Investigating the effect of low-dose sulfonylureas on glucagon secretion in diabetes mellitus

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Ultimately, this project could not have been completed without the support, patience and encouragement of my wife Katja, and that of my parents Yorgo and Bessie, for which I will always be grateful. Αἰὲν ἀριστεύειν.

Contents

Abstract

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Ioannis I. Spiliotis, Harris Manchester College, DPhil thesis, Trinity 2018

Background: Diabetes is a multi-hormonal disorder characterised by insufficient insulin secretion and aberrant glucagon secretion with fasting hyperglucagonaemia leading to increased rates of hepatic glucose production, which further exacerbates hyperglycaemia. Sulfonylureas used at low concentrations have been shown to partially restore appropriate glucose-regulated glucagon secretion in isolated islets from donors with type 2 diabetes (T2DM). The main objective of this thesis was to investigate whether low doses of the sulfonylurea glibenclamide could reduce fasting hyperglucagonaemia in patients with T2DM. In addition, the effect of age and sex on islet hormone secretion was examined in human islets, as was the impact of low-dose gliclazide (another sulfonylurea) on glucagon secretion in MODY patients.

Methods: I performed a pilot, dose-finding (0.3mg – 6mg/day) clinical trial (LEGEND-A) of a novel oral glibenclamide suspension in sixteen patients with T2DM (diet controlled or on metformin alone). Fasting plasma glucagon, glucose, insulin, C-peptide and glibenclamide were measured at each dose-change, and continuous glucose monitoring was used throughout. For the human islet hormone secretion study, a database analysis was performed which covered an 11-year period (2006-2017). Finally, a pilot clinical study ("Glucagon in MODY") involving an oral glucose tolerance test before and after the omission of gliclazide for 72h was performed in three patients diagnosed with HNF1-α MODY (study ongoing).

Results: Glibenclamide at 0.3mg/day was able to reduce fasting glucagon levels by 30% in four T2DM patients who had hyperglucagonaemia, without causing insulin secretion and with no adverse effects. This effect was not observed at higher glibenclamide concentrations, nor in the twelve T2DM patient who had normal-range fasting glucagon levels. In addition, the islet hormone database analysis revealed the novel finding that islets from older donors (especially males) secreted twice as much insulin at euglycaemic conditions as those from younger donors, but only 40% as much glucagon when challenged with hypoglycaemic conditions. Finally, the preliminary results of the "Glucagon in MODY" study suggest that HNF1-α MODY patients may not display appropriate glucose-dependent glucagon suppression during an oral glucose tolerance test, and that this may be reinstated by using low doses of gliclazide .

Conclusions: Using low-dose sulfonylureas it may be possible to normalise aberrant glucagon secretion patterns in patients with diabetes, perhaps by subtly altering the activity of the alpha-cell ATP-sensitive potassium channel. This novel approach has the potential to be used both as a standalone therapy and as an adjunct to other medications, such as SGLT2 inhibitors, in which a reduction in glucagon could increase overall efficiency. I have also demonstrated that age and sex can impact the secretion of insulin and glucagon in *ex vivo* human islets, and this may in part provide a mechanistic explanation for the vulnerability of older people to sulfonylurea-induced hypoglycaemia episodes.

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Abbreviations

AE	Adverse events
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CI	Chief Investigator
CRU	Clinical Research Unit
DPP4	dipeptidyl peptidase-4
EPAC	exchange protein activated by cAMP
GIRK	G protein-coupled inwardly-rectifying potassium channels
GLP-1, -2	glucagon like peptide-1, -2
HNF1-α, /4-α	hepatic nuclear factor 1-alpha / 4-alpha
IMP	interventional medicinal product
K_{ATP}	ATP-sensitive potassium channel
Kir6.2	inwardly-rectifying potassium channel 6.2
MHRA	Medicines and Healthcare products Regulatory Agency
MODY	maturity onset diabetes of the young
NAFLD	Non-alcoholic fatty liver disease
NEFA	Non-esterified fatty acids
OCDEM	Oxford Centre for Diabetes, Endocrinology and Metabolism
OGTT	oral glucose tolerance test
PC-1, -2, -3	prohormone convertase-1, -2, -3
PIP	patient involvement project
SGC	Structural Genomics Consortium
SGLT2	sodium glucose co-transporter 2
SSTR2	Somatostatin Receptor 2
SU	Sulfonylureas
SUR1	sulfonylurea receptor 1
T2DM	Type 2 diabetes mellitus
T1DM	Type 1 diabetes mellitus

Chapter 1: Introduction

1.1. Diabetes mellitus

Diabetes mellitus is a complex metabolic disorder involving abnormal processing of carbohydrates, proteins and fat, which ultimately leads to chronic hyperglycaemia. This in turn contributes to an increased risk of developing microvascular disease (nephropathy, retinopathy, neuropathy) and macrovascular disease (myocardial infarction, stroke, peripheral vascular disease) through a variety of mechanisms including endothelial dysfunction, oxidative stress and altered platelet metabolism [1-3]. It is estimated that the worldwide prevalence of diabetes is 425 million (around 1 in 11 adults), and as such it is the fourth largest cause of death from non-communicable diseases accounting for 1.6 million deaths annually [4, 5]. These figures are increasing year on year, and when taking into account medication costs, treatment of complications etc., diabetes makes up about 12% of total global health expenditure [4].

In the UK it is estimated that 6.6% of the population have a diagnosis of diabetes, and direct healthcare costs make up about 10% of the NHS budget [6]. However, the true prevalence is unknown as 1 in 2 adults with the condition may be undiagnosed [4].

There are multiple aetiologies for the development of diabetes, most of which are incompletely understood. The majority of cases (around 90%) are classified as type 2 diabetes (T2DM) with type 1 diabetes making up around 10%, though the proportions change depending on age-group. Around 1% of cases are caused by specific genetic mutations, or other conditions such as cystic fibrosis, pancreatitis etc. However, diabetes is largely defined by its effect rather than its cause. The World Health Organization (WHO) criteria for diagnosing diabetes rely on the detection of hyperglycaemia (≥ 7.0 mmol/L fasting, ≥ 11.1

mmol/L random or post glucose tolerance test) or raised glycated haemoglobin (≥ 48mmol/mol, 6.5%) [7]. While this is a sensible and pragmatic approach from a clinical perspective, the emergence of hyperglycaemia is likely a late sign which indicates significant beta-cell dysfunction [8]. Furthermore, a recent study has highlighted the existence of discrete subgroups of diabetes beyond the "type 1" or "type 2" classification, each with specific clinical features and progression [9].

From the perspective of the pancreatic islets, diabetes is a multi-hormonal disorder characterised by insufficient insulin secretion, aberrant glucagon secretion and dysregulated somatostatin signalling [10, 11]. Taken together, these factors contribute to hyperglycaemia but also a vulnerability to hypoglycaemia episodes. Of particular interest is the role alpha-cells play in the pathogenesis of diabetes, as inappropriately high secretion of glucagon appears to be a feature shared in both type 1 and T2DM [12-15], and small increases can have a significant effect on plasma glucose levels [16]. It is therefore key to examine the properties of this hormone, how its secretion is regulated, and the impact pharmacological therapy has on alpha-cell function.

1.2. Glucagon production

Glucagon is a 29 amino acid peptide hormone (3,485 Da) which adopts a mainly alpha-helical tertiary structure with no disulphide bonds (Figure 1; [17]). It was first identified nearly 100 years ago by Kimball and Murlin in 1923, a year after the discovery of insulin, as a substance in pancreatic extracts which induced hyperglycaemia when injected into pancreatectomised dogs [18]. They termed the compound "glucagon" (i.e. **gluc**ose **agon**ist). It's full amino acid sequence was described by Bromer and colleagues [19], and this was later found to be identical in all mammals (except for guinea-pigs [20]).

The glucagon peptide is the result of post-translational cleavage of the preproglucagon (*Gcg*) gene product. This 160 amino acid pro-hormone is expressed in the alpha-cells of the pancreatic islets, the enteroendocrine L-cells in the intestine, and a specific group of neurons in the nucleus solitarius in the brainstem [21]. In addition to glucagon, it contains the sequences of other biologically active hormones including glucagon like peptide-1 and -2 (GLP-1, GLP-2) and oxyntomodulin (Figure 2), as well as other peptides whose function is still not fully understood (glicentin, glicentin-related pancreatic polypeptide and major proglucagon fragment). The combination of preproglucagon fragments available in each tissue is determined by the relative abundance of the three isoforms of the enzyme prohormone convertase (PC-1, -2, and -3), which are differentially expressed in various tissues.

In the alpha-cells, the high levels of PC-2 expression leads to the production of glucagon as the main bioactive end-product; in the intestine and brainstem PC-1 and -3 are mainly expressed, resulting in GLP-1, GLP-2 and oxyntomodulin production. While all these peptides have important roles in energy homeostasis, they can exert opposing influences. The main role of glucagon, as part of the counter-regulatory mechanism, is to prevent hypoglycaemia by stimulating hepatic glucose production (glycogenolysis and gluconeogenesis) during periods of starvation and exercise [22, 23]. Conversely, GLP-1 acts to enhance nutrient-stimulated insulin release and suppresses glucagon secretion [24]. The less abundant PC isoforms may also play a role in a tissue-specific manner, with mounting evidence that GLP-1 is also present in islets and that glucagon can be produced in the bowel [25].

1.2.1. Glucagon receptor distribution and signalling

The glucagon receptor belongs to the class B (secretin) G-protein coupled receptor family, a group which also includes the receptor for GLP-1. As such, both receptors are structurally similar with seven transmembrane domains at the cell surface [26]. Most glucagon receptors are located in the liver, but they are also expressed at lower levels in the kidney, heart, adrenal glands, adipose tissue, brain, gastrointestinal tract [27], as well as the pancreas where they are found on both alpha- and beta-cells [28]. However, most of these data are derived from mRNA expression patterns in rodents. In humans, glucagon receptor mRNA expression seems to follow similar distribution patterns, though there are inconsistencies in the relative abundance reported in various tissues (Figure 1c).

The wide range of tissues expressing glucagon receptors indicates that the hormone's physiological action extends beyond the prevention of hypoglycaemia, and that abnormal secretion patterns can have significant systemic consequences. In clinical practice, glucagon is used for both reveral of severe hypoglycaemia and in the management of beta-blocker overdose, due to its effects on cardiomyocytes [29].

Signal transduction via the glucagon receptor occurs through activation of two G protein classes: G_{sa} which stimulates adenylyl cyclase to produce cAMP, and G_q which stimulates phospholipase C to produce inositol trisphosphate (IP_3) [26]. The latter results in increased calcium release from the endoplasmic reticulum, while cAMP activates protein kinase A and EPAC 1 & 2 (exchange protein activated by cAMP), which induce changes in gene transcription.

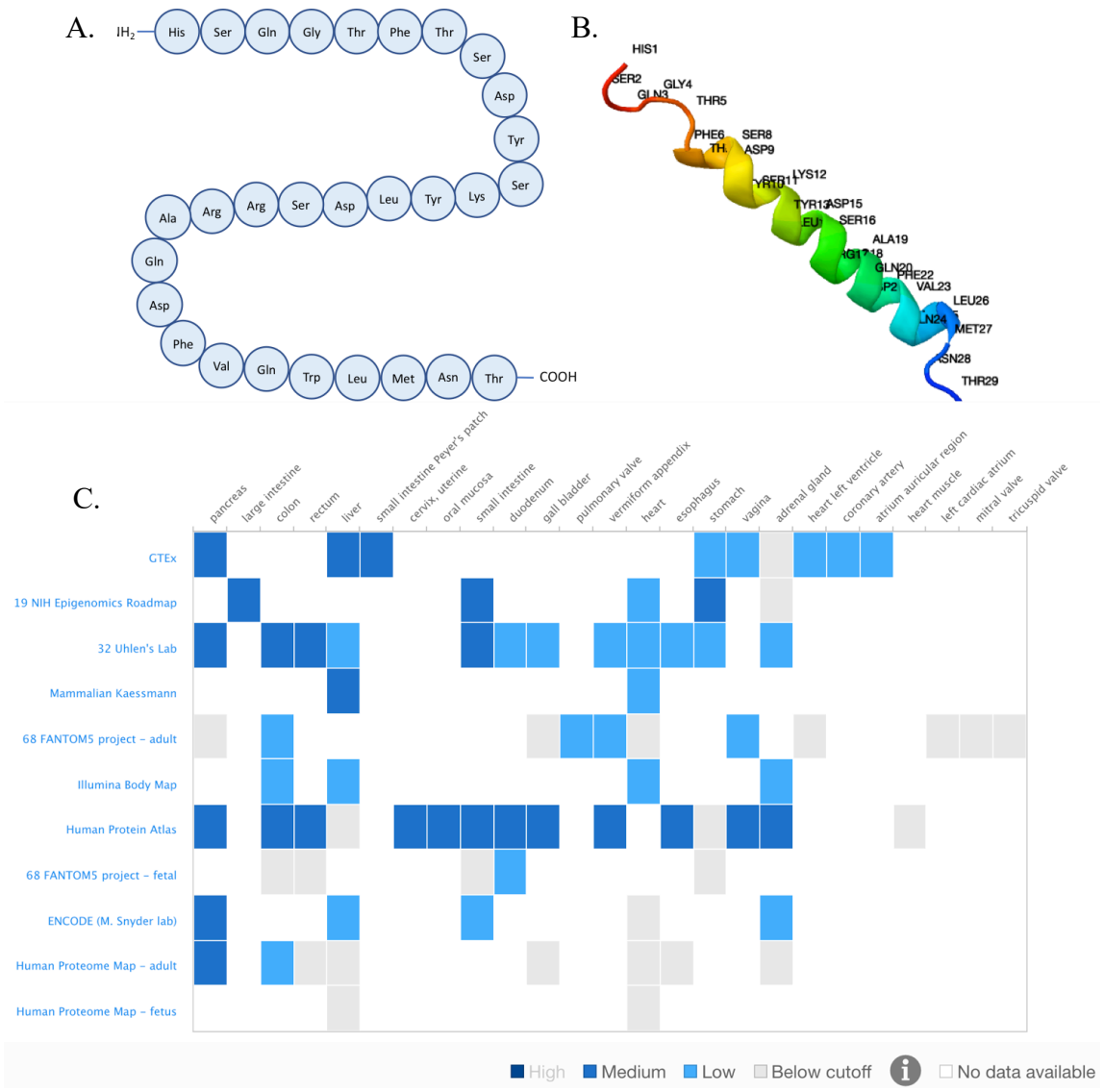
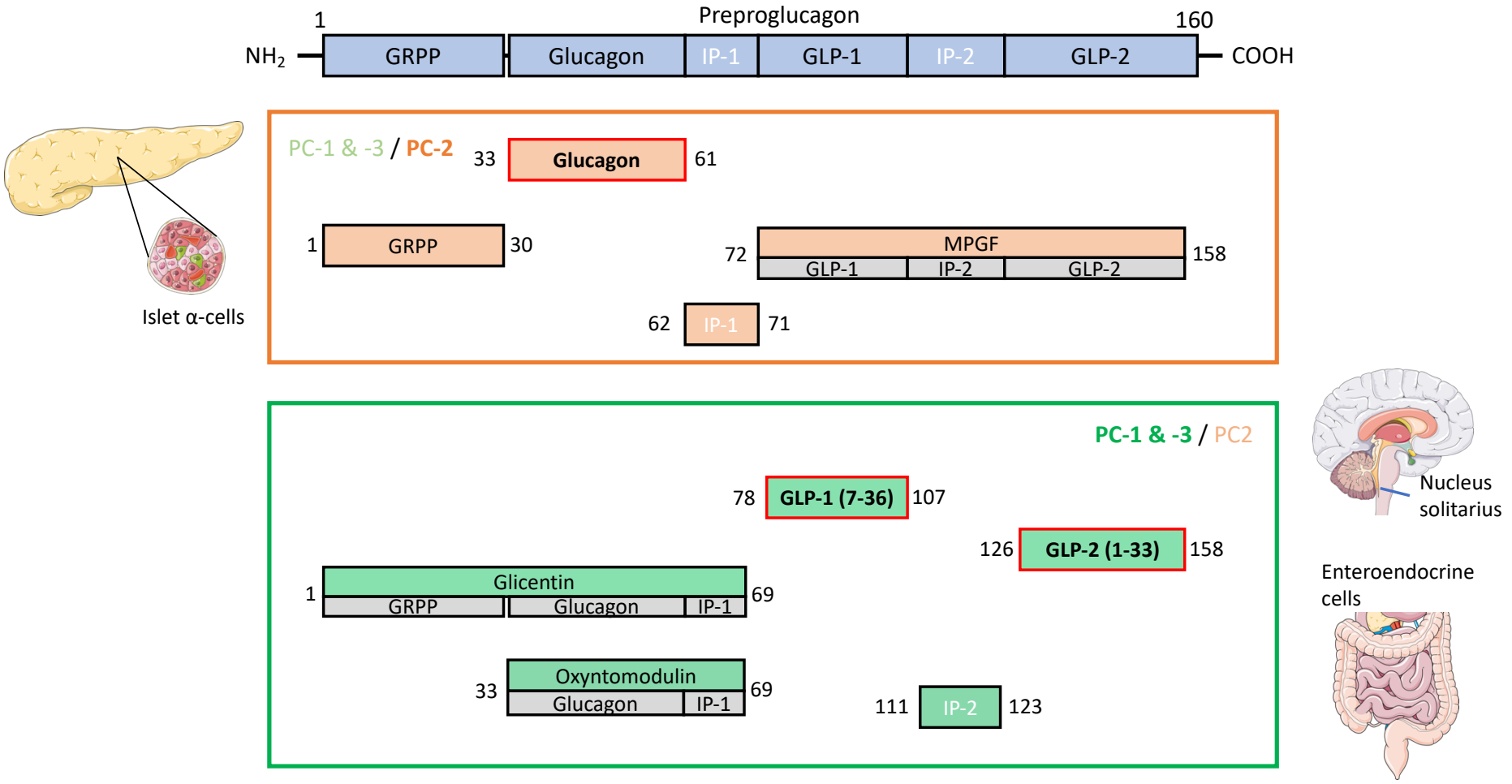


Figure 1 A: Glucagon amino acid sequence and B: tertiary structure. C: tissue distribution of glucagon receptor mRNA expression in humans. 3D illustration produced in JSmol and expression table produced in Expression Atlas [30]. Used under creative commons license.



PC-1, -2, -3 = prohormone convertase 1, 2, 3
GRPP = glicentin-related pancreatic polypeptide
MPGF = major proglucagon fragment
IP-1, -2 = intervening peptide 1, 2

Figure 2 Preproglucagon peptide processing. Note that the amino acid sequence of glucagon is shared with both glicentin and oxyntomodulin.

1.3. Regulation of glucagon secretion

It is well established that under normal conditions glucagon secretion is inhibited in euglycaemia and hyperglycaemia, and stimulated in hypoglycaemia [31, 32]. However, there is currently no over-arching model which explains the behaviour of alpha-cells in health and disease. Attempts at dissecting this problem have revealed two main processes which appear the play important roles and are not mutually exclusive. These are:

- alpha-cell intrinsic
- paracrine signalling

1.3.1. Intrinsic alpha-cell mechanisms

One of the fundamental puzzles in the field of islet research is how alpha- and beta-cells, which share a number of physiological and metabolic properties, have such a dramatically different response to glucose. Both are electrically excitable cells and respond to action potential firing via increases in the intracellular calcium concentration ($[Ca^{2+}]$), which triggers exocytosis of hormone-containing secretory granules [33, 34]. However, while beta-cells show no action potential activity during low glucose conditions, alpha-cells fire high-amplitude action potentials which activate voltage-gated calcium channels, leading to an influx of calcium into the cell and glucagon release (Figure 3). This is achieved because the ATP-sensitive potassium channel (K_{ATP}) is sub-maximally closed, allowing voltage-gated sodium channels (particularly $Na_v1.3$) and calcium channels (P/Q type) to be activated and contribute to the action potential upstroke [35, 36].

Under hyperglycaemic conditions, glucose enters via both the glucose transporter (Glut) and sodium glucose co-transporter 2 (SGLT2) present on the alpha-cells [37]. The higher

production of ATP has been proposed to cause maximal closure of the K_{ATP} channel, which leads to higher frequency but lower amplitude action potentials. This is because the voltage-gated sodium channels undergo voltage-dependent inactivation and can no-longer contribute to the action potential upstroke [38]. Calcium influx via the P/Q-type voltage-gated calcium channels is therefore inhibited, as these channels principally activate at the peak of the action potential.

There are also other intrinsic mechanisms thought to be involved in the regulation of glucagon secretion. It was demonstrated that genetic ablation of glucokinase in alpha-cells leads to loss of the inhibitory effect of hyperglycaemia [39]. Interestingly however, a high rate of glucagon secretion persisted at low glucose. While the exact mechanism(s) remain unclear, it has recently been shown that this is in part attributable to lipid metabolism [40].

1.3.2. Paracrine signalling

Islet cells are able to tightly coordinate their function, suggesting a high level of paracrine interaction. Previous studies in rodent pancreas perfusion models and in isolated human islets have suggested that the pulsatile release of insulin and somatostatin is synchronised in-phase, whereas the secretion of glucagon is anti-synchronous [41], and more recently it was discovered that beta- and delta-cells are electrically coupled via gap junctions [42].

The alpha-cell milieu contains a number of compounds that have been postulated to act as paracrine signals including beta-cell-derived GABA [43], zinc ions [44], or insulin itself [45]. However, an important argument against the beta-cell regulation of alpha-cells is that glucagon is maximally inhibited at glucose concentrations which do not stimulate significant

insulin secretion. It is therefore more likely that the delta-cells play a key role in the coordination of glucagon secretion [11, 46].

Somatostatin is a potent inhibitor of both insulin and glucagon release, and signals to the alpha-cell via somatostatin receptor 2 (SSTR2) [47]. When somatostatin binds to SSTR2 it acts via three separate mechanisms (Figure 4):

1) it activates G-protein coupled inwardly rectifying potassium channels (GIRK) which transiently repolarises the cell membrane [48]
2) it inhibits adenylyl cyclase leading to reduced cyclic AMP levels, thereby inhibiting glucagon release [49].
3) it inhibits exocytosis by a direct effect, possibly mediated by activation of the protein phosphatase calcineurin [50]

Taken together, these models of glucagon secretion suggest that the alpha-cells are able to respond to a number of different stimuli, both metabolic and electrical, and that there might be multiple mechanisms behind the aberrant glucagon secretion observed in diabetes.

