis or diagram (flowchart) of protocol in non technical language [Flow chart of Protocol]	1	24 September 2021

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Learning

We are pleased to welcome researchers and research staff to our HRA Learning Events and online learning opportunities– see details at:
https://www.hra.nhs.uk/planning-and-improving-research/learning/

IRAS project ID: 305555	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

Charlotte Miller

pp.

Professor Catherine Urch
Chair

Email: westlondon.rec@hra.nhs.uk

Enclosures: "After ethical review – guidance for researchers" [SL-AR2]

Copy to: Dr Patricia Burns

Figure A-0-2: Ethical approval letter (5 pages)

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