SGLT2 = sodium glucose cotransporter 2
Glut = glucose transporter
K_{ATP} = ATP sensitive potassium channel
Na_v = voltage-gated sodium channel
SSTR2 = somatostatin receptor 2
GIRK = G protein coupled inward rectifying potassium channel
AC = adenylyl cyclase

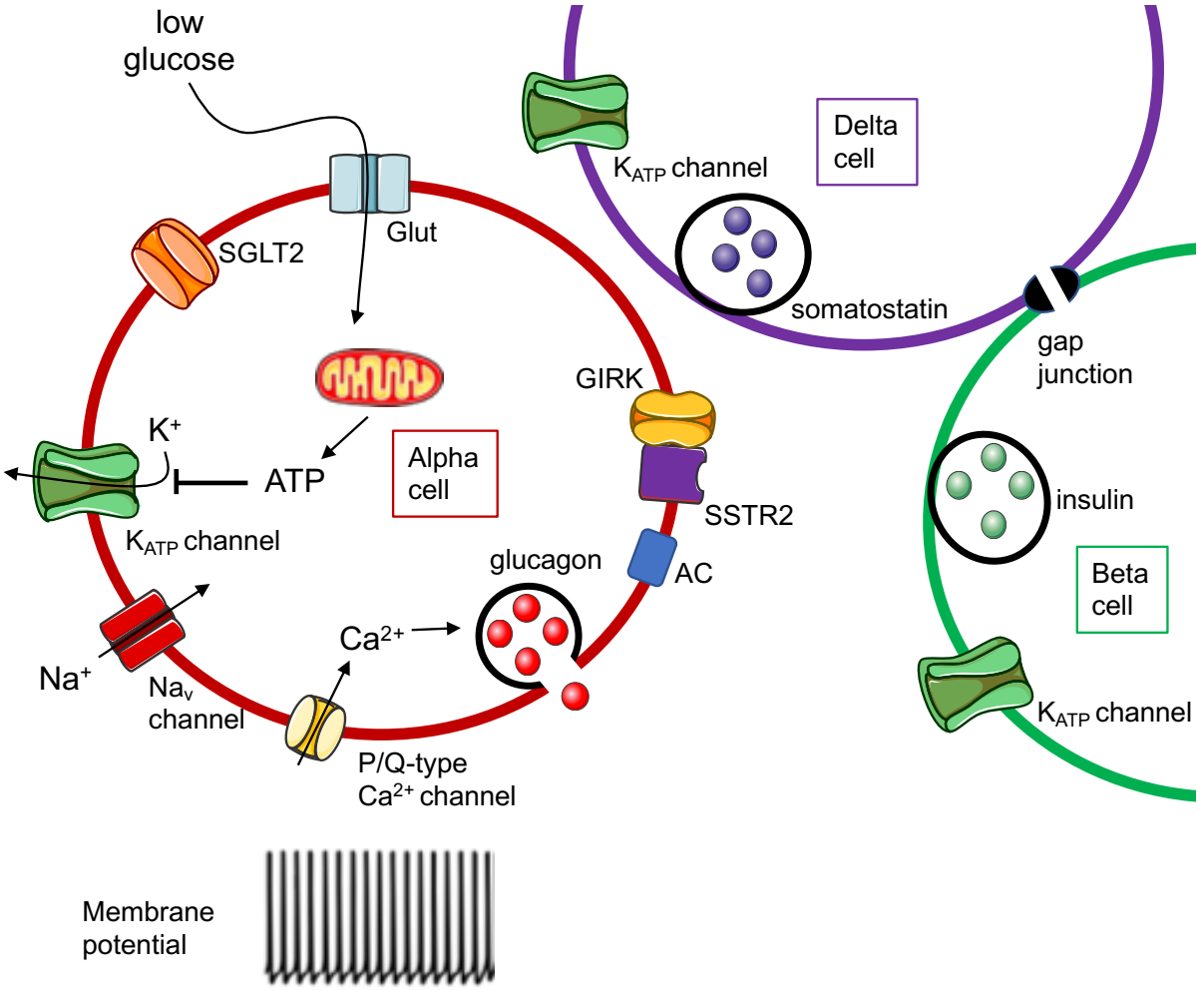


Figure 3 Model of glucagon secretion during low glucose conditions. High-amplitude action potentials activate voltage-gated sodium channels and calcium channels (P/Q-type) leading to glucagon release. Modified from [10]

SGLT2 = sodium glucose cotransporter 2
Glut = glucose transporter
K_{ATP} = ATP sensitive potassium channel
Na_v = voltage-gated sodium channel
SSTR2 = somatostatin receptor 2
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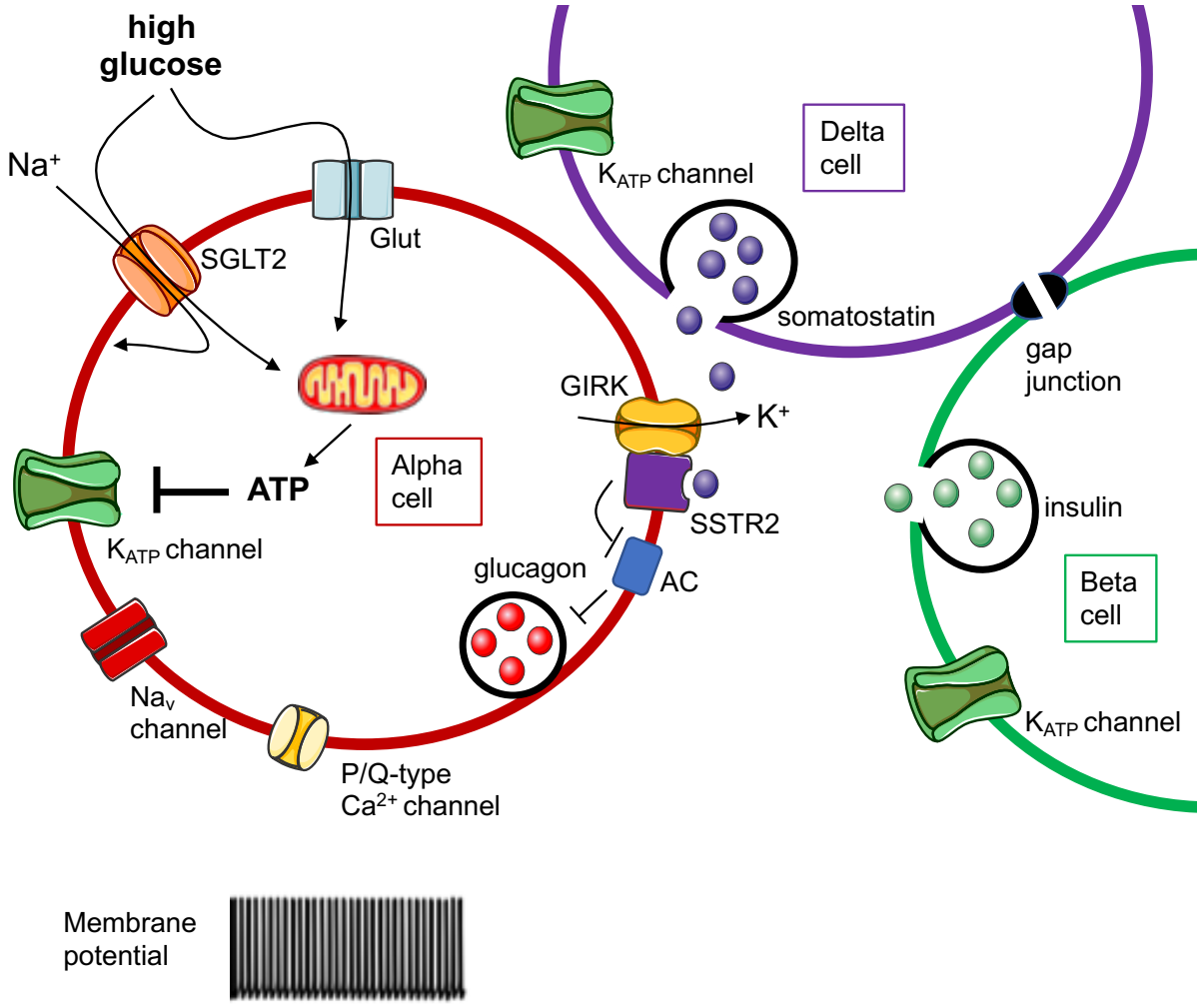


Figure 4 Model of glucagon secretion during high glucose conditions. Membrane depolarisation due to K_{ATP} channel closure increases action potential frequency but the amplitude is reduced of inactivation of Na_v channels, leading to reduced activation of P/Q-type calcium channels that are linked to glucagon exocytosis. Somatostatin inhibits glucagon release via adenylyl cyclase inhibition and activation of GIRK channels. Modified from [10].

1.4. Glucagon secretion in type 2 diabetes

It was demonstrated 50 years ago that fasting glucagon levels in patients with T2DM were comparable to those in non-diabetic individuals despite having fasting hyperglycaemia, suggesting an impairment of glucose-mediated glucagon suppression [51], and over the years the importance of aberrant alpha-cell function in the pathogenesis of diabetes has become more widely recognised [52]. Indeed, a recent clinical trial involving glucagon receptor blockade demonstrated that hyperglucagonaemia is a major contributor to the hyperglycaemia of T2DM [53]. However, it is still unclear exactly how and in whom this dysfunction manifests.

As discussed in Section 1.2, glucagon is a key hormone in the maintenance of energy homeostasis, with effects throughout the body including on appetite [54, 55], thermogenesis [56] and heart rate [57-59], in addition to its role in blood glucose and lipid metabolism [60]. As such, any disruption in its physiological regulation can have widespread consequences.

A number of studies are consistent with the original observation [61] that fasting glucagon levels in patients with T2DM are generally the same as in non-diabetic individual [62-67]. However, hyperglycaemia fails to decrease (or even stimulates) glucagon secretion after an oral glucose challenge [68]. While these fasting levels might appear “normal”, they are inappropriately raised given the higher fasting blood glucose [69]. Significantly elevated plasma glucagon levels after overnight fast [15, 70] and during an oral glucose tolerance test [71] have been reported in adult and paediatric cases [72] of T2DM compared to controls. This (relative or absolute) hyperglucagonaemia results in increased rates of hepatic glucose production [73, 74], thus further exacerbating hyperglycaemia.

On the other hand, the abnormal response of alpha-cells seen in diabetes also leads to an inappropriately low glucagon response to falling glucose levels, and a vulnerability to developing hypoglycaemia. This too was described nearly 50 years ago in patients with type 1 diabetes [75], and has more recently been documented in T2DM [76]. In this latter study, patients with longstanding T2DM failed to increase glucagon secretion during a hyperinsulinaemic – hypoglycaemic clamp compared to non-diabetic controls, and their glucagon levels were further blunted following hypoglycaemia events during the previous day. While there are limitations to this experimental set-up (exogenous hyperinsulinaemia inhibits both insulin and glucagon secretion [77]), it suggests that there is a failure of appropriate glucose-dependent hormone response rather than an overall increase in glucagon secretion. There are also indications that islet composition may play a role in aberrant hormone secretion, as a shift in alpha- / beta-cell ratio has been implicated in the pathogenesis of T2DM [78].

Taken together, the above evidence supports the concept that alpha-cells in T2DM are functionally impaired, resulting in over-secretion of glucagon during eu-/hyperglycaemia and under-secretion during hypoglycaemia. Therefore, strategies to improve the glucagon response, especially in combination with glucose-lowering medications, may be clinically useful and have attracted increasing interest in recent years [68, 79].

1.5. Role of sulfonylureas

The possible role of sulfonylureas in the restoration of appropriate glucose-dependent glucagon secretion was highlighted in recent studies performed in isolated human islets from donors with T2DM [35]. All three major islet cell types express the same isoforms of the K_{ATP} channel (Kir6.2, SUR1), and binding of sulfonylureas to the SUR1 subunit leads to

closure of the channel. As discussed in Section 1.3.1, the alpha-cell K_{ATP} channel is sub-maximally inhibited at low glucose condition which leads to high amplitude action potentials and results in glucagon release. A rise in ambient glucose concentration reduces K_{ATP} channel activity further, leading to suppression of hormone release (Figure 5). In T2DM however, K_{ATP} channel activity is increased leading to reduced glucagon secretion at low glucose, and a physiologically inappropriate stimulation when glucose is elevated [38]. This abnormal pattern of glucagon secretion was also reproduced when islets from non-diabetic mice were treated with the ATP-synthase inhibitor oligomycin [35].

When islets from donors with T2DM were treated with a low concentration of the sulfonylurea tolbutamide (10μM), the inhibitory effect of glucose was restored. This is thought to be achieved by slightly reducing alpha-cell K_{ATP} channel activity such that the starting point is shifted to the physiological left side of the bell-shaped glucagon-response curve (Figure 6), leading to appropriate glucose-induced glucagon suppression.

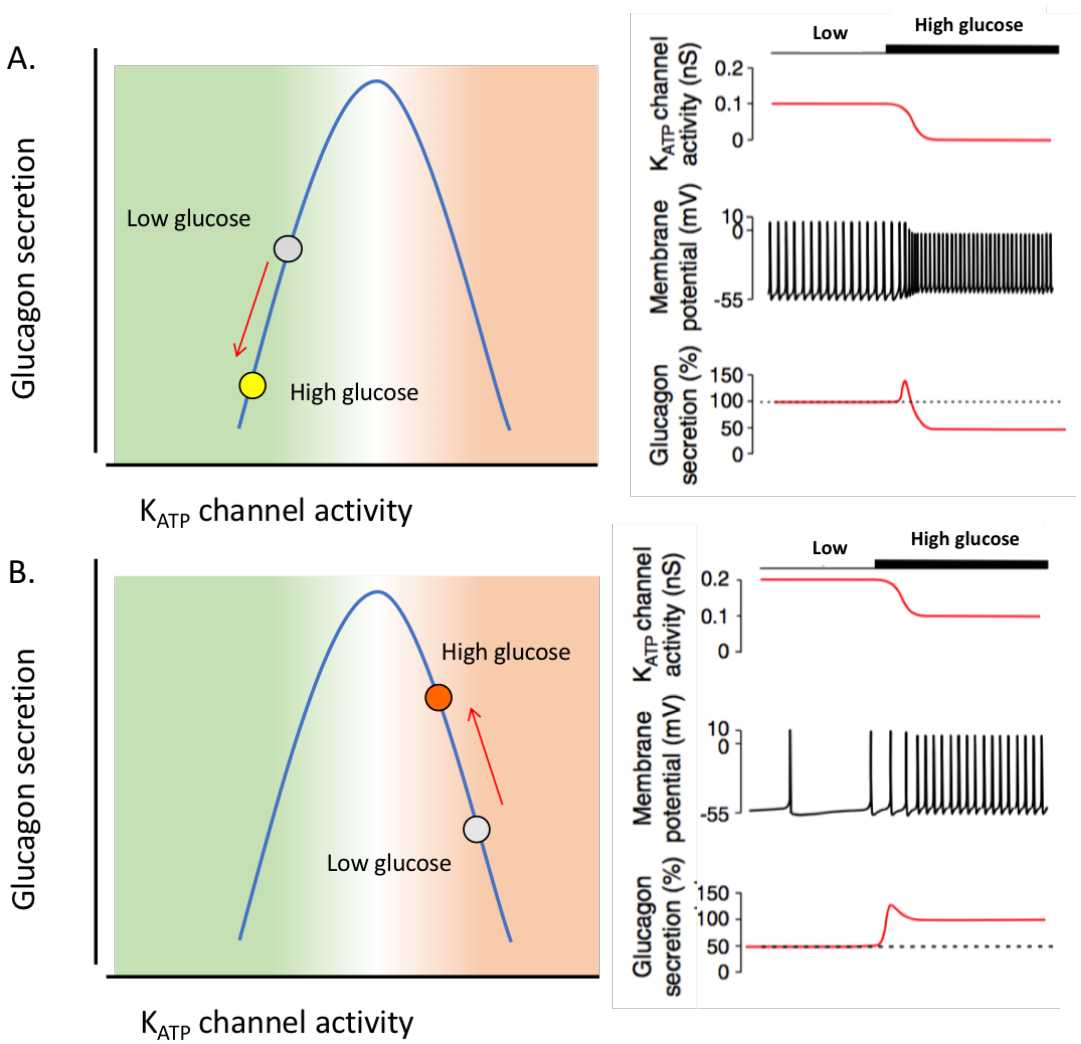


Figure 5 Schematic representation of the bell-shaped relationship between glucagon secretion and alpha-cell ATP-sensitive potassium (K_{ATP}) channel activity during hypoglycaemic and hyperglycaemic conditions in healthy individuals (A) and in patients with T2DM (B). Also represented on the right is membrane potential showing individual action potentials. Adapted from [38].

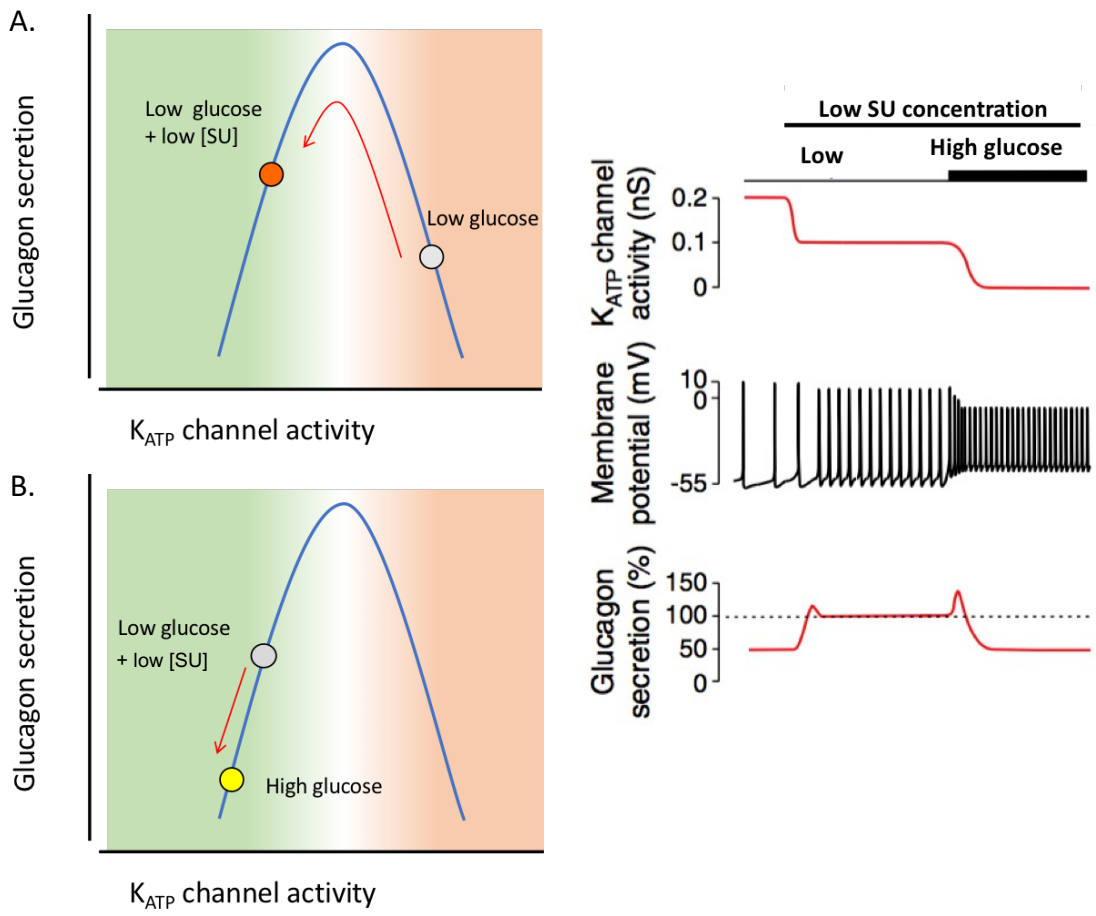


Figure 6 Schematic representation of the bell-shaped relationship between glucagon secretion and alpha-cell ATP-sensitive potassium (K_{ATP}) channel activity during hypoglycaemic (A) and hyperglycaemic (B) conditions in patients with T2DM who have been treated with low concentrations of sulfonylurea (SU). Also represented on the right is membrane potential showing individual action potentials. Adapted from **[38]**.

1.5.1. Gut-derived glucagon

While there have previously been studies investigating extra-pancreatic glucagon secretion, it has only recently been confirmed that full-size glucagon (29 amino acids) is secreted in patients who have undergone total pancreatectomy [25]. The origin of this is likely to be the enteroendocrine L-cells, via alternative processing of the pro-glucagon peptide (Figure 1). Plasma levels of extra-pancreatic glucagon were shown to be lower than in healthy controls at baseline, however they increased significantly during an oral glucose tolerance test (OGTT). This increase was not observed in the control group (in which glucagon levels were appropriately suppressed), nor was it seen after an intravenous glucose infusion, suggesting that direct stimulation of the intestinal mucosa is important. These findings raise the possibility that the inappropriately high levels of glucagon observed in T2DM may at least in part be due to secretion from the gut.

1.6. Technical aspects of glucagon measurement

It is important to recognise that there have been significant improvements over the years in the measurement of glucagon since the first radio-immunoassay was developed [80], coupled with better understanding of the lability of this peptide hormone in plasma samples and the different tissues in which it is produced. These facts may go some way to explaining the inconsistencies that are observed in some older (but also in more recent) studies of glucagon in T2DM, however this still remains a controversial topic.

There are currently 100 active clinical trials listed on clinicaltrials.gov which use glucagon measurement as an endpoint, with over 600 registered in the entire database (see Figure 7). Many of these involve repeated measurements of plasma glucagon following interventions or

challenges over a period of time, however there is still no worldwide consensus regarding how blood samples should be processed in this context.

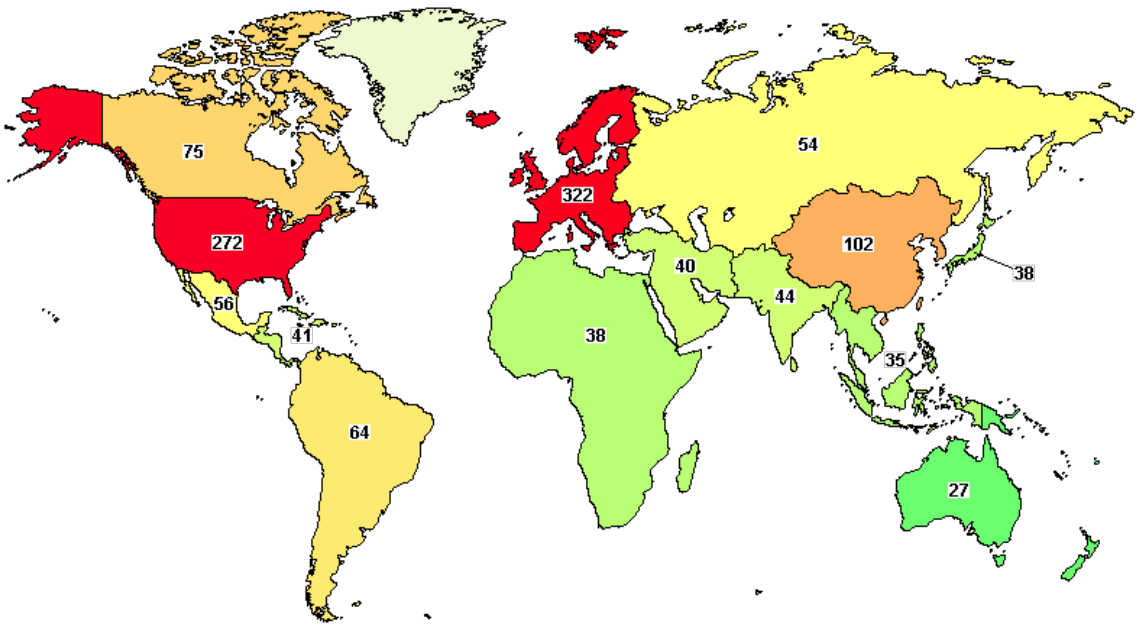


Figure 7 Worldwide distribution of clinical trials (active and completed) which list glucagon measurement as a study endpoint (source: www.clinicaltrials.gov , last accessed 22/1/18).

1.6.1. Glucagon degradation and clearance

Glucagon is mainly degraded via its G protein-coupled receptors, through a process of internalisation [81], and the insulin-degrading enzyme located in the liver and kidneys [82-84]. Glucagon is not as sensitive to proteolysis by the serine protease dipeptidyl peptidase 4 (DPP4) as the closely related glucagon-like peptide 1 (GLP-1) under physiological conditions [85], however it does undergo significant degradation in the circulation.

Previous studies in participants with and without T1DM indicate that glucagon is cleared from the circulation at around 9 ml/kg/min, and has a half-life ($t_{1/2}$) in the circulation of around 5 min [86, 87]. Both hepatic and renal extraction account for the majority of glucagon

clearance from the circulation [88, 89]. It is also not clear whether and to what extent glucagon clearance is altered in patients with T2DM, and this is currently the focus of a recent clinical trial (NCT02475421).

1.6.2. Methodological considerations

Early studies during the development of the glucagon radioimmunoassay (RIA) concluded that glucagon was significantly degraded during venous blood collection unless a protease inhibitor (Trasylol®, the tradename of aprotinin) was added [90]. Since then, commercial glucagon assay kits and hormone reference laboratories generally recommend that blood is drawn-up in pre-chilled EDTA tubes (usually containing a trypsin inhibitor such as aprotinin), kept on ice and centrifuged within 10 minutes at low temperature before storage in a -20^{o}C freezer (or -80^{o}C if extended storage time is required) [91]. This process makes blood collection for glucagon analysis a substantial undertaking during clinical trials, particularly if they involve distal collection sites or home visits.

Recently however, this process has been re-examined with the benefit of more sensitive and specific modern assays (including commercially available RIA, ELISA and mass spectrometry). These include the development of "sandwich" immunoassays, which rely on antibodies binding to two different epitopes on glucagon (at the N- and C-terminus) and are therefore very sensitive at detecting the intact peptide.

In small-scale studies, Emmen and colleagues concluded that plasma glucagon is stable even at room temperature for up to 3 days using a RIA kit (Millipore, USA) [92], while Cegla and colleagues also noted that the addition of aprotinin, a DPP4 inhibitor, or a combination of the

two had no impact on the detectable glucagon levels using liquid chromatography tandem mass spectrometry (LC-MS/MS) and two sandwich immunoassays (Homogenous Time Resolved Fluorescence HTRF©, Cis-Bio, France; Milliplex MAP Human Metabolic Hormone Panel, Merck Millipore, Germany) [93]. Furthermore, storage at -80ºC for up to 9 months and two freeze/thaw cycles did not appear to have a significant effect on glucagon degradation, although there was a trend towards lower values in the mass spectrometry data. The authors suggested that the apparent lability of glucagon in early studies may have been due to cross-reactivity of the immunoassays with GLP-1, which is much more susceptible to DPP4-mediated proteolysis.

In contrast to the above studies, Wewer Albrechtsen and colleagues reported a significant impact of the freezing process on glucagon (but not GLP-1) levels, however they also confirmed that the addition of DPP4 inhibitor was unnecessary for glucagon measurement [94]. As part of the experimental set-up they used samples which were spiked *in vitro* with glucagon, rather than measuring endogenous glucagon, used various enzyme inhibitors, stored the samples at -20ºC or -80ºC for up to 1 year and performed up to 3 freeze/thaw cycles. Glucagon measurements were made using in-house RIAs developed by the same group, which were specific for either the intact C-terminus or N-terminus and had previously been validated [95]. They concluded that glucagon is particularly vulnerable to long-term storage, with up to 50% loss occurring within 1 month (possibly via non-enzymatic fibrillation of the protein) regardless of method of collection or storage, however this then remained stable for up to 1 year. The implication of this work is that studies which batch-process their samples may be underestimating the absolute concentration of plasma glucagon. However, this would not necessarily affect projects investigating between-group differences,

nor does it explain the wide range of "normal" absolute values which are present in the literature.

1.6.2.1. Accuracy of modern glucagon assays

As described above and in Section 1.2, glucagon shares significant amino-acid sequence homology with other proglucagon products which can result in cross-reactivity when using antibody-mediated assays, leading to a loss of specificity. This becomes even more important when considering that glucagon concentrations in the peripheral circulation are in the picomolar range [96] (often close to the detection limit of many assays), as the majority of alpha-cell-derived glucagon is secreted into the portal vein and remains in the liver [97].

A variety of studies have reported inconsistencies between different types of glucagon assays [95, 98-100], and have highlighted potential pitfalls when interpreting results of dynamic studies. Bak and colleagues examined the accuracy of 10 commercially available assays in detecting plasma glucagon collected during hypoglycaemic and hyperglycaemic conditions (n=5 normoglycaemic controls), and synthetic glucagon in phosphate buffered saline by comparing the results to those obtained by quantitative amino acid analysis and their own validated in-house RIA [101]. They noted that the Millipore RIA (Billerica, MA, USA) was the most sensitive and specific, however the absolute values were more than twice those measured using their in-house RIA. Importantly, the Millipore assay did not detect a significant drop in glucagon levels during hyperglycaemia. The same group then went on to examine the ability of assays (their own RIA and 3 different ELISA-based products) to detect the suppression of endogenous glucagon in healthy volunteers [99] during a euglycaemic clamp (glucose = 6 mmol/L) with and without the administration of atropine (which further inhibits glucagon secretion by blocking muscarinic receptors). Of those tested, a reduction in

glucagon values was detected only by their in-house RIA and the Mercodia ELISA (Uppsala, Sweden). The authors commented that the more advanced assay techniques employing electrochemiluminescence (MSD, USA) and homogenous time-resolved fluorescence (Cis-Bio, France) did not in fact perform as well as the standard spectrophotometry-based ELISA, and that particular care must be taken when deciding on which assay to use in clinical research.

This discrepancy between assays using one antigenic site (RIA) versus two (sandwich ELISA) was independently supported by a different group using OGTTs in patients with T2DM [100], and by one investigating glucagon secretion patterns in young women with a history of gestational diabetes [98]. Finally, it was also noted that the hyperglucagonaemia seen in patients with end-stage renal disease when measured by RIA is an artefact, possibly caused by cross-reactivity with other proglucagon derivatives, whereas sandwich ELISA measurements showed levels in the normal range [102].

Overall, there remains a high degree of uncertainty when measuring plasma glucagon levels and this has significant implications for both clinical research and practice. At present, best practice (which errs on the side of caution) appears to involve a combination of early sample centrifugation at low temperatures, storage at -80C for the least amount of time and without freeze-thaw cycles, and measurement using a “sandwich” assay in a validated laboratory.

1.7. Aims of thesis

The overall aim of this thesis was to test the hypothesis that low-dose sulfonylureas can reduce aberrant glucagon secretion in diabetes mellitus. The aims of the individual projects were as follows:

a) Investigate the donor factors which influence insulin and glucagon secretion from human islets *in vitro.*

b) Construct a dose-response curve of glibenclamide for insulin and glucagon in human islets and establish a dose-range for use in the clinical trial.
c) Identify the dose of glibenclamide which leads to a reduction in fasting hyperglucagonaemia in patients with T2DM.

d) Examine the impact low-dose gliclazide has on fasting and post-glucose-challenge glucagon secretion in patients with HNF1-α and HNF4-α MODY (Maturity Onset Diabetes of the Young).

1.8. Summary of chapters

Chapter 2 describes the methodology used in the preliminary *in vitro* experiments, and the protocols of the clinical studies.

Chapter 3 describes the analysis of the human islet hormone secretion database in Oxford, and discusses the results regarding the impact of donor factors.

Chapter 4 describes the purpose and results of the *in vitro* hormone secretion experiments performed initially in mouse islets and then in islets from diabetic and non-diabetic human donors.

Chapter 5 describes the set-up and results of the "Low DosE GlibENclamide in Diabetes – part A" (LEGEND-A) pilot clinical trial.

Chapter 6 describes the set-up and preliminary findings of the "Glucagon in MODY" pilot clinical study.

Chapter 7 provides an overall summary of the experimental work and a discussion of the conclusions that can be drawn.

Chapter 8 briefly describes the plans to continue the on-going work and extend the clinical studies.

2. Chapter 2: Methods

2.1. *In vitro* methods

The following methods were used during the hormone secretion assays for both mouse and human islets.

2.1.1. Reagents

2.1.1.1. KRB (Krebs Ringer Buffer)

Krebs Ringer Buffer was made up according to the recipe in Table 1 and using double distilled water. The pH of the final solution was adjusted to 7.35 – 7.45 at room temperature using 1M NaOH, and refrigerated until use.

Table 1 KRB solution

KRB (1000 ml)	mass (g)	MW	Molarity (mM)
NaCl	8.18	58.44	139
KCl	0.268	74.55	3.5
$MgSO_4.7H_2O$	0.123	246.48	0.5
NaH_2PO_4	0.059	119.98	0.5
$NaHCO_3$	0.168	84.01	2.0
HEPES	1.19	238.3	5.0
$CaCl_2$	0.288	110.99	2.6

2.1.1.2. RIA dilution buffer

The following buffer was used to dilute the samples from the hormone secretion experiments during the radio immunoassays (RIA) and other assays which did not involve horseradish peroxidase:

- Hanks balanced salt solution (HBSS) (Sigma H8264)
- 0.025% w/v Bovine serum Albumin (BSA) suitable for RIA (Sigma A7888)
- approximately 10mg Sodium azide (Sigma A438456)

2.1.1.3. Acidified ethanol solution

Ice cold acidified ethanol was added to the islets during the hormone secretion experiments in order to extract the intracellular content:

- 74% w/w ethanol
- 24% w/w double distilled water
- 1% w/w HCl (Sigma 435570)

2.1.2. Islet isolation (mouse)

For the preliminary *in vitro* work, islets were isolated from either NMRI or C57BL/6J mice (female only) ranging from 13 – 23 weeks old. The mice were killed by Schedule 1 technique. The abdomen was then opened and a suture placed at the ampulla of Vater. A 30G needle was inserted caudally into the common bile duct and 2ml of ice cold Hanks buffered salt solution (HBSS) containing 1mg/ml of Liberase™ TL (Sigma 05401020001) were injected to distend the pancreas. The pancreas was dissected and transferred to a 15ml tube on ice.

The pancreas was then transferred to a shaking water bath and incubated at 37°C for 13-14 minutes (depending on experience with the age of the animals and the batch of Liberase™). Ten ml of ice cold HBSS containing 2mg/ml BSA (Sigma A8806, essentially fatty acid free) was added, and the tube was shaken vigorously for approximately 1 minute. The tube was then inserted upright into a bucket of ice and the islets were allowed to sediment by gravity for 3 minutes. The supernatant was aspirated off and the pellet re-suspended by shaking in 10ml of ice cold HBSS with BSA as above, before being allowed to sediment again for 3 min. The washing process was repeated a total of 3 times. The isolated islets were then hand-picked into a cell-suspension dish containing RPMI culture medium with 5mM glucose, and transferred to a CO_2 incubator at 37°C for 30-60 minutes.

2.1.3. Human islets

Human islets which became available for research purposes (i.e. with appropriate consent for use in research) were obtained from the Oxford Human Islet Isolation Facility, as part of the Islet Cell Transplantation programme. These included islets isolated in Oxford or at King's College London. Islets obtained from this source were either kept in UW (cryopreservation) medium at 4°C, or in CMRL culture medium at 37°C. As part of the transplantation programme, these islets were cultured for a minimum of 24 hours in CMRL containing human albumin and 5mM glucose, prior to release for research.

Prior to the hormone secretion experiments, islets stored in UW were hand-picked into RPMI with 5mM glucose and transferred to a CO_2 incubator at 37°C for 30-60 minutes. Otherwise, islets were used directly from CMRL medium.

In addition, certain human islet preparation were available from a collaboration between Dr. Quan Zhang and Dr. Patrik MacDonald at the IsletCore facility, University of Alberta,

Edmonton, Canada. These islets were obtained with appropriate consent for use in research, and were shipped via courier in CMRL medium at room temperature. Upon arrival were hand-picked into RPMI medium with 7.5mM glucose and kept in a CO_2 incubator at 37°C overnight.

2.1.4. Static incubation

All islets used were hand-picked, preferentially selecting visually intact, small to medium-sized islets. This was done to minimise the variation in islet cell composition, as the ratio of alpha- to beta-cells is significantly different in larger islets [103].

Depending on the experimental protocol used, 13-20 islets per condition were transferred to 500µl tubes containing 300µl of pre-incubation buffer (KRB solution containing 2mg/ml BSA and 3mM glucose). The tubes were then incubated at 37°C for one hour, either using an air incubator with a humidified chamber, or a water bath. The pre-incubation buffer was then replaced with test-condition incubation solution and returned to the incubator for one hour. Care was taken so as to cause as little mechanical disturbance to the islets as possible. The supernatant from each tube was then transferred to a new 500µl tube ("supernatant"), and 100µl of ice-cold acidified ethanol was added to each original tube containing on the islets ("contents"). All tubes were stored at -20°C, or -80°C. The test-condition solutions varied depending on the experiment, but all contained KRB solution with 2mg/ml BSA (essentially fatty acid free).

2.1.5. Serial incubation

Islets were hand-picked as described above (Section 2.1.4)

A variation on the standard “static incubation” method was developed in order to accommodate experimental protocols (such as the glibenclamide dose-response curve) which included a large number of experimental conditions. This “serial incubation” method was identical to the “static” method up until the end of the pre-incubation stage, after which the islets were exposed to the test-condition incubation solutions in succession, with the supernatant being stored separately on dry ice after each condition change.

The experimental protocols which used this technique were designed so that exposure to the compound of interest would increase with each condition change (e.g. increasing concentration of glibenclamide). This was particularly important for compounds which were dissolved in DMSO, as carry-over from condition to condition would be likely. In addition, a “time control” using the basal condition was used in certain protocols to take into account the variation in hormone secretion over time.

2.2. Assays

2.2.1. RIA, MSD

Radio-immunoassays (RIA) were performed for rodent insulin (Millipore RI-13K), human insulin (Millipore HI-14K), glucagon (Euro Diagnostica RB310) and somatostatin (Euro Diagnostica RB306) according to the manufacturers’ protocols, and after appropriate training in “Radiation Safety for Laboratory Workers”.

Insulin and glucagon were also assayed using the duplex Meso Scale Discovery kit (MSD K15145C), according to the manufacturer's protocol. While the kit is marketed as specific for mouse and rat hormones, the standards used are synthetic human glucagon and recombinant human insulin, and both the capture and detection antibodies have cross-reactivity between human and rodent. Therefore, this kit was used in experiments involving either mouse and human islets. The technical characteristics of each assay, including cross-reactivity, can be found in Table 2.

Prior to assaying, all "contents" samples were individually sonicated for 10 seconds on ice.

Table 2 Product characteristics of the assays used in this project. Data are derived from the manufacturers' technical specifications.

Assay	%CV		detection limit	Cross-reactivity	
Euro Diagnostica Glucagon RB310 RIA	low	8.1	3 pmol/L	Glucagon pancreatic, human Glicentin Secretin Cholecystokinin -39 Vasoactive intestinal peptide Gastric inhibitory peptide GLP-1 Oxyntomodulin	100 % < 0.1 % < 0.02 % < 0.02 % < 0.02 % < 0.02 % < 0.1 % < 0.1 %
	high	4.5			
Millipore Rodent Insulin RI-13K RIA	low	2.2	0.081ng/mL	Rat insulin I Rat insulin II Human insulin Human Proinsulin Mouse insulin Glucagon Somatostatin Pancreatic polypeptide IGF-I Human IGF-I Human IGF-II	100 % 100 % 100 % 69 % 100 % 0 % 0 % 0 % 0 % 0 % 0 %
	high	4.6			
human insulin Millipore HI-14K RIA	low	3.1	2.715 µU/mL	Human insulin Human proinsulin Rat insulin IGF Glucagon Somatostatin Pancreatic polypeptide	100 % < 0.2 % 0.1 % 0 % 0 % 0 % 0 %
	high	4.4			
MSD K15145C) (Glucagon)	low	7	29 pg/mL	Capture antibody Species cross-reactivity: Detection antibody Isoforms recognised: Species cross-reactivity:	 Human, mouse, rat pancreatic glucagon, reacts weakly to gut glucagon Human, mouse, rat
	high	6			
MSD K15145C) (Glucagon)	low	8	45 pg/mL	Capture antibody Isoforms recognised: Species cross-reactivity: Detection antibody Isoforms recognised:	 Does not react with human proinsulin, rat or human c-peptide Human, mouse, rat Does not react with human proinsulin, rat
	high	3			

				Species cross-reactivity:	or human c-peptide Human, mouse, rat
Somatostatin (Euro Diagnostica RB306)	low	8.3	6 pmol/L	Somatostatin, cyclic Tyr^{1}-somatostatin Linear somatostatin Tyr^{11}-somatostatin Des-ala-gly-somatostatin	100 % 100 % 50 % 38 % 25 %
	high	2.8			

2.2.2. Glibenclamide assay (mass spectrometry)

In collaboration with Dr. Rod Chalk (Structural Genomics Consortium, SGC, University of Oxford), a mass spectrometry assay was developed to quantify low concentrations of glibenclamide in human plasma. This was based on a previous protocol used to determine the concentration of glibenclamide in rat blood and CSF samples [104]. Both the facilities in OCDEM (Oxford Centre for Diabetes, Endocrinology and Metabolism) and the SGC had appropriate standard operating procedures in place for handling and storing human plasma (-80^{o}C), and all samples stored in the SGC were rendered acellular (after centrifugation at 2,000 x g for 10 minutes) and therefore contained no tissue relevant to the Human Tissue Act 2004.

2.2.2.1. Preparation of glibenclamide standards

For the glibenclamide calibration standards, a stock solution of glibenclamide (Santa Cruz Biotechnology) was dissolved in methanol (1mg/ml). Human plasma (donated by Dr. Ioannis Spiliotis) was spiked with the stock glibenclamide solution, and using serial dilutions the following standards were prepared: no drug, 1 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml, 30 ng/ml, 40 ng/ml, 50 ng/ml, 100 ng/ml 150 ng/ml. Quality control (QC) samples were also prepared at 50 ng/ml.

Deuterated glibenclamide (d_{11}-glibenclamide, Santa Cruz Biotechnology) was used as the internal standard, and was added at a concentration of 333 ng/ml into all the calibration, QC and study samples.

2.2.2.2. Study sample preparation and extraction

Study plasma samples were thawed and centrifuged at 14,000 x g for 10 minutes prior to use, and 60µl was added to 20µl of internal standard (at 1µg/ml) and acidified with 80µl of 4% orthophosphoric acid (from 85% w/w stock). The samples were then diluted by adding 800 µl of buffer A (2% acetonitrile, 0.1% formic acid), processed by reverse phase solid phase extraction (C18-SPE, Biotage Isolute C18). Samples were washed with a further 1 ml buffer A followed by 1 ml 10 % acetonitrile and then eluted using two volumes of 150 µl buffer B (80% acetonitrile, 0.1% formic acid). The eluates were then transferred to labelled 1.5ml Eppendorf tubes and evaporated using a Rotovac vacuum centrifuge at 35^{o}C for 1-2 hours. The residue was re-suspended in 12µl of 100% methanol and 48µl of 0.5mM ammonium formate using an ultrasonic bath for 10 minutes. Finally, the samples were centrifuged for 10 minutes and the supernatant was transferred to the liquid chromatography tandem-mass spectrometry (LC-MS/MS) instrument.

2.2.2.3. Liquid chromatography tandem-mass spectrometry

LC-MSMS was performed using a Dionex U3000 nano HPLC coupled to a Bruker Esquire HTC ion trap mass spectrometer. Chromatographic separation was performed using a 50 mm x 2.1 mm, 2.6µm Accucore™ C18 RP column and pre-column (ThermoFisher Scientific 17626-052130). A gradient of 20-35 % buffer B was developed over 1 minute, then 35-90 % B over 5 minutes, followed by isocratic elution at 90 % B for 3 minutes and equilibration at

20 % B for 2 minutes at 200 µl/min flow rate. The mass spectrometer was operated in positive ion mode with a scan range 300-600 m/z and scan speed 26,000 m/z/sec. The source parameters were: nebuliser gas 10 psi, drying gas 5 l/min, drying gas temperature 300°C, voltage 4000V. Multiple reaction monitoring parameters were programmed for transition 494.4 m/z to 369.0 m/z (glibenclamide) and transition 505.1 m/z to 369.0 m/z (d11 glibenclamide) with isolation widths 3 Da and 4 Da respectively. Sample injection volume was 2 µl. All samples were preceded by a blank injection and analysed in triplicate. Data analysis was performed using QuantAnalysis 2.0 software (Bruker Daltonik).

2.3. LEGEND-A trial

2.3.1. Pre-trial patient involvement project

As part of the preparation for the clinical trial protocol, a questionnaire was sent out by post to 68 patients with type 2 diabetes who had previously given their consent to have their details stored on the Clinical Research Unit (CRU) database for the purposes of being contacted regarding future research. The letter introduced the planned clinical trial and included a specific section on why this research was being carried out, how the investigation would take place, what the aims were and how the participants could help plan this study. It also included information about the continuous glucose monitoring (CGM) system.

Participants were asked how likely they would be to take part in the study if CGM was compulsory, the usability of meal/exercise diaries and the dosing schedule. They were also encouraged to provide comments about each of the questions, and had the opportunity to register an interest in taking part in this study when it began.

2.3.2. Clinical trial design

This investigator-initiated clinical trial of an investigational medicinal product (CT-IMP) was a single-centre, open-label, non-randomised, dose finding study. It was conducted in the CRU at the OCDEM at the Churchill Hospital, Oxford.

2.3.3. Sample size calculation

No human trials had previously used doses of glibenclamide under 5mg (the normal starting dose in the management of T2DM) for the measurement of insulin and glucagon secretion. The sample size calculations were based on data from isolated human islets from T2DM donors [35], which suggested that 15 participants (allowing for a 15% dropout) would give the study 80% power to detect a 57% reduction in baseline plasma glucagon (the primary endpoint) with an alpha error of 5%. It was also predefined that if additional drop-outs occurred then further participants would be recruited in order to avoid loss of statistical power.

2.3.4. Statistical plan

Two-way repeated measures Analysis of Variance (Two-way RM ANOVA) with post-hoc multiple comparisons (Holm-Sidak method) was used to compare baseline (visit 3) plasma concentrations of glucagon, glucose, insulin and c-peptide, with those at each dose change. Bonferroni correction was performed for the existence of two subgroups: 'Normal' and 'High' fasting plasma glucagon, defined in this study as >15pmol/L (normal range 6-12pmol/L, [96]). This strategy was predefined in the trial protocol.

Analyses were performed using SigmaPlot version 13 (Systat Software, London, UK) and GraphPad Prism version 7 (CA, USA) statistical software. All statistical significance was assessed using a p value of 0.05 (95% confidence interval).

It is known from previous observational studies that fasting glucagon levels in both obese and non-obese patients with T2DM can be 50% higher than in non-diabetic controls [15]. Therefore a 57% reduction would be in keeping with normalisation of fasting plasma glucagon levels.

2.3.4.1. Subgroup inclusion

Participants and research staff were blinded to the baseline values and therefore also to subgroup inclusion ('Normal' or 'High'), as glucagon measurements were only performed after completion of the trial. Two baseline fasting samples were collected: prior to (visit 2) and at the beginning of the trial (visit 3), and the mean was used to determine group inclusion for analysis.

2.3.5. Recruitment and screening

Potential participants with T2DM were recruited to the study from existing clinical trial registers at the CRU, OCDEM, and the Oxford Biobank. All individuals had previously consented to being contacted regarding this type of research, and were sent a trial invitation pack. This included a covering letter, asking them whether they would like to join the trial, the Participant Information Leaflet, a reply slip and a prepaid envelope. They were able to indicate whether they preferred a telephone, email or face-to-face discussion to have the trial protocol explained to them. Everyone who indicated an interest in participating was invited to a screening visit at the CRU.

Screening visits were performed by the research nurse and the Chief Investigator (CI, Dr. Ioannis Spiliotis), and involved obtaining informed consent, checking inclusion/exclusion criteria (see below), concomitant medication, demographics and clinical observations (heart rate and blood pressure). Blood samples were taken for HbA1c, full blood count, renal function tests, electrolytes and liver function tests, and a pregnancy test was performed in all women of childbearing age (18-49 years old) unless they were using some form of contraception.

2.3.5.1. Informed consent

The participants personally signed and dated the Informed Consent Form (Appendix A.1) before any trial-specific procedures were performed. It was clearly stated that the participants were free to withdraw from the trial at any time and for any reason without prejudice to future care, and with no obligation to give the reason for withdrawal. The CI obtained consent from all participants, and a letter was sent to their GP indicating they were taking part in the trial.

Participants were also given the options of consenting to wearing a continuous glucose monitor (see Section 2.3.5.4), and to storage of an aliquot of their blood samples for future research. Such samples were deemed a "gift" to the University of Oxford and would remain anonymised.

2.3.5.2. Inclusion criteria

Potential participants were deemed eligible for the trial if all the following criteria applied:

- Diagnosis of T2DM.
- Age 18 years or over.
- Diet controlled or on metformin only for diabetic control.
- Body mass index 40 kg/m^2 or less.
- HbA_{1c} 6.0% to 9.5% (42mmol/mol to 80mmol/mol) inclusive.

2.3.5.3. Exclusion criteria

Potential participants were excluded from the trial if any of the following applied:

- Taking anti-diabetic therapies other than metformin
- Pregnancy or women of childbearing age without adequate contraception
- Women who were breastfeeding
- Major psychiatric disease including diagnosed eating disorders, history of drug or alcohol abuse
- Known sight-threatening retinopathy
- Renal impairment (eGFR < 60 ml/min; CKD Stage 3)
- Abnormal liver function tests (> 1.5 x upper limit of normal range)
- Known ischaemic heart disease or heart failure
- Known history of a stroke
- Known history of porphyria
- Concomitant use of miconazole or other oral antifungal medication.
- Known or suspected allergy to trial product or related products
- Oral steroid treatment 30 days prior to the start or at any time during the trial period.

- Known malignancy or any other condition or circumstance which, in the opinion of the investigator, would affect the patient's ability to participate in the protocol.
- Ketoacidosis
- Felt to be unsuitable to participate in the trial in the opinion of the Chief Investigator.
- Receipt of any investigational trial drug within 3 month prior to participation in the current trial.

2.3.5.4. Continuous glucose monitoring

Participants were asked during the consent process whether they agreed to having a continuous glucose monitor (CGM) fitted, as an optional part of the study. The CGM system used was the FreeStyle Navigator II (Abbott Diabetes Care), which measured glucose in the interstitial fluid every 10 minutes and lasted up to 5 days. The monitor was set to a masked "professional" mode so that the glucose trend did not influence the behaviour of the participants, however it would display the value if they used a capillary blood glucose testing strip. This allowed them to test for and verify any possible hypoglycaemia episodes. The sensors were replaced at every visit and data from the monitor were downloaded at the final visit.

2.3.6. Oral Glibenclamide suspension and dosing schedule

Participants self-administered doses of the oral glibenclamide suspension GlibenTek [105] (AMMTeK, Paris, France). The doses of glibenclamide investigated were 0.3mg, 0.6mg, 1.2mg, 1.8mg, 3mg and 6mg per day, split between morning and evening, and making use of the two different strengths available of the oral suspension (Table 3). A personalised dosing schedule was designed for each participant taking into account their start date, weekends / bank holidays, and minimising inconvenience to participants while still allowing for a

minimum of 3 days at each dose-step. Therefore a typical trial timeline would involve a dose change every 3-4 days, and a total treatment duration of 21 days (see Appendix A.3).

The GlibenTek was distributed by Pharma Services (Paris, France) according to Good Manufacturing Practices (GMP) and regulation, and dispensed by the Oxford University Hospitals NHS Foundation Trust Clinical Trials Pharmacy (Churchill Hospital, Oxford). The suspension was classified as an investigational medicinal product (IMP) because glibenclamide is not licensed in the UK in this formulation.

Table 3 Oral glibenclamide suspension doses

Total dose	Strength	AM	PM	Total (max 4 days per dose-step)
0.3mg	0.6mg/ml	0.25ml	0.25ml	26ml
0.6mg	0.6mg/ml	0.5ml	0.5ml	
1.2mg	0.6mg/ml	1ml	1ml	
1.8mg	0.6mg/ml	1.5ml	1.5ml	
3mg	6mg/ml	0.25ml	0.25ml	6ml
6mg	6mg/ml	0.5ml	0.5ml	

2.3.7. Fasting blood sampling

Prior to each dose change, the participants would attend the CRU for fasting blood tests for plasma insulin, glucagon, C-peptide, glucose and glibenclamide concentration. All fasting blood samples were collected in appropriate tubes (those for glucagon measurement were collected in pre-chilled tubes containing EDTA and aprotinin, BD Vacutainer, 361017), centrifuged at 2,000 x g for 10 minutes, and the plasma stored on dry ice prior to transfer to a designated -80^{o}C freezer at OCDEM.

Participants who were unable to attend the CRU on certain days were visited at home by the CI for blood sample collection. After venepuncture, plasma was collected on site using a mobile centrifuge and stored on dry ice before transfer to the -80^{o}C freezer within 1 hour.

All samples stored in the designated -80^{o}C freezers were of plasma or serum only, i.e. they were not classified as "relevant material" under the Human Tissue Act 2004.

2.3.8. Glucagon, insulin, C-peptide and glucose assays

Blood samples were transferred in batches to the Royal Devon and Exeter NHS Foundation Trust biochemistry laboratory for assaying (Mercodia Glucagon ELISA, 10-1271-01). This was done to ensure a high degree of reproducibility, as there is significant variability between different glucagon assays (see Section 1.6.2). The glucagon assay used has little or no cross-reactivity to oxyntomodulin, glicentin, mini-glucagon, GLP-1, GLP-2 and GRPP (glicentin-related pancreatic polypeptide) according to the manufacturer's information.

Samples for fasting insulin, C-peptide and glucose measurements were assayed using the i200 Immunology Analyzer (Abbott Diagnostics) at the Oxford University Hospitals NHS

Foundation Trust biochemistry laboratory. Plasma samples for glibenclamide measurement were analysed as described in Section 2.2.2.

3. Chapter 3: Effect of donor factors on insulin and glucagon secretion from human islets

3.1. Introduction

The availability of isolated human islets for research purposes, alongside the development of clinical islet transplantation programmes worldwide, has allowed much more refined studies into the pathophysiology and genetics of diabetes to be conducted [106]. However, as translational research moves from using rodent islets to those from humans, there comes with it much more complexity both in terms of tissue structure and architecture, but also with respect to donor variables. While the influence of donor factors on the isolation process (such has islet yield, purity, viability etc.) has been widely investigated [107, 108], less is known for certain about the impact that donor age, sex and BMI have on the function of isolated islets *in vitro* and after transplantation [109-111].

Advancing age is known to have a strong correlation with the development of T2DM [112], however it is unclear whether this is due to an increase in peripheral tissue resistance to insulin-mediated glucose disposal [113], reduced glucose-stimulated insulin secretion [114], or a combination of the two. Equally, while obesity is a known risk factor for T2DM and is associated with insulin resistance [115], adaptive changes within the islets attempt to compensate for increasing BMI by expanding beta-cell mass and increasing basal and stimulated insulin secretion [116, 117]. And yet, studies examining these factors using isolated islets have produced conflicting results.

There is mounting evidence that donor age has a significant impact on the function of transplanted islets, and suggests that islets from older donors are associated with poorer outcomes [111, 114]. This is in keeping with reports of age-related decrease in beta-cell secretory function, possibly due to impaired mitochondrial metabolism or coordination of electrical activity [109, 118, 119]. In contrast, other studies have shown no impact of donor

age on insulin secretion capacity using static incubation or perifusion methods [110, 111], with one study demonstrating an age-related enhancement of insulin secretion due to expression of the senescence factor p16Ink4a, possibly leading to increased mitochondrial activity [120]. These latter reports are not necessarily at odds with human *in vivo* studies, as the decreased glucose tolerance observed in older subjects may be due to defects of intra-islet capillaries leading to reduced insulin permeability and therefore also plasma concentrations [121].

The impact of rising BMI appears to be an increase in average islet size, and while previous studies have not reported a corresponding increase in the content of insulin [109, 122], more recent perifusion experiments using larger number of islets suggest a positive correlation with BMI [110]. These also demonstrated no difference in insulin secretion capacity between islets from male and female donors.

While the above studies demonstrate the heterogeneity in our current understanding of factors influencing insulin secretion in isolated human islets, they also highlight the lack of data available regarding glucagon secretion. As discussed in Section 1.4, physiologically inappropriate glucagon secretion is a feature of T2DM which is also observed in isolated islets [35]. Therefore, better understanding of factors that impact alpha-cell function could shed new light on this pathological process, which may not be apparent from whole-body physiology studies.

3.2. Aims

The aims of this project were to:

a) evaluate the overall results of insulin and glucagon secretion experiments at 1mM, 6mM and 20mM glucose conditions
b) investigate the effects of donor age, sex and BMI on glucose-dependent insulin and glucagon secretion

This was done by analysing all available data over an 11-year period (2006-2017) from experiments performed on human islets (obtained from the facility in Oxford as well as national and international collaborators).

3.3. Methods

3.3.1. The Human Isolation Facility in Oxford

The Diabetes Research & Wellness Foundation (DRWF) Human Islet Isolation Facility in Oxford (OCDEM, Churchill Hospital, University of Oxford) opened in 2006. It forms one of the three national islet isolation centres (along with King's College London and Edinburgh), which together contribute to the UK clinical islet transplantation programme. Every year the facility processes on average 53 pancreases using a standard islet isolation protocol [123], however a substantial proportion do not result in islets of sufficient quality or yield to proceed to transplantation. This is in keeping with data from other centres around the world [124]. As part of the quality assessment process, a small sample of isolated islets undergo glucose-stimulated insulin secretion experiments. Islets which cannot be used for transplantation are used in diabetes research studies, provided appropriate consent was obtained (see Section 2.1.3).

3.4. Hormone secretion data collection

All hormone secretion experiments were performed using the static incubation method (as described in Section 2.1.4). The results were reported on the Human Islet Isolation Facility database, as part of quality assessment procedure. This database was searched for all islet preparations which had hormone secretion data at 1mM, 6mM or 20mM glucose (or a combination of the three), and in which the results were reported as percentage of content. Additional data were included from experiments I performed over the course of my DPhil project using islets obtained from national and international collaborators (with appropriate research consent). Data were excluded if details of donor age, sex and BMI were missing from the record, or if the donor had diabetes.

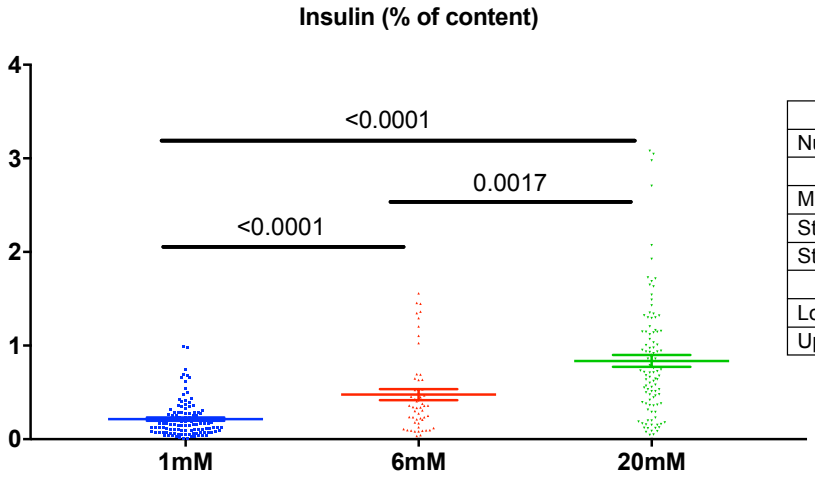
3.5. Statistical analysis

Statistical analysis was performed using Prism 7 (GraphPad) and R version 3.3.2 (The R Foundation for Statistical Computing). D'Agostino & Pearson normality tests were used to check for Gaussian distribution of data, and one-way or two-way ANOVA was performed using the Kruskal-Wallis nonparametric test. Post-hoc analysis was performed using Tukey's multiple comparisons test. Linear regression was assessed using an F-test. Linear mixed effects models were constructed using the following parameters: fixed effects= age, sex and BMI; random effect= islet preparation ID. Data are presented as mean ±SEM, or as linear trend with 95% confidence interval. Statistical significance was set at $p<0.05$.

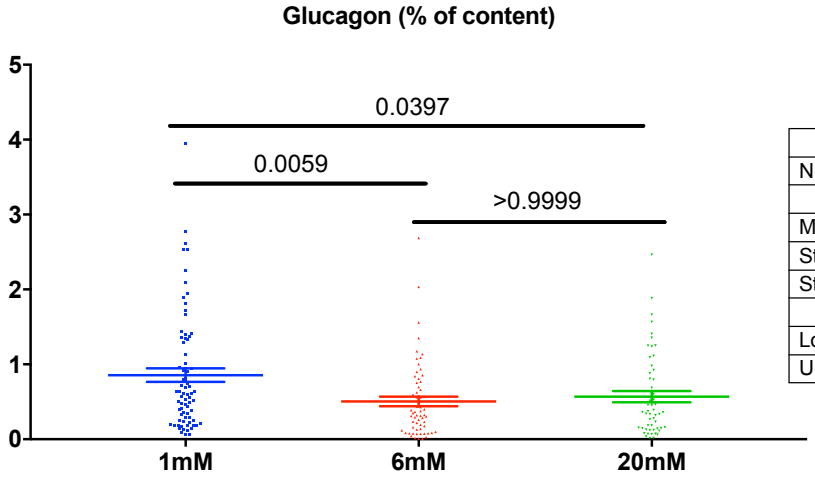
3.6. Results

3.6.1. Glucose-dependent insulin and glucagon secretion

Not all islet preparations had data for each glucose condition (1mM, 6mM and 20mM), therefore these were analysed as separate categories (i.e. not as repeated measures). Insulin and glucagon secretion values (expressed as percentage of content) showed evidence of non-gaussian distribution within each group ($p<0.005$), therefore the Kruskal-Wallis nonparametric test was used. As shown in Figure 8, there was a two-fold increase in insulin secretion between 1mM and 6mM glucose, and a four-fold increase between 1mM and 20mM. With regards to glucagon, there was a 40% decrease in secretion between 1mM and 6mM, with no further inhibition at 20mM.



	1mM	6mM	20mM
Number of values	116	53	102
Mean	0.2153	0.4763	0.8365
Std. Deviation	0.1905	0.4235	0.6422
Std. Error of Mean	0.01769	0.05817	0.06358
Lower 95% CI of mean	0.1803	0.3596	0.7103
Upper 95% CI of mean	0.2504	0.593	0.9626



	1mM	6mM	20mM
Number of values	74	62	54
Mean	0.8564	0.5048	0.5681
Std. Deviation	0.784	0.5088	0.5518
Std. Error of Mean	0.09114	0.06461	0.07509
Lower 95% CI of mean	0.6747	0.3756	0.4175
Upper 95% CI of mean	1.038	0.634	0.7187

Figure 8 Overall insulin and glucagon secretion (expressed as percentage of content) at each glucose condition. Individual values are plotted as well as mean ±SEM, and the numbers above the plots indicate p values (one way ANOVA).

3.6.2. Effect of donor age, sex and BMI

Linear regression analysis did not demonstrate a significant correlation between age and insulin or glucagon secretion at any glucose concentration ($p>0.05$, Figure 9), nor between hormone secretion and BMI ($p>0.05$, Figure 10). However, it did suggest a small positive correlation between age and BMI ($p<0.05$, Figure 11). While the correlation between age and BMI has been demonstrated previously [125], the purpose of this analysis was to demonstrate that there was no selection bias in the islet preparations, as donor factors can influence the islet yield during isolation [126].

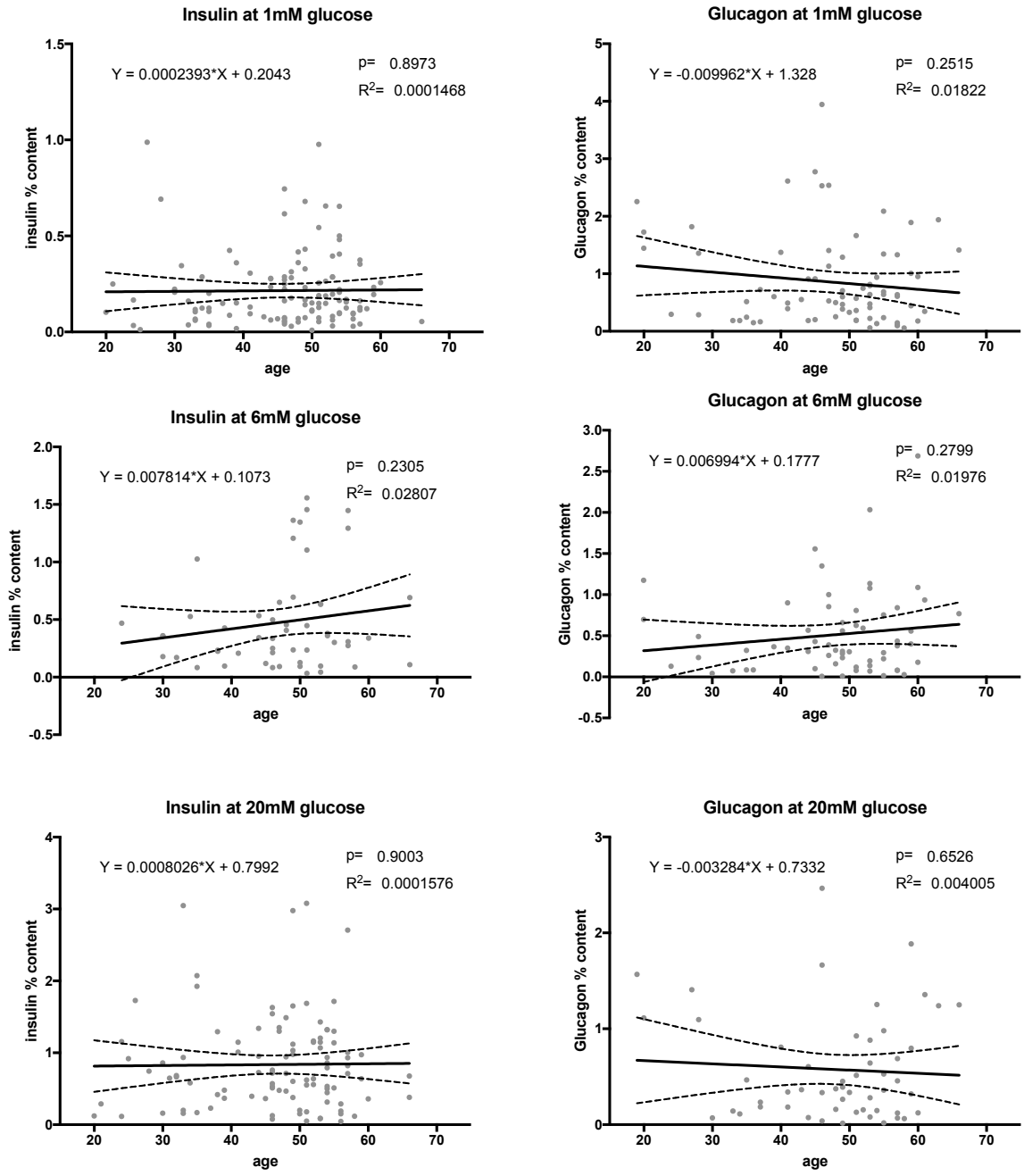


Figure 9 Linear regression analysis of hormone secretion and age.

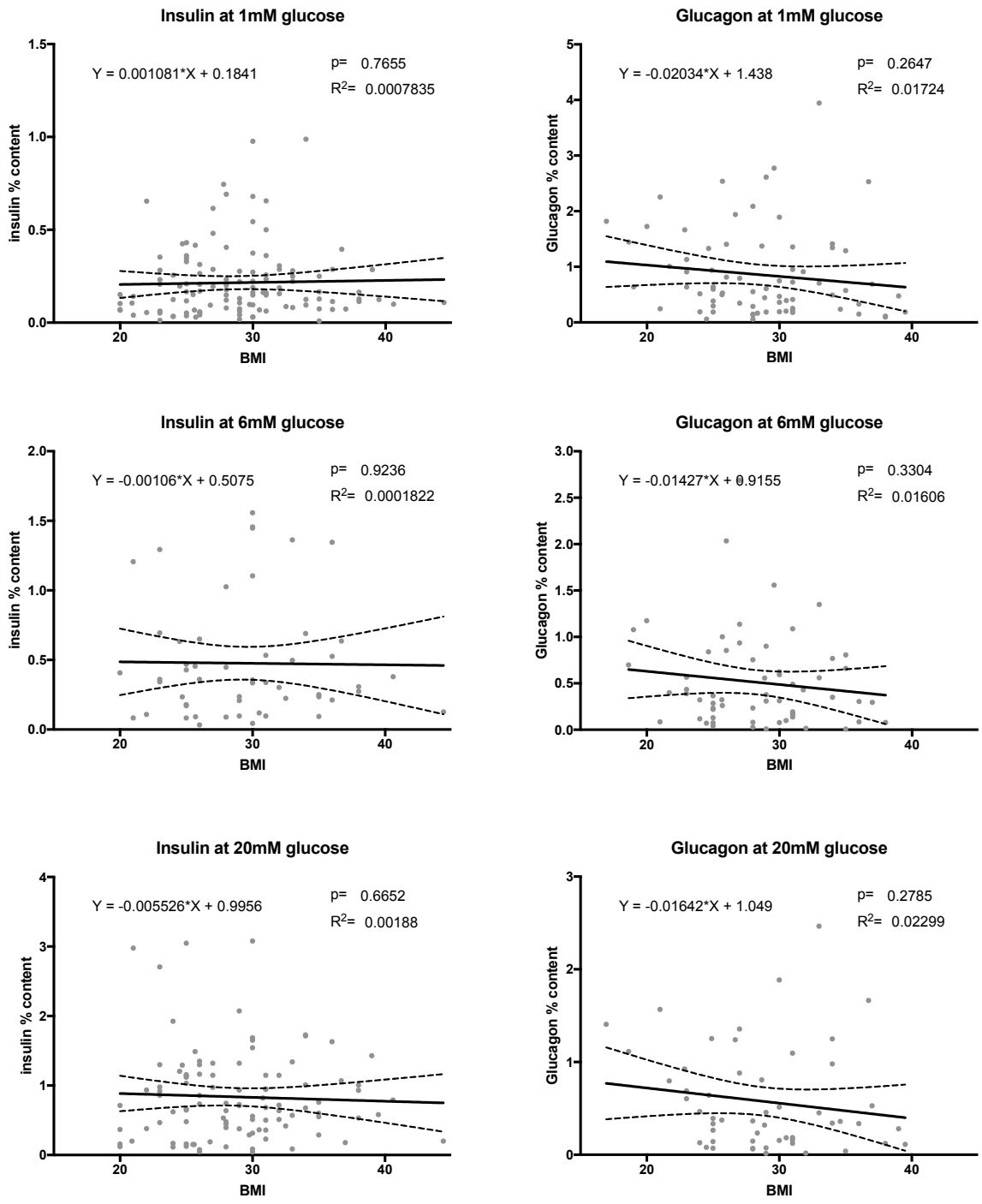


Figure 10 Linear regression analysis of hormone secretion and BMI.

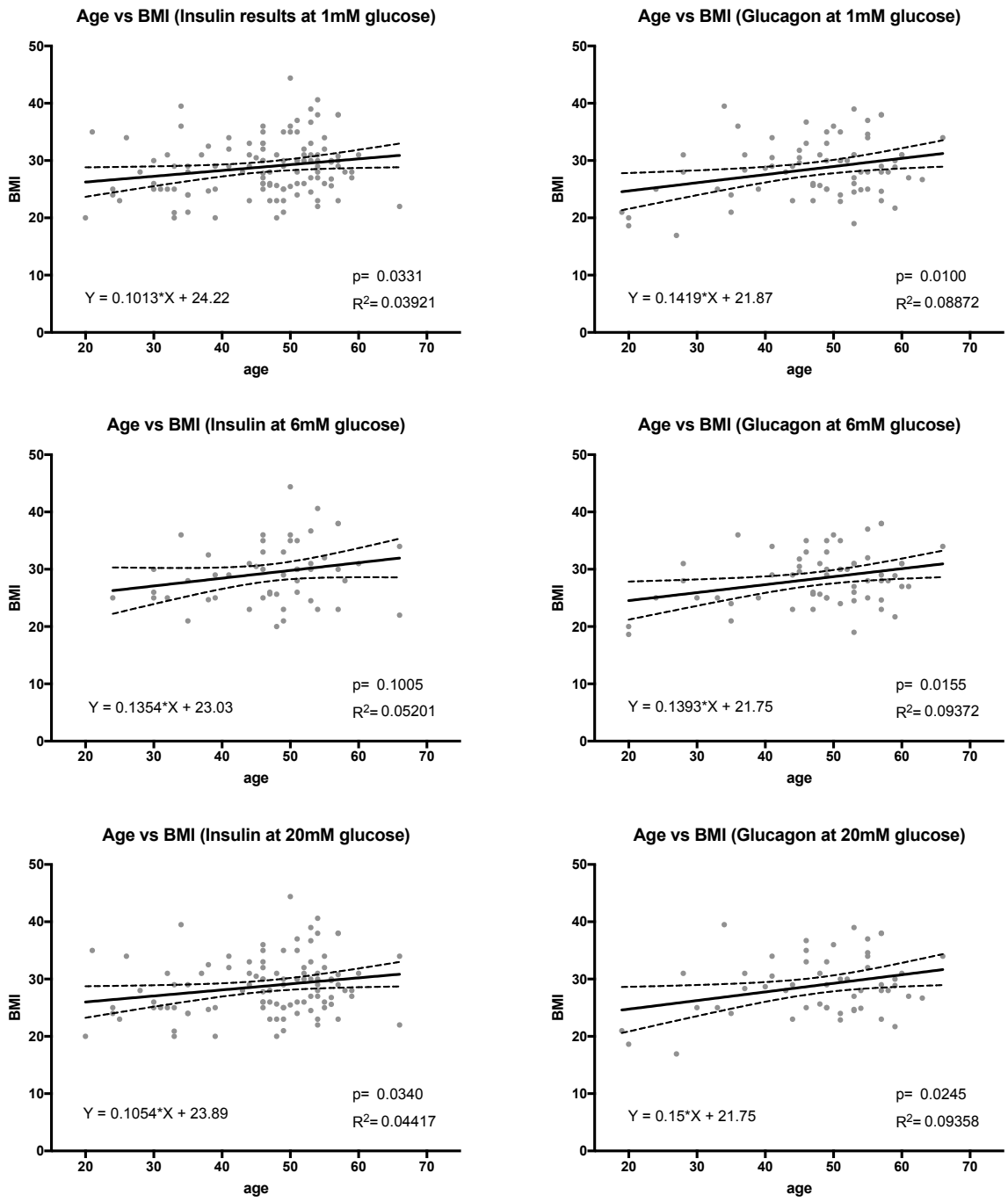


Figure 11 Linear regression analysis of BMI and age

In order to further explore the relationship between hormone secretion and each donor factor (age, sex and BMI), linear mixed effects models were constructed for each glucose concentration, which could take into account the variability of each islet preparation (islet preparation ID was set as the random effect). This also did not reveal a significant association ($p>0.05$, Table 4). However, when donors were separated into two age-groups (median = 48 years, therefore "young" was defined as < 48 years), the models suggested an association between age-group and sex but not BMI (Table 5).

Table 4 Results of linear mixed-effects model analysis, constructed using age, sex and BMI as fixed effect factors.

Linear mixed-effects model
Random effects = islet prep. ID
Fixed effects = age, sex, BMI

Insulin			
1mM glucose			
	Value	Std.Error	p-value
age	0.000	0.002	0.831
sexM	0.054	0.037	0.149
BMI	0.003	0.004	0.506
6mM glucose			
	Value	Std.Error	p-value
age	0.011	0.007	0.114
sexM	0.198	0.126	0.122
BMI	0.000	0.012	0.988
20mM glucose			
	Value	Std.Error	p-value
age	0.002	0.007	0.806
sexM	0.111	0.134	0.410
BMI	-0.004	0.014	0.755

Glucagon			
1mM glucose			
	Value	Std.Error	p-value
age	-0.007	0.009	0.449
sexM	0.134	0.186	0.474
BMI	-0.012	0.020	0.525
6mM glucose			
	Value	Std.Error	p-value
age	0.087	0.097	0.377
sexM	-0.675	1.899	0.724
BMI	0.026	0.220	0.905
20mM glucose			
	Value	Std.Error	p-value
age	-0.001	0.008	0.882
sexM	0.009	0.159	0.955
BMI	-0.016	0.016	0.338

Table 5 Results of linear mixed-effects model analysis, constructed using age-group ("young" defined as <48 years), sex and BMI as fixed effect factors.

Linear mixed-effects model
Random effects = islet prep. ID
Fixed effects = age group, sex, BMI

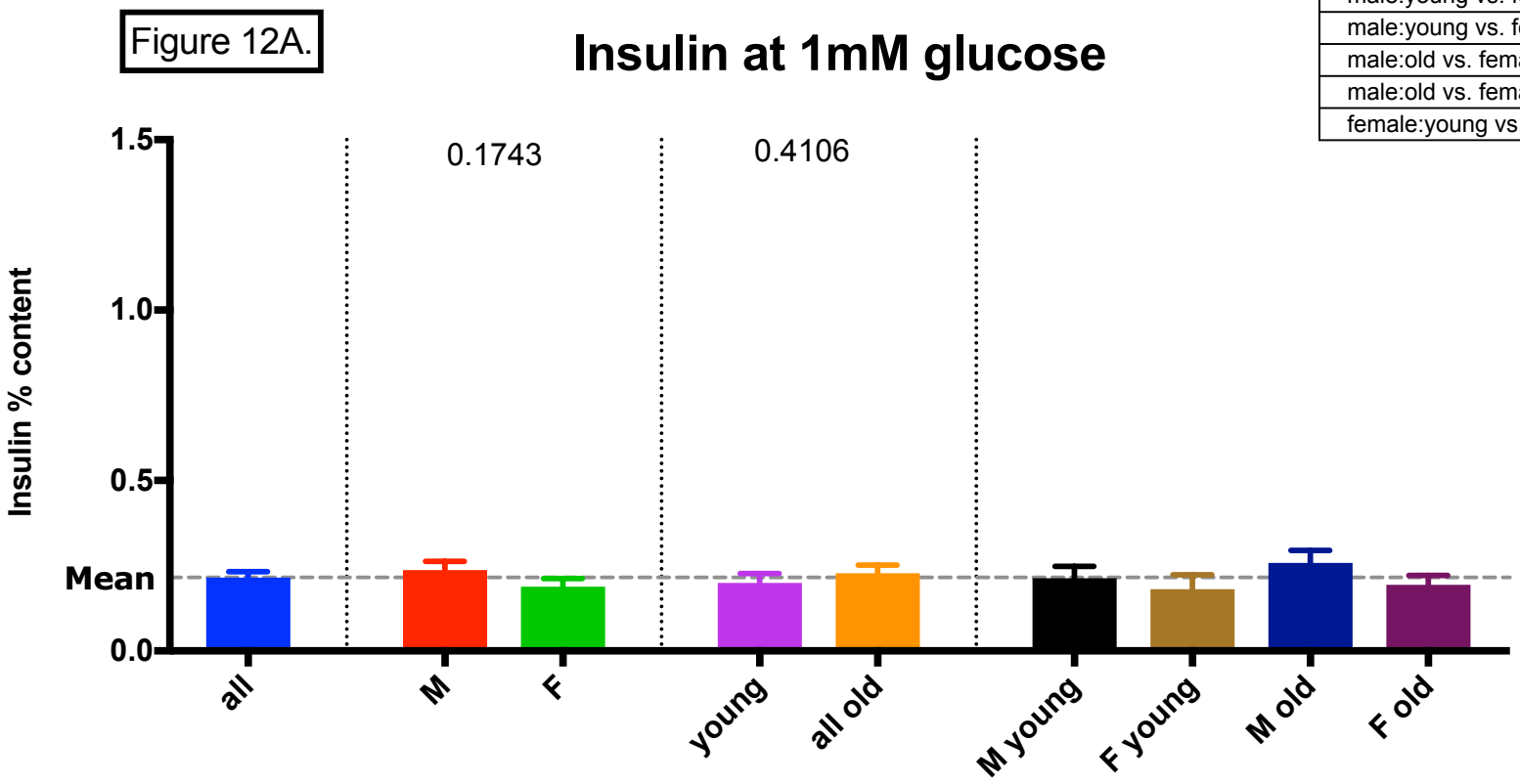
Insulin			
1mM glucose			
	Value	Std.Error	p-value
group.47young	-0.032	0.036	0.381
sexM	0.056	0.037	0.132
BMI	0.002	0.004	0.550
6mM glucose			
	Value	Std.Error	p-value
group.47young	**-0.356**	**0.117**	**0.004**
sexM	**0.261**	**0.121**	**0.035**
BMI	0.000	0.011	0.990
20mM glucose			
	Value	Std.Error	p-value
group.47young	-0.068	0.132	0.604
sexM	0.113	0.134	0.402
BMI	-0.004	0.014	0.744

Glucagon			
1mM glucose			
	Value	Std.Error	p-value
group.47young	**0.395**	**0.186**	**0.037**
sexM	0.089	0.182	0.627
BMI	-0.011	0.018	0.559
6mM glucose			
	Value	Std.Error	p-value
group.47young	-0.086	0.139	0.537
sexM	0.143	0.133	0.288
BMI	-0.016	0.015	0.280
20mM glucose			
	Value	Std.Error	p-value
group.47young	0.128	0.161	0.428
sexM	0.022	0.159	0.892
BMI	-0.015	0.015	0.336

When the data were re-examined using two-way ANOVA (grouped according to sex and age-group), the analysis supported the findings of the linear mixed effect models. Specifically, it demonstrated that at 6mM glucose (euglycaemic condition) islets from older donors secreted almost twice as much insulin (corrected for islet content) as those from younger donors (Figure 12B). Furthermore, this effect was primarily observed in males. With regards to glucagon, at 1mM glucose (hypoglycaemic condition) islets from older donors secreted 37% less glucagon (corrected for islet content) than those from younger donors without an effect of gender (Figure 13A).

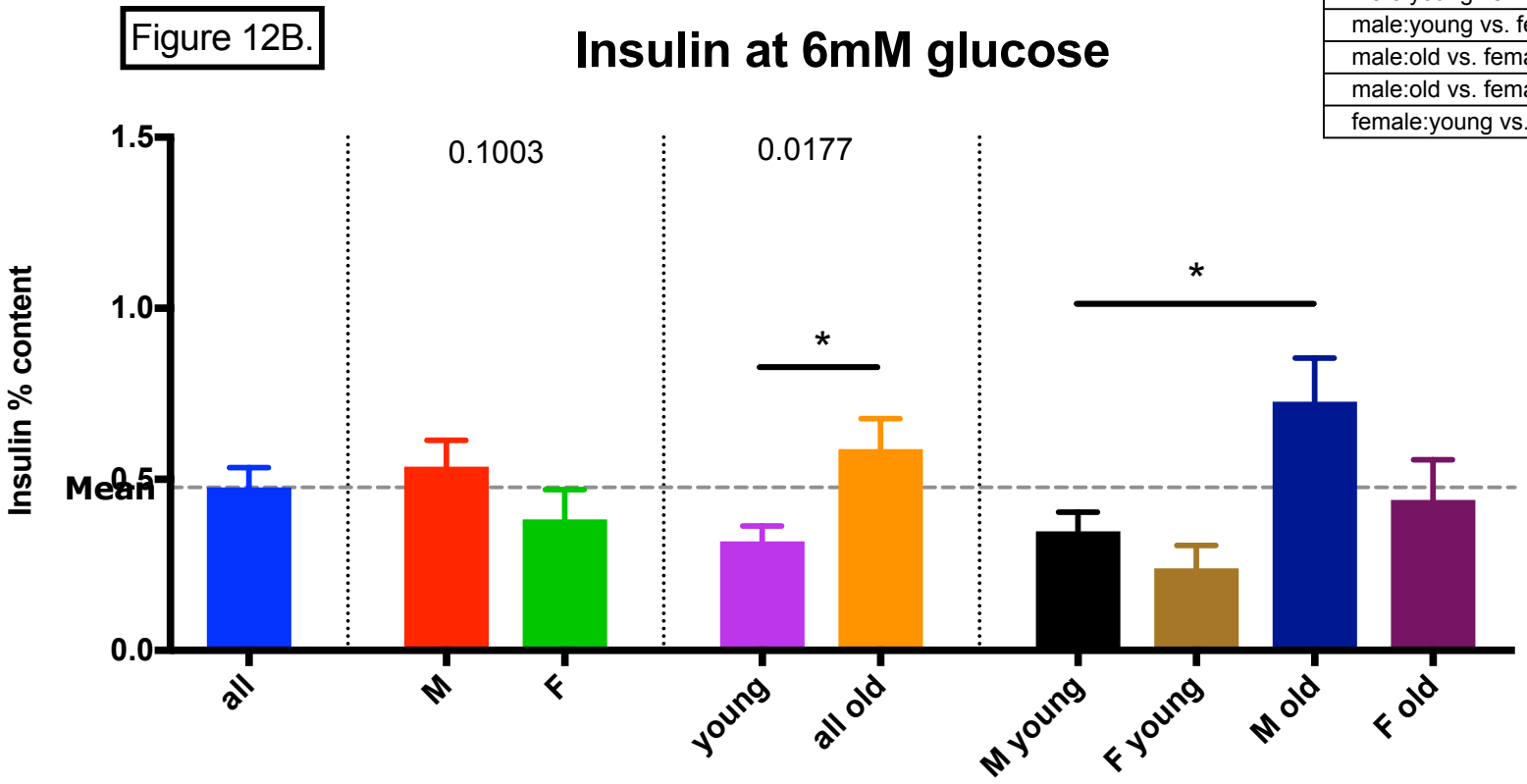
male:young vs. male:old	0.7828
male:young vs. female:young	0.9222
male:young vs. female:old	0.9791
male:old vs. female:young	0.4364
male:old vs. female:old	0.5349
female:young vs. female:old	0.9933

Figure 12A.



	all	M	F	young	all old	M young	F young	M old	F old
Number of values	116	64	52	52	64	30	22	34	30
Mean	0.2153	0.2371	0.1885	0.1997	0.228	0.2136	0.1808	0.2579	0.1942
Std. Deviation	0.1905	0.2039	0.1709	0.1947	0.1877	0.1916	0.2017	0.2148	0.1477
Std. Error of Mean	0.01769	0.02548	0.0237	0.027	0.02346	0.03498	0.043	0.03683	0.02697

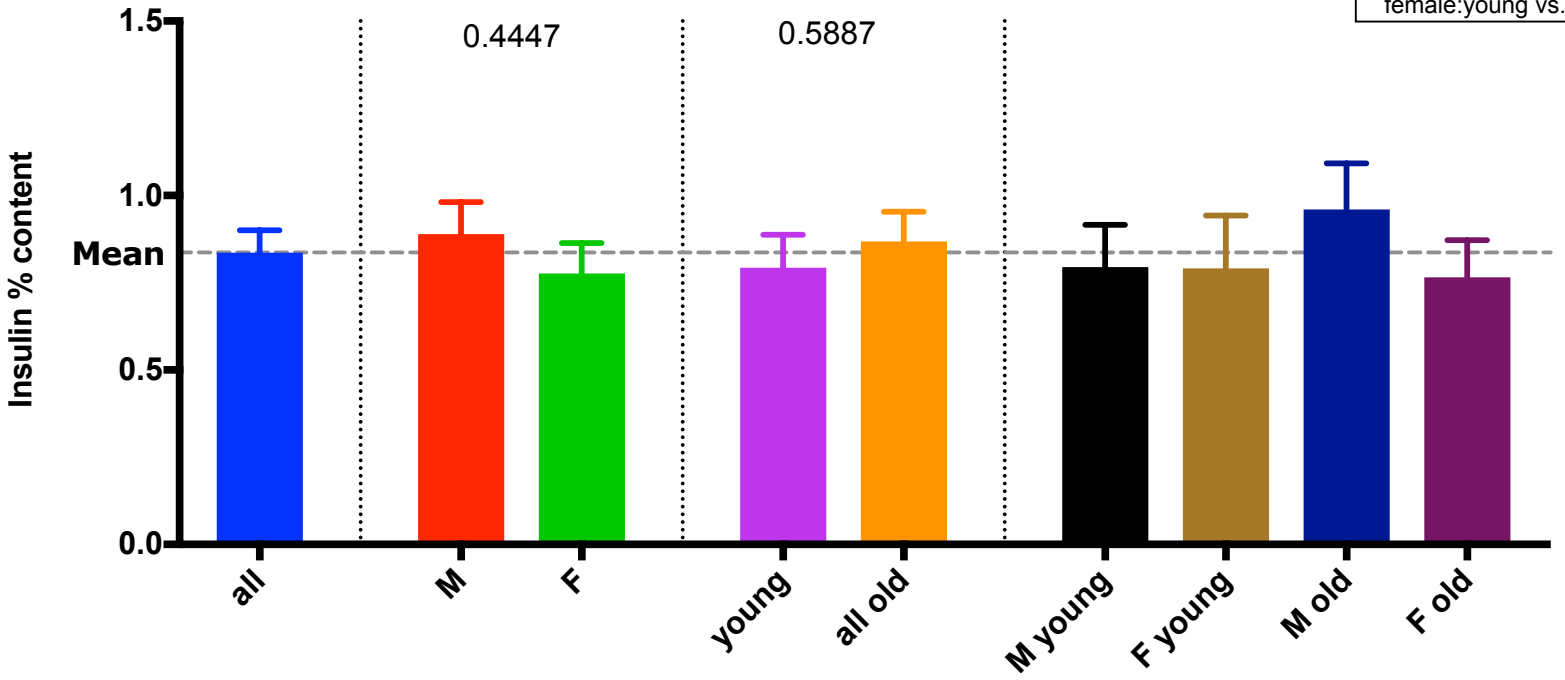
Figure 12B.



male:young vs. male:old	0.0431
male:young vs. female:young	0.9398
male:young vs. female:old	0.9151
male:old vs. female:young	0.0600
male:old vs. female:old	0.1923
female:young vs. female:old	0.7206

	all	M	F	young	all old	M young	F young	M old	F old
Number of values	53	32	21	22	31	16	6	16	15
Mean	0.4763	0.5376	0.3829	0.3186	0.5882	0.3481	0.2402	0.7271	0.44
Std. Deviation	0.4235	0.433	0.4006	0.2124	0.4981	0.2257	0.1629	0.5099	0.4556
Std. Error of Mean	0.05817	0.07655	0.08742	0.04528	0.08947	0.05642	0.0665	0.1275	0.1176

Figure 12C.

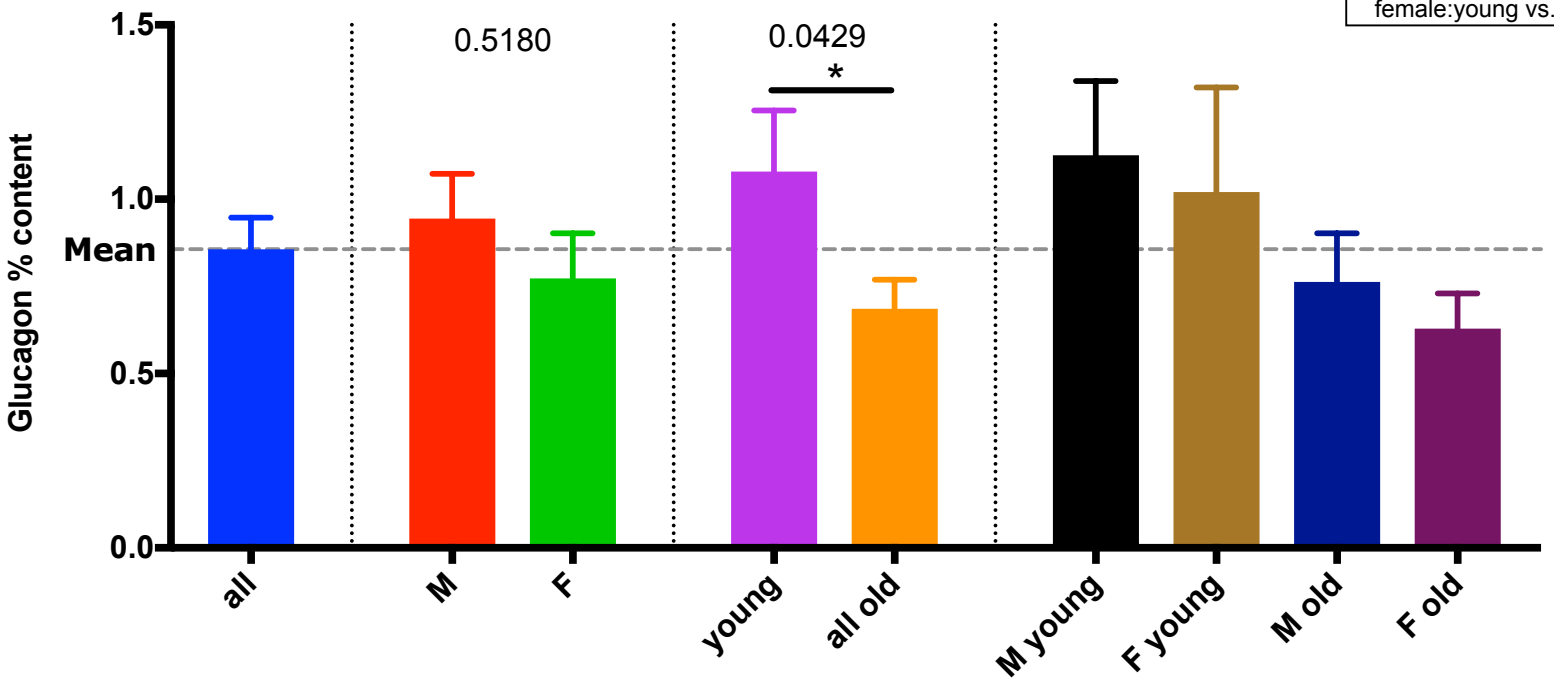


male:young vs. male:old	0.7838
male:young vs. female:young	>0.9999
male:young vs. female:old	0.9985
male:old vs. female:young	0.7955
male:old vs. female:old	0.6504
female:young vs. female:old	0.9990

	all	M	F	young	all old	M young	F young	M old	F old
Number of values	102	54	48	43	59	23	20	31	28
Mean	0.8365	0.8898	0.7764	0.7932	0.868	0.7946	0.7916	0.9605	0.7656
Std. Deviation	0.6422	0.6736	0.6062	0.6203	0.6611	0.5842	0.6747	0.7343	0.5648
Std. Error of Mean	0.06358	0.09167	0.0875	0.09459	0.08607	0.1218	0.1509	0.1319	0.1067

Figure 12 Insulin secretion analysis at 1mM, 6mM and 20mM glucose (A, B, C). Data are plotted as mean ±SEM, and number above plots indicate p values (two-way ANOVA).

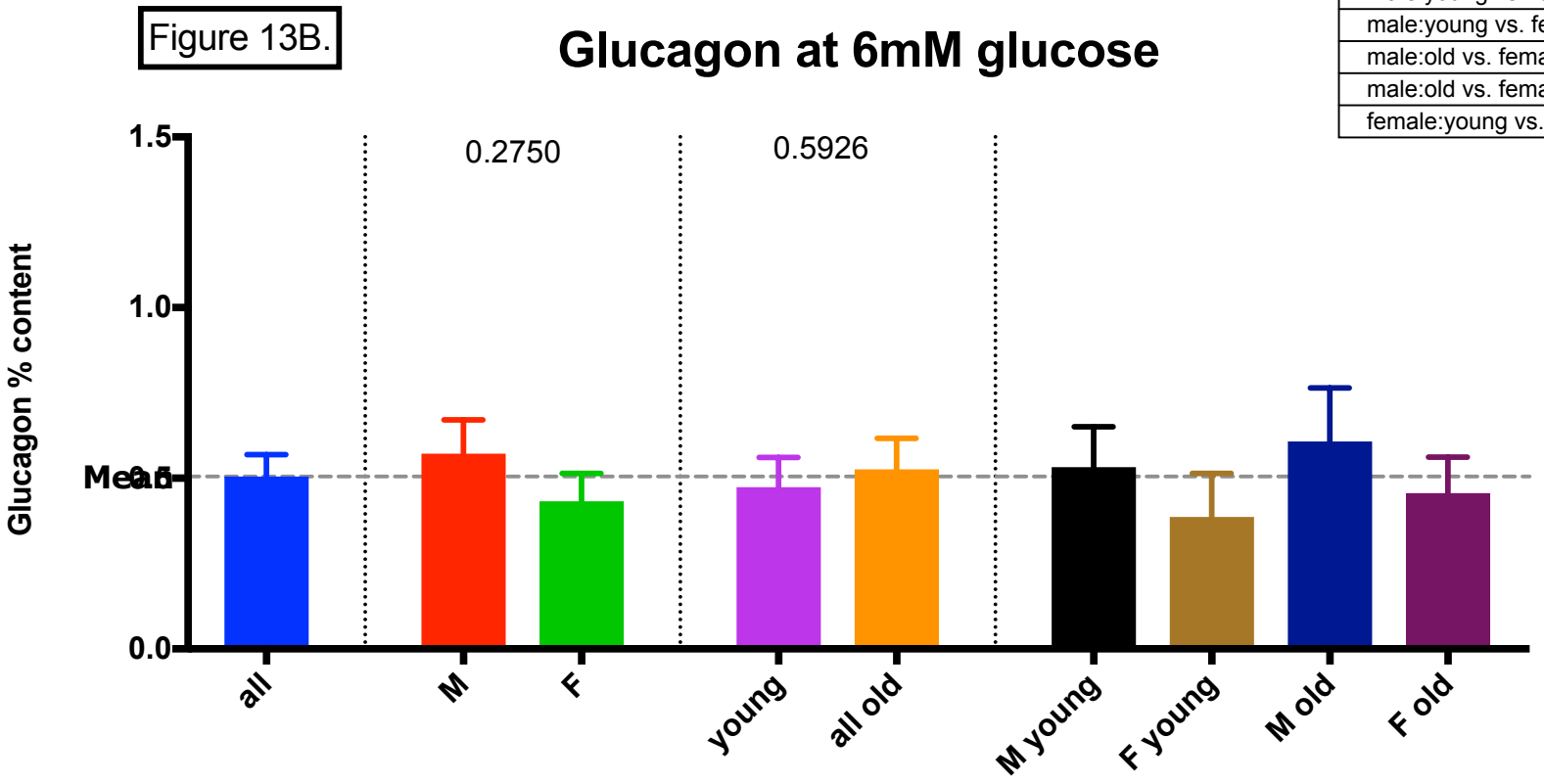
Figure 13A.



male:young vs. male:old	0.4992
male:young vs. female:young	0.9815
male:young vs. female:old	0.1767
male:old vs. female:young	0.7842
male:old vs. female:old	0.9449
female:young vs. female:old	0.4376

	all	M	F	young	all old	M young	F young	M old	F old
Number of values	74	36	38	32	42	18	14	18	24
Mean	0.8564	0.944	0.7733	1.08	0.6862	1.125	1.021	0.7626	0.6289
Std. Deviation	0.784	0.7736	0.7951	0.9888	0.5355	0.9013	1.124	0.5916	0.4944
Std. Error of Mean	0.09114	0.1289	0.129	0.1748	0.08262	0.2124	0.3003	0.1394	0.1009

Figure 13B.

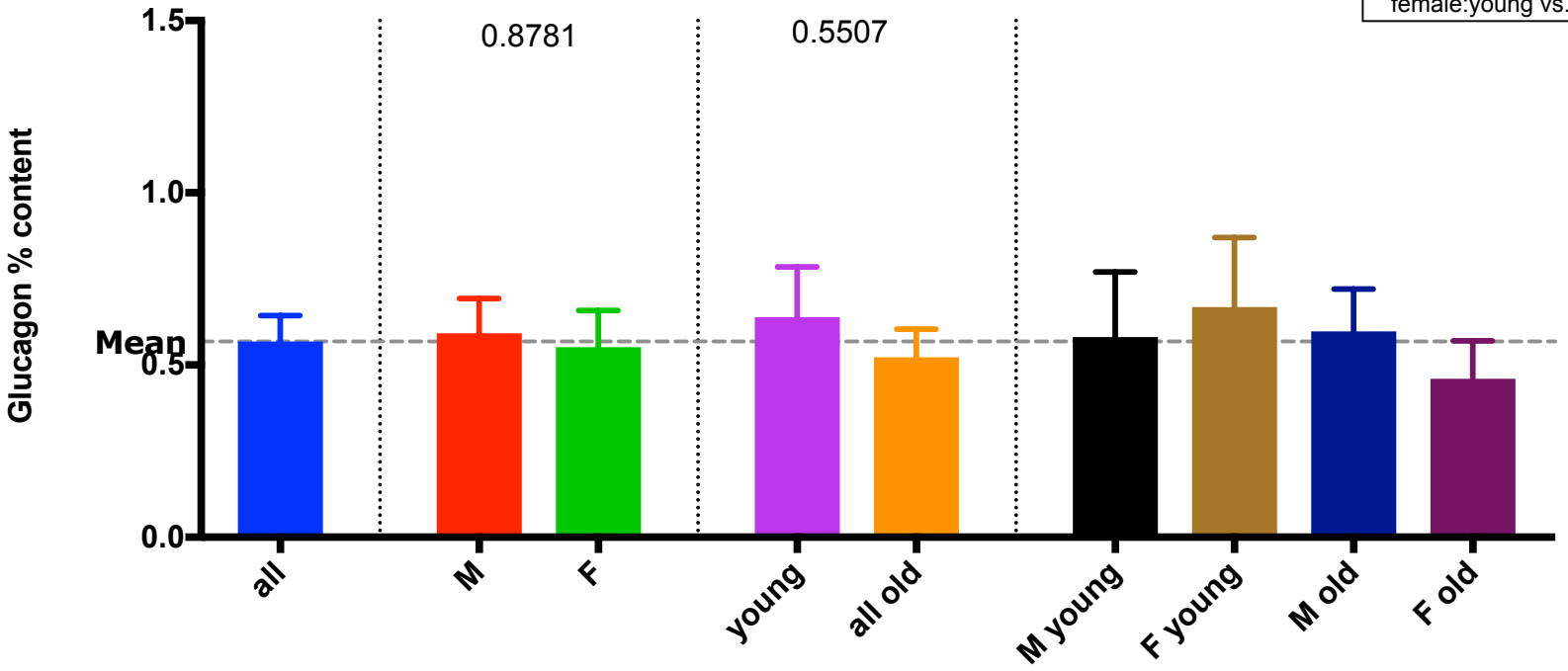


male:young vs. male:old	0.9761
male:young vs. female:young	0.8980
male:young vs. female:old	0.9725
male:old vs. female:young	0.7030
male:old vs. female:old	0.8093
female:young vs. female:old	0.9849

	all	M	F	young	all old	M young	F young	M old	F old
Number of values	62	32	30	25	37	15	10	17	20
Mean	0.5048	0.5724	0.4328	0.4739	0.5257	0.5323	0.3863	0.6078	0.456
Std. Deviation	0.5088	0.5606	0.4451	0.4365	0.5572	0.4606	0.4049	0.6484	0.4723
Std. Error of Mean	0.06461	0.0991	0.08127	0.08731	0.0916	0.1189	0.128	0.1573	0.1056

Figure 13C.

Glucagon at 20mM glucose



male:young vs. male:old	0.9999
male:young vs. female:young	0.9855
male:young vs. female:old	0.9606
male:old vs. female:young	0.9853
male:old vs. female:old	0.8907
female:young vs. female:old	0.7121

	all	M	F	young	all old	M young	F young	M old	F old
Number of values	54	22	32	21	33	7	14	15	18
Mean	0.5681	0.5923	0.5514	0.6393	0.5227	0.5809	0.6684	0.5976	0.4603
Std. Deviation	0.5518	0.4733	0.6067	0.6684	0.4686	0.5007	0.7542	0.478	0.4649
Std. Error of Mean	0.07509	0.1009	0.1072	0.1459	0.08157	0.1893	0.2016	0.1234	0.1096

Figure 13 Glucagon secretion analysis at 1mM, 6mM and 20mM glucose (A, B, C). Data are plotted as mean ±SEM, and number above plots indicate p values (two-way ANOVA).

3.7. Limitations

Due to the exploratory and retrospective nature of this analysis, sample size calculations were not performed and a number of statistical approaches were used. These were not pre-specified in the statistical plan and therefore represent a limitation of this study.

The original linear regression analysis was unable to take into account donor-specific factors, therefore more in-depth linear mixed effects models were contructed, which could also account for the variability of each islet preparation. This analysis did not demonstrate a statistically significant difference, possibly due to the large spread of ages within the study population which were not normally distributed. It was therefore decided to simplify the structure of the data by dichotomising it along the median age of islet donors (47 years old). This simplified dataset was used to construct the second set of linear mixed effects models (Table 5). Finally, a further analysis of the simplified dataset using two-way ANOVA was performed to test whether the findings of the mixed effects model could be reproduced using a statistical method which was more familiar to our research group.

With regards to the glucagon and insulin values included in the dataset, these were produced using three different assay kits. While all the data prior to 2015 were produced using the Eurodiagnostica Glucagon RIA and Millipore Human Insulin RIA, from 2015 onwards some data were produced using the Meso Scale Discovery insulin and glucagon duplex kit (see section 2.2.1). This was not taken into account during the analysis. However, many of the limitations of radio-immunoassay based glucagon kits (described in section 1.6) do not apply in this experimental set-up, as the use of isolated islets excludes any interferrance from gut-derived pro-glucagon products.

3.8. Discussion

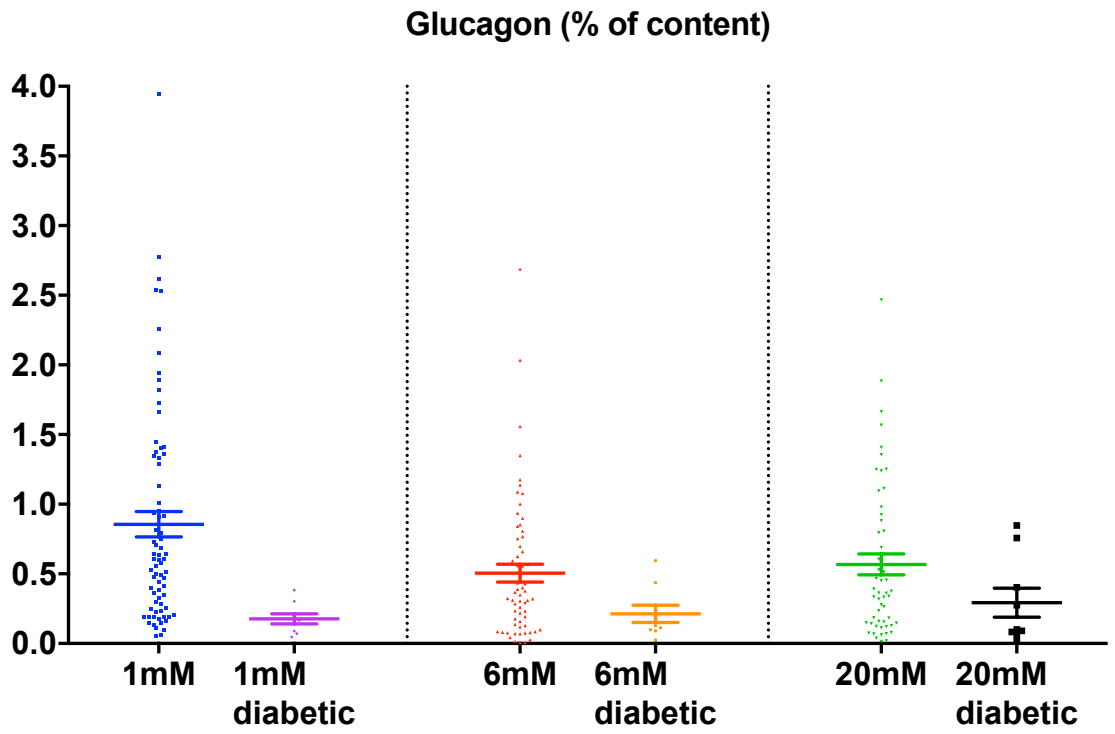
The current dataset of human islet insulin and glucagon secretion results is one of the largest available and includes data from 116 non-diabetic donors over an 11-year period. Throughout this time the experiments were performed using a consistent technique (1 hour static incubation), and hormone levels were tested using validated assays (see Section 2.2.1). One advantage of this analysis over others of similar sized datasets [109] is the use of a more refined statistical approach (linear mixed effects model) to examine the relative contributions of each factor simultaneously, which has provided novel insights into the effect both age and sex of the donor have on islet hormone secretion.

3.8.1. Variability of hormone secretion

It is known that isolated human islets display a high degree of variability when glucose-stimulated insulin secretion is tested [127], even in preparations which are deemed high enough quality for transplantation [128]. Currently this information is not used to determine lot release, as it remains unclear whether it can help predict transplant outcomes. Our results are in keeping with findings from other large islet isolation centres in the USA [128], and demonstrate a four-fold increase of insulin secretion between low and high glucose conditions (Figure 8). While this is considerably less than the secretory response reported by the research-focused Edmonton group in Canada (average fold-increase 11.0 ± 1.0, [109]), it highlights the variability within the international literature. Indeed, the Clinical Islet Transplantation Consortium in the USA reported a median fold-increase of 2.3 (interquartile range = 1.6), suggesting that certain transplanted preparations showed no glucose-stimulated insulin secretion at all [128].

Our dataset also included information on glucagon from up to 74 donors, which has not been reported by other centres. Analysis of secretion levels at 1mM, 6mM and 20mM glucose

conditions demonstrates that the large degree of variability between preparations extends to alpha-cells as well, and suggests that glucagon secretion is maximally inhibited by approximately 40% at 6mM glucose (Figure 8). In addition, these results were compared with those of 9 donors with T2DM for whom results were also available at all three glucose conditions (**Figure 14**). While the differences are not statistically significant ($p>0.05$), there was a trend towards much lower glucagon secretion (normalised to content) overall and a non-physiological increase at 6mM and 20mM glucose, indicating a defective mechanism for glucose processing and hormone release. These observations are in keeping with model of abnormal alpha-cell function in T2DM discussed in Section 1.4.



Non-diabetic

	1mM	6mM	20mM
Number of values	74	62	54
Mean	0.8564	0.5048	0.5681
Std. Deviation	0.784	0.5088	0.5518
Std. Error of Mean	0.09114	0.06461	0.07509

T2DM

1mM diabetic	6mM diabetic	20mM diabetic
9	9	9
0.1769	0.2137	0.2937
0.1101	0.1873	0.3128
0.03671	0.06243	0.1043

Figure 14 Overall insulin and glucagon secretion (expressed as percentage of content) at each glucose condition in non-diabetic donors, and islets from 9 donors with T2DM. Individual values are plotted as well as mean ±SEM.

Taken together, our data confirm the significant variability in hormone secretion data observed by other groups [109, 127] and emphasise the importance of taking this into consideration when designing or interpreting studies involving small numbers of human islet preparations. They also suggest that glucagon measurement has the potential to add an extra dimension to the evaluation of islet quality and function, as inappropriate secretion patterns are present in donors with T2DM.

3.8.2. Influence of donor factors on hormone secretion

The population included in the dataset (n=116) had a median age of 48 years (mean = 46 ± 0.1, range 20 – 66 years) and a median BMI of 29 kg/m^2 (mean = 29 ± 0.1, range 20 – 44.4 kg/m^2), with a total of 62 males (53%) and 52 females (46%). As such, the donor characteristic are comparable to those in the most recently published analyses from Belgium [110] and Canada [109]. In contrast to these other studies though, analysis of our dataset identified a significant effect of both age and sex on insulin secretion which was present at euglycaemic conditions (6mM glucose). This was revealed when the population was divided into "young" and "old" along the median, and suggest that islets from older males secreted twice as much insulin as those from younger males (Figure 12B). Importantly, these results were not confounded by the amount of insulin stored in the islets as all values were expressed as percentage of insulin content, and BMI did not appear to affect this relationship at 6mM glucose (Table 5) as was the case in the study by Lyon and colleagues [109]. Results across both age-groups and genders were similar at maximal inhibition (1mM glucose) and stimulation (20mM glucose) of insulin release, suggesting preservation of the normal glucose-sensing and insulin release mechanisms at the extreme conditions. Whether the threshold of glucose sensing is shifted with age is unclear, however the findings that the senescence factor p16Ink4a could lead to increased glucose uptake into the beta-cell is

particularly interesting [120]. The apparent enhancement of insulin secretion in older males at euglycaemic conditions is surprising and seems to be at odds with studies which have demonstrated the positive effect of androgen-receptor agonists on glucose-stimulated insulin secretion in isolated human islets [129]. However, it is possible that testosterone levels do not simply decline with age but instead increase in their variability after 40 years of age [130].

Our analysis also identified an association between age and glucagon secretion which to our knowledge has not previously been reported. At hypoglycaemic conditions (1mM glucose) islets from older donors secreted 40% less insulin than those from younger donors (Figure 13A), and this was not influenced by sex or BMI (Table 5). Glucagon was maximally inhibited at 6mM and 20mM glucose, and absolute secretion values were similar in both age-groups. These finding too appear to be at odds with a recent report which demonstrated an increase in alpha-cell mass with increasing weight and age (though the latter was not statistically significant) [131], however it is interesting to speculate on their physiological or clinical importance. When taken together, our analysis suggests that older individuals secrete higher levels of insulin under conditions of euglycaemia, but are unable to mount the same level of glucagon counter-regulatory response when challenged with hypoglycaemia. This echoes the clinical findings that older people are particularly sensitive to the effects of insulin secretagogues such as sulfonylureas [132].

In summary, the 11-year analysis of human islet hormone secretion experiments at OCDEM demonstrates the following:

a) Baseline (1mM glucose) secretion of insulin is 0.2% of islet content per hour; there is a two-fold increase at 6mM and at four-fold increase at 20mM glucose.

b) There is maximal glucose-dependent glucagon suppression at 6mM by 40%.

c) The range of values obtained during human islet hormones secretion experiments is wide.

d) Insulin secretion is affected by age and sex, with islets from older males secreting twice as much insulin as those from younger males.

e) Glucagon secretion is affected by age, with islets from older donors secreting approximately 40% less glucagon than those from younger donors.

4. Chapter 4: Effect of glibenclamide on insulin & glucagon secretion in isolated islets.

4.1. Background

Glibenclamide and other sulfonylureas has been extensively studied over the years in a number of different patient populations. On the one hand, their function as potent insulin secretagogues has been well established since the discovery of hypoglycaemic sulfonamides by Loubatieres in the 1940s [133, 134]. This was confirmed both *in vitro* [135] and *in vivo* in participants with and without T2DM [136], and these studies demonstrated that the effect on beta-cells differed between euglycaemic and hyperglycaemic conditions. On the other hand, their effect on glucagon secretion is more complex with studies reporting an increase, decrease or no effect on plasma glucagon levels depending on the experimental set-up [137]. This is compounded by the variability in the measurement of plasma glucagon (as discussed in section 1.6.2). *In vitro* studies exploring the relationship between glibenclamide and glucagon secretion in rats suggested that both insulin and glucagon secretion was stimulated by glibenclamide in the absence of glucose [138]. However, some clinical studies in patients with T2DM showed no effect of glibenclamide on post-parandial or 24-hour plasma glucagon [139-141], while other demonstrated a significant decrease in secretion following insulin-induced hypoglycaemia [142].

As their use in the management of T2DM became part of standard clinical practice, and newer second generation versions became available, attempts were made to better characterise the sulfonylurea dose-response curve under physiological conditions in humans [143]. These studies challenged the (albeit pragmatic) clinical rationale of simply increasing the dose of glibenclamide from 5mg/day up to 30mg/day when glycaemic control was not achieved, as they suggested that the medication was effective over a rather narrow range of plasma concentrations (50-200nmol/L), equivalent to oral doses less than 10mg/day. Interestingly, while glucagon levels were unaffected by glibenclamide in these studies (which

were carried out on healthy volunteers), a more recent study in patients with type 1 diabetes (C-peptide negative) demonstrated that a single glibenclamide dose of 10.5mg caused a significant rise in plasma glucagon levels during a hyperinsulinaemic euglycaemic clamp [144].

4.2. Purpose of *in vitro* hormone secretion experiments

The main objective of this project was to determine the effect low doses of glibenclamide have on fasting plasma glucagon levels in patients with T2DM, without stimulating insulin secretion. Therefore, a key element in this study was to decide on the dose-range to investigate. However, this presented a challenge as no previous human trials have reported plasma glucagon values when using doses of glibenclamide under 5mg.

To better understand the dose-response relationship between glibenclamide and glucagon, a series of *in vitro* experiments were constructed using isolated islets, which would complement and inform the clinical trial set-up. These initially involved the use of wild-type mouse islets, with a view to refining the hormone secretion methodology and saving the less abundant human islets for more critical experiments. The human islets were isolated from deceased donors with and without diabetes and were provided by the Oxford Human Islet Isolation Facility, as well as national and international collaborators.

4.3. Glibenclamide dose-response curve (insulin) - mouse

The secretion pattern of insulin and glucagon from mouse islets at low and high glucose concentrations is well characterised, as is the influence of a number of pharmacological

agents, including sulfonylureas [145, 146]. Therefore, a glibenclamide dose-response curve for insulin was constructed in order to assess both the validity of my experimental technique, and to provide a starting point for future studies on human islets, which would focus on glucagon secretion.

As previous experiments had focused on the effect tolbutamide (a first-generation sulfonylurea) at concentrations of 10μM - 100μM [38], and in view of the difference in the doses used clinically (the starting dose for tolbutamide is 500mg, 100-fold higher than the 5mg for glibenclamide), the glibenclamide dose-response curve was constructed using concentrations ranging from 1nM to 100μM.

Glibenclamide (G0639, Sigma-Aldrich) was diluted in DMSO (dimethyl sulfoxide). Islets were isolated from wildtype C57Bl6 mice (as described in Section 2.1.2) and hormone secretion experiments were performed using a static incubation technique (see Section 2.1.4) at 10mM glucose. This concentration of glucose was chosen as it would sub-maximally stimulate insulin secretion. The results of the glibenclamide dose-response curve are presented in Figure 15, and demonstrate appropriate glucose-stimulated insulin secretion (between 1mM glucose and 20mM glucose) with a calculated EC50 for glibenclamide of 34nM. This is in keeping with data suggesting that insulin secretion is significantly stimulated at concentrations above 50nM [143].

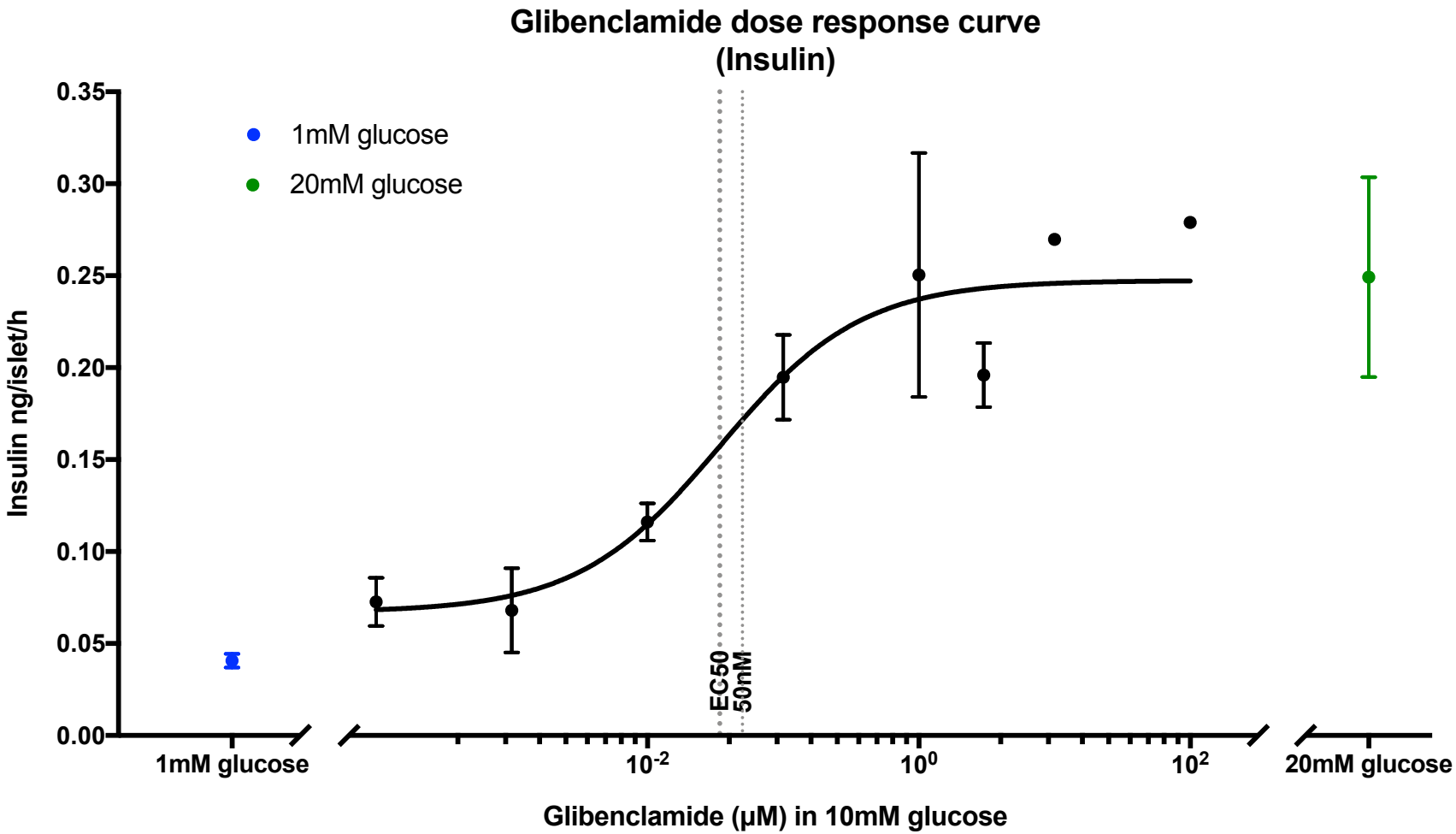


Figure 15 Glibenclamide dose response curve for insulin. The EC50 was calculated to be 34nM, and concentrations above 1μM glibenclamide led to maximal insulin secretion, as compared to 20mM glucose. All data-points are mean ± SEM.

4.4. Technical limitations and modification to technique

In the above experiments, islets from 2-3 animals (but of the same strain, age and sex) were pooled, and groups of size-matched islets from the common pool were used as replicates in the experimental conditions. Due to the number of conditions required for the dose response curve, and the number of islets required for each condition (around 10-15 per replicate) in order to reliably detect insulin and glucagon, it was not possible to test all dose-conditions during each experiment.

This presented a significant challenge when planning experiments using human islets as there was usually a limited supply. Therefore, a modification of the hormone secretion technique was developed in order to maximise the number of experimental conditions that could be performed using a single human islet preparation. This involved exposing the same group of islets (representing one replicate) to each condition sequentially (as described in Section 2.1.5), reducing the volume of experimental solution from 300µl to 200µl, and decreasing the incubation time per condition from 1h to 30min.

In order to validate the serial incubation method, a series of experiments were first performed using wildtype mouse islets (

Figure 16). These demonstrated that appropriate insulin and glucagon secretion changes could be identified using this set-up, suggesting more results could be generated with fewer islets. Furthermore, this method allowed more reliable detection of glucagon (due to higher concentration in the solution) using the duplex MSD assay (Section 2.2.1). By moving away from the previously used radio-immunoassay (RIA) technique, the samples could be assayed for both insulin and glucagon simultaneously, thereby avoiding repeated freeze/thaw cycles.

Figure 16

Serial incubation method validation (mouse)

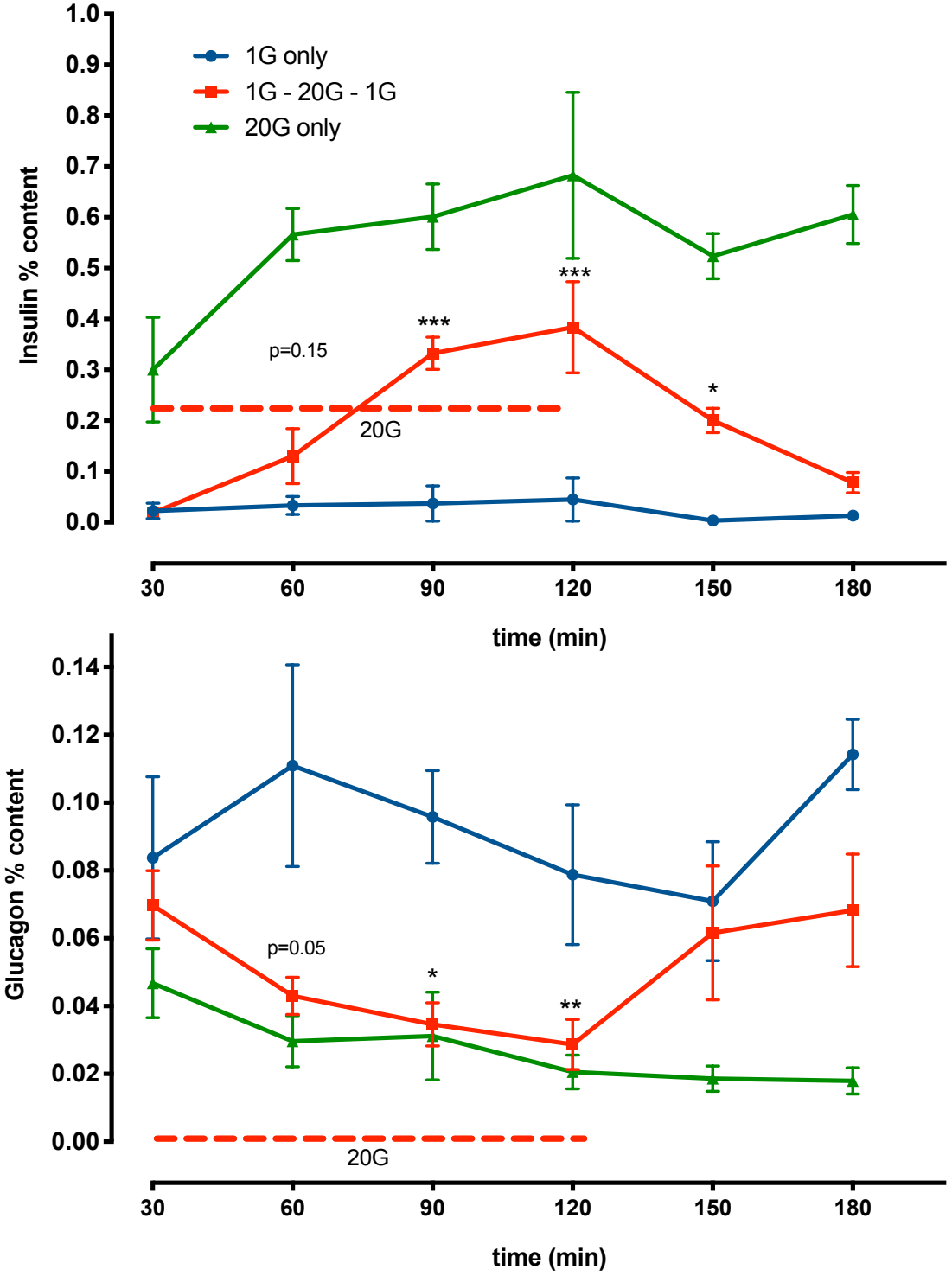


Figure 16 Validation of the serial incubation methodology using mouse islets (3-5 replicates per group), which were sequentially exposed to the glucose conditions at 30min intervals. In the blue and green groups, islets were exposed to only 1mM glucose (1G) or 20mM glucose (20G) for the duration of the study. In the red group, islets were initially in 1mM glucose for 30min, followed by 20mM glucose for 1.5 hours (indicated by the dashed line), and then in 1mM glucose again for 1h. Data were analysed using one-way ANOVA (comparison made with baseline values at 30min in each group), and the graphs show mean ± SEM (*$p<0.05$, **$p<0.001$, ***$p<0.0001$). Of note, there appears to be a time-delay before reaching maximal stimulation / inhibition when glucose conditions are changed, however this seems to affect insulin secretion more than glucagon. In the groups with unchanged glucose conditions, while there was variation with time this did not reach statistical significance.

4.5. Glibenclamide dose response curve - human

Having validated the serial incubation method in mouse islets, a series of experiments using human islets was performed to construct the glibenclamide dose-response curve for glucagon, using glibenclamide concentrations from 1nM to 1μM concentration (see Section 2.1.5).

A list of the human islet preparations which were used in this project is provided in Table 6. All human islets were obtained with appropriate consent for use in research (see Section 2.1.3). In addition to islets from non-diabetic donors, one preparation was obtained from a donor with T2DM who shared characteristics with the target population of the clinical trial (HbA1c 6.7%, on metformin alone).

Table 6 Details of human islet preparation used in the glibenclamide dose-response curve experiments.

Islet prep. identifier	Donor Age (years)	Donor Sex	Donor BMI (kg/m^2)	Additional details
HP16-23	32	Female	31	
HP16-24	33	Male	21	
HP16-26	63	Male	23	
HP16-32	55	Female	26	
HP16-38	59	Female	59	
HP16-42	32	Male	26	
P425	31	Male	35	isolated at King's
R184	59	Female	21.5	isolated in Edmonton
T2DM donor				
R173	77	Male	35.7	T2DM for 4 years, on metformin, HbA1c 6.7%. Isolated in Edmonton

4.5.1. Glucagon

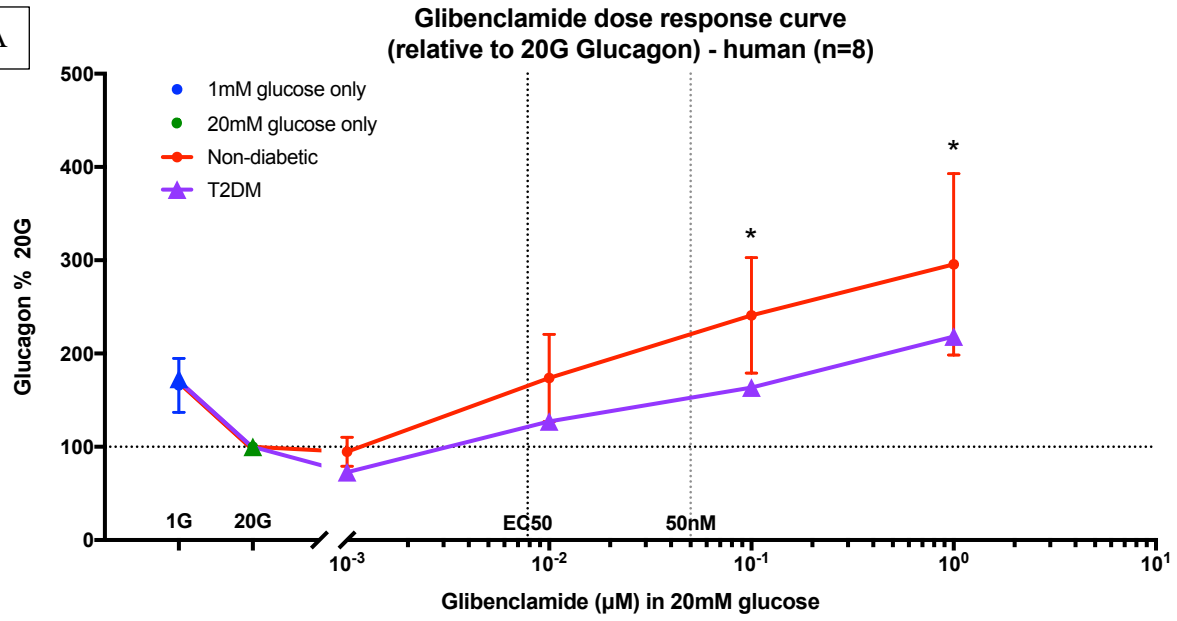
The results of the glibenclamide dose-response curve for glucagon are presented in Figure 17. While overall there appeared to be an inhibition of glucagon secretion between 1mM and 20mM glucose (as would be expected), this did not reach statistical significant when compared by one-way repeated measures analysis of variance (one-way RM ANOVA). The reason for this may in part be the heterogeneity that human islet preparations exhibit with respect to hormone secretion experiments (see Section 3.8.1). As these were pilot experiments, no sample size calculations were performed prior to the execution.

Unexpectedly, concentrations of glibenclamide ≥100nM in the presence of 20mM glucose stimulated glucagon secretion. The calculated EC50 was around 8nM. This glibenclamide-stimulated glucagon secretion was also reproduced when comparing the results of the single T2DM islet preparation. Intriguingly, although concentrations >10nM stimulated glucagon secretion, 1nM glibenclamide was inhibitory (by ~30%).

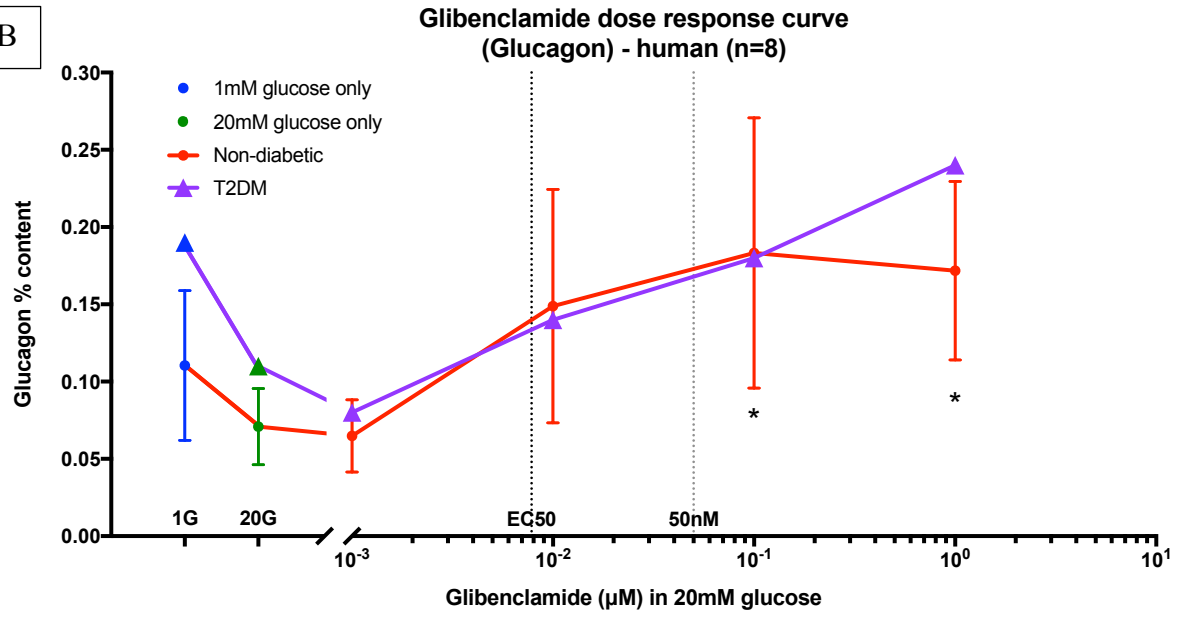
While these results are purely descriptive, due to the lack of other preparations from donors with T2DM, they indicate that during the clinical trial it would be important to investigate doses of glibenclamide that were 1-2 orders of magnitude lower than those normally used clinically.

Figure 17

A



B



One way RM ANOVA

Condition		mean % content (n=8)	SEM	adjusted p value (vs 20G)	T2DM (n=1)
1G		0.111	0.04855	0.367	0.19
20G		0.071	0.02473		0.11
Glibenclamide (µM) at 20mM	0.001	0.065	0.02337	>0.999	0.08
	0.01	0.149	0.07563	>0.999	0.14
	0.1	0.183	0.08745	0.041	0.18
	1	0.172	0.05774	0.022	0.24

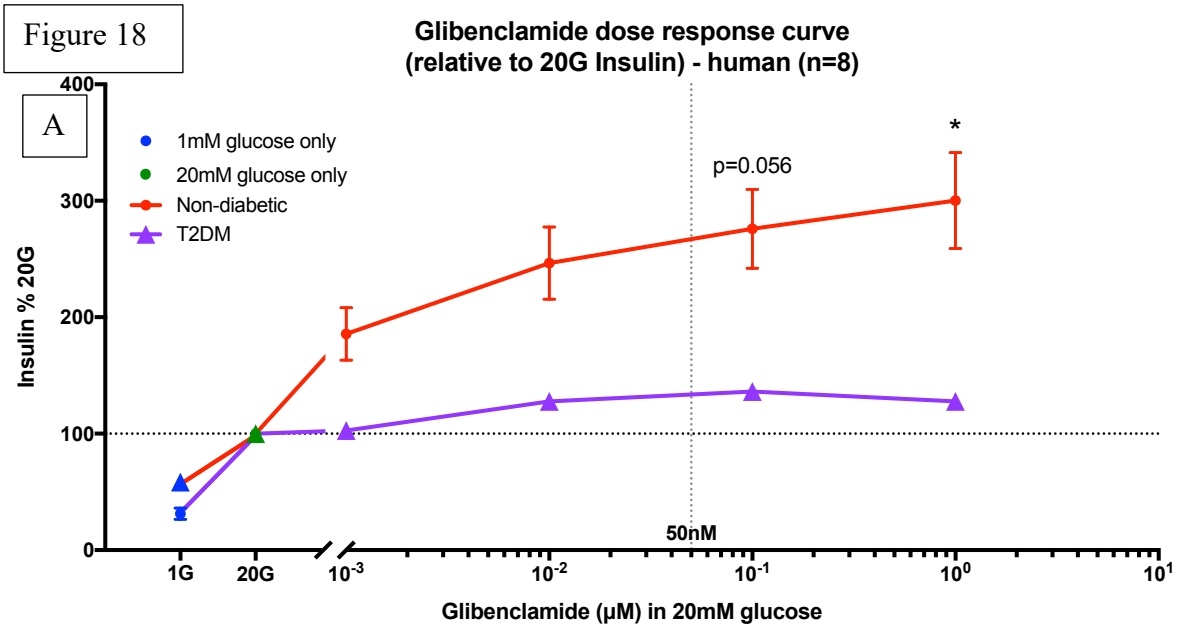
Figure 17 Glibenclamide dose-response curve for glucagon at 20mM (20G) using human islets. Values are expressed as either: **(A)** percentage of baseline (20mM glucose) for each islet preparation or **(B)** percentage of content. Preparations from 8 donors without diabetes (circles), and one from a donor with T2DM (triangles) were used in these experiments. Blue circle / triangle indicates results at 1mM, green circle / triangle indicates results at 20mM (without glibenclamide). Groups of islets were exposed to increasing concentrations of glibenclamide sequentially using 30min incubations. Data presented are mean ± SEM (*$p<0.05$, **$p<0.001$). The results were analysed using one way repeated measures ANOVA (table), comparing all conditions to 20mM glucose. The calculated EC50 was around 8nM, while concentrations above 50nM resulted in a significant stimulation of glucagon secretion, as indicated by the adjusted p values.

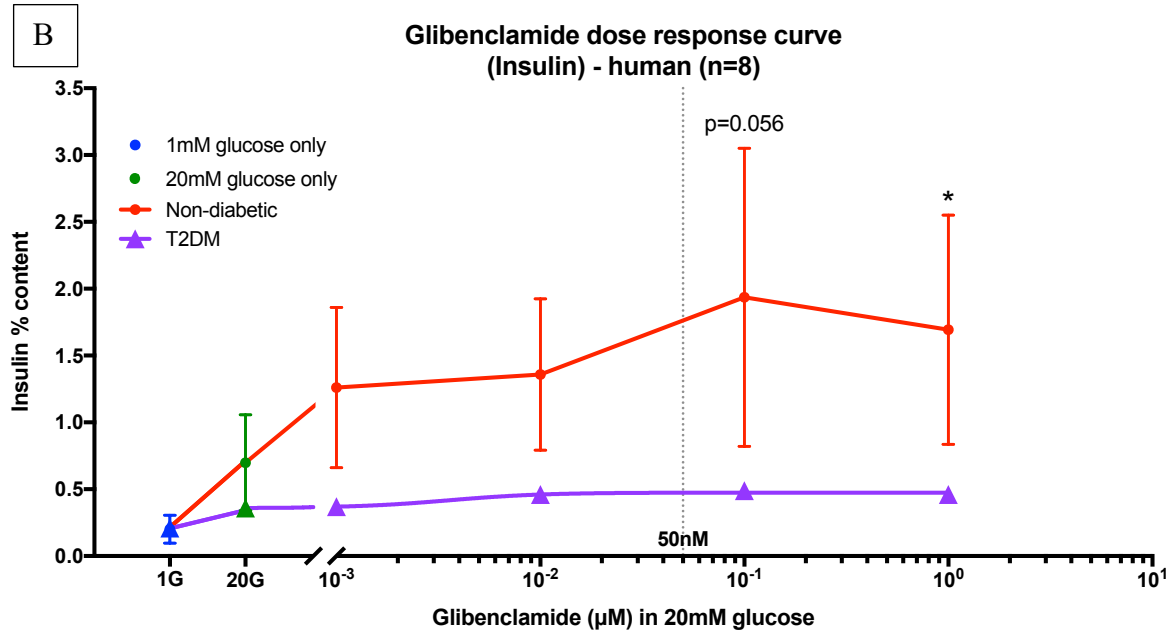
4.5.1. Insulin

The corresponding glibenclamide dose response curve for insulin is presented in Figure 18. Similarly to the glucagon results, while there is an overall 3.5-fold increase in insulin secretion from 1mM to 20mM glucose, this does not reach statistical significance when compared using One way RM ANOVA. The results from the glibenclamide conditions are particularly heterogeneous (as indicated by the large standard errors), however concentrations above 50nM resulted in statistically significant increases of insulin secretion compared to the 20mM glucose condition alone.

Interestingly, the islets from the donor with T2DM did not demonstrate significant glucose-stimulated, nor glibenclamide-stimulated insulin secretion. This response is reminiscent of the hyperinsulinaemic clamp studies performed in diet-controlled T2DM patients, before and after sulfonylurea therapy (gliclazide), and non-diabetic controls [147]. These showed an impaired response of insulin and C-peptide secretion at hyperglycaemic conditions (15mmol/L), which was not significantly increased after sulfonylurea treatment. Moreover, impairment of glucose-stimulated insulin secretion has been noted in a large series of islets isolated from donors with T2DM [109].

Figure 18





One way RM ANOVA

Condition		mean % content (n=8)	SEM	adjusted p value (vs 20G)	T2DM (n=1)
1G		0.20	0.10	>0.999	0.21
20G		0.70	0.36		0.36
Glibenclamide (µM) at 20mM	0.001	1.26	0.60	>0.999	0.37
	0.01	1.36	0.57	0.224	0.46
	0.1	1.94	1.12	0.056	0.49
	1	1.69	0.86	0.015	0.46

Figure 18 Glibenclamide dose-response curve for insulin at 20mM glucose (20G), using human islets. Values are expressed as either: **(A)** percentage of baseline (20mM glucose) for each islet preparation or **(B)** percentage of content. As in Figure 17, preparations from 8 donors without diabetes (circles), and one from a donor with T2DM (triangles) were used in these experiments. Blue circle / triangle indicates results at 1mM, green circle / triangle indicates results at 20mM (without glibenclamide). Groups of islets were exposed to increasing concentrations of glibenclamide sequentially using 30min incubations. Data presented are mean ± SEM. The results were analysed using one-way repeated measures ANOVA (table), comparing all conditions to 20mM glucose.

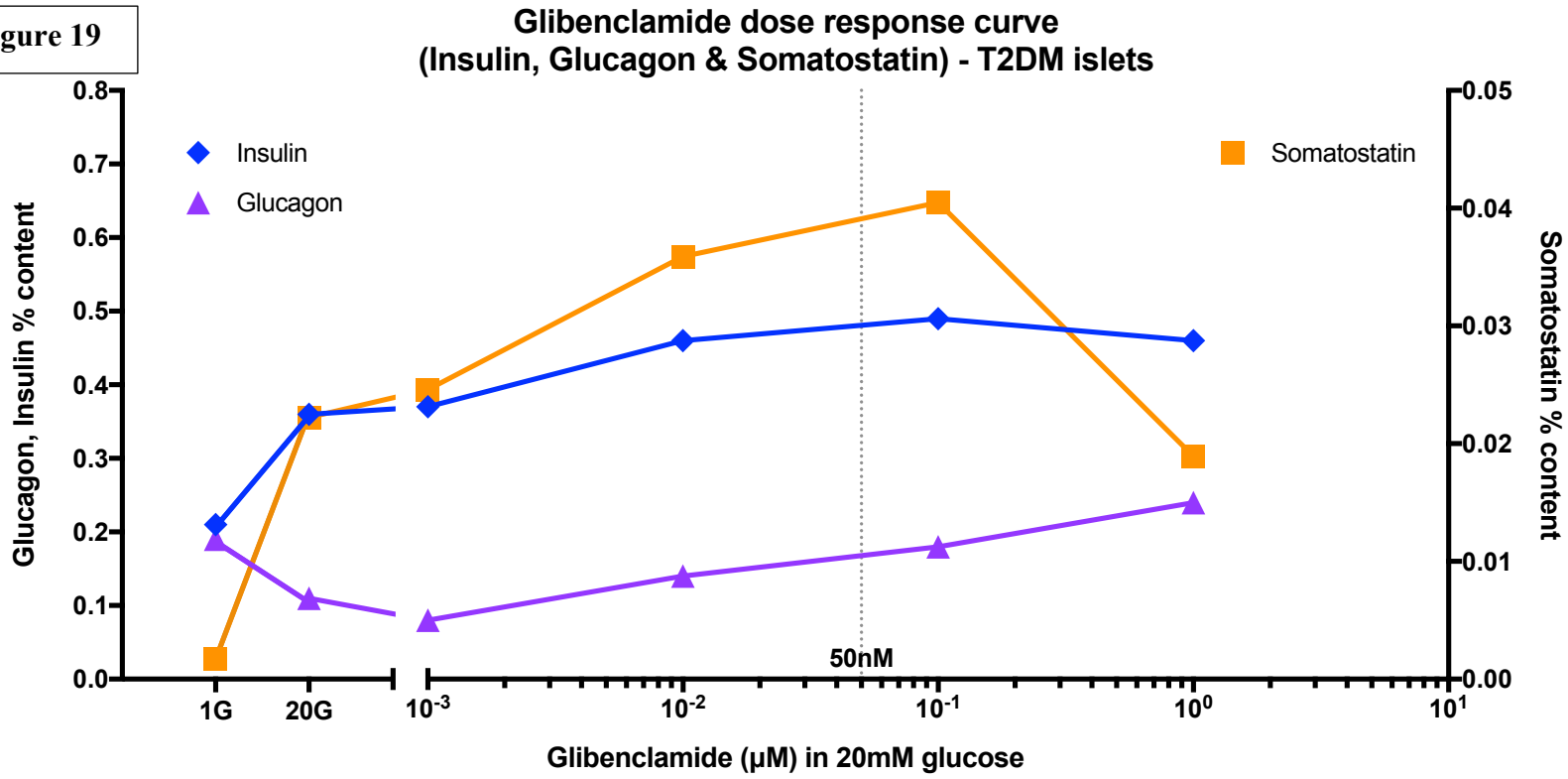
4.5.2. Somatostatin

In addition to insulin and glucagon, a separate sample aliquot for measurement of somatostatin was prepared during the glibenclamide dose-response experiments using the diabetic islets (R173). This was not part of the original set-up, and therefore not performed in the experiments using non-diabetic islets. However, it provided an opportunity to examine the effect low and high concentrations of glibenclamide had on delta-cells, and how this might impact the secretion of the other islet hormones.

The effect of somatostatin is particularly relevant in the context of alpha-cell regulation, as previous studies on pancreatic sections from donors with and without T2DM have reported significant differences in islet beta- / alpha-cell ratio but an unchanged delta-cell fraction [148]. Moreover, it was recently demonstrated that beta- and delta-cells are electrically coupled via gap junctions, and that this mechanism contributes around 20% to the glucose-induced glucagon suppression in human islets [42].

The somatostatin, insulin and glucagon secretion results, expressed as percentage of content, are presented alongside each other in Figure 19. Interestingly, there was a 20-fold increase in somatostatin secretion between 1mM and 20mM glucose but just under 2-fold increase in insulin secretion (as discussed in Section 4.5.1). Somatostatin appears to have a bi-phasic secretion pattern as glibenclamide concentration increases: it rises by about 80% at 100nM glibenclamide before falling to 15% below baseline at 1μM. This change in behaviour occurs at concentrations which significantly increased insulin secretion in non-diabetic islets (Figure 18) and are also known to do so clinically [143], however the mechanism behind this is unclear.

Figure 19



Condition		Somatostatin	Glucagon	Insulin
1G		0.002	0.19	0.21
20G		0.022	0.11	0.36
Glibenclamide (µM at 20mM)	0.001	0.025	0.08	0.37
	0.01	0.036	0.14	0.46
	0.1	0.040	0.18	0.49
	1	0.019	0.24	0.46

Figure 19 Glibenclamide dose-response curve for insulin, glucagon and somatostatin at 20mM glucose, using human islets from a single donor with T2DM (R173). Somatostatin secretion was appropriately suppressed at 1mM glucose (1G) and significantly stimulated by 20mM glucose (20G). Glibenclamide appears to cause a bi-phasic response as concentrations increase from 1nM to 1μM, with a shift in behaviour occurring at concentrations known to stimulate insulin secretion in vivo (>50nM, dotted line) [143].

4.6. Conclusions from *in vitro* experiments

Isolated islet hormone secretion experiments are unique tools to investigate physiological and pharmacological stimuli, without the complexities of *in vivo* models. They do however pose their own challenges, both in the way the experiments have to be performed and in the interpretation of the results.

In this chapter I have demonstrated the valuable role that mouse islets can play during the optimisation of a new experimental protocol, and that when using a limited number of sequential (un-selected) human islet preparations it is possible that the mean hormone secretion values at baseline (1mM and 20mM glucose) will not reach a statistically significant difference, even if the same conditions achieve the expected results in isolated mouse islets. This is likely due to the range of values that are obtained under normal circumstances (see Section 3.8.1). However, these experiments can give insights into possible interactions, which can aid in the preparation of larger experiments or pilot translational clinical trials.

The overall conclusions from these pre-clinical experiments are as follows:

a. Glibenclamide at concentrations >100nM stimulates both insulin and glucagon secretion.

b. Glibenclamide concentrations below 10nM (i.e. < 10% the concentration which stimulates insulin secretion) may reduce glucagon secretion in patients with T2DM by about 30%, thereby bringing the values in range with those in non-diabetic individuals.

This information was taken into account during the planning of the LEGEND-A clinical trial. Based on these findings we decided to test glibenclamide using doses as low as 0.3mg/day, less than 10% of the normal starting dose of glibenclamide which is 5mg/day. Given the practicalities of the available oral glibenclamide suspension (see Section 2.3.6), the dose-range selected was as follows: 0.3mg, 0.6mg, 1.2mg, 1.8mg, 3mg and 6mg total daily dose.

5. Chapter 5: LEGEND-A trial

Diabetes is a multi-hormonal disorder characterised by insufficient insulin secretion and aberrant glucagon secretion, with fasting hyperglucagonaemia leading to increased rates of hepatic glucose production [73, 74], which further exacerbates hyperglycaemia. Sulfonylureas used at low concentrations have been shown to partially restore appropriate glucose-regulated glucagon secretion in isolated islets from donors with type 2 diabetes (T2DM) [35].

The "Low-dosE GlibENclamide in Diabetes – part A" (LEGEND-A) trial was a 4 week, open-label, non-randomised, dose-finding clinical trial of an oral glibenclamide suspension (GlibenTek [105]). Sixteen participants with type 2 diabetes (T2DM; diet-controlled or on metformin alone) were given increasing doses of glibenclamide (0.3mg – 6mg/day), and fasting blood samples were taken prior to each dose change. The primary aim of this trial was to determine whether and at what dose the sulfonylurea glibenclamide could safely reduce fasting glucagon levels in this patient population (see Appendix A.2).

Participants were recruited through the CRU at OCDEM and the Oxford Biobank study databases at the Churchill Hospital, Oxford. After a screening visit and baseline fasting blood samples at two consecutive study visits, participants were given the oral glibenclamide suspension, a dosing syringe, and a personalised dosing schedule. Participants self-administered the glibenclamide suspension (half the total daily dose in the morning, half in the evening), and the dose was increased every 3-4 days (see Appendix A). Further details of the study design can be found in Section 2.3.

Funding for this trial was provided by the NIHR Biomedical Research Centre, based at Oxford University Hospitals NHS Foundation Trust, Oxford.

5.1. Choice of sulfonylurea and sourcing of IMP

All doses of sulfonylureas (SU) used clinically primarily aim to increase the secretion of insulin from beta-cells, however the dose that exclusively affects glucagon secretion is believed (based on *in vitro* data) to be approximately 10% of that which causes insulin secretion [38]. In order to reliably deliver doses as low as this, it was decided to use either intravenous or oral suspension (i.e. non-tablet) formulations.

The original experiments in mouse and human islets were performed using tolbutamide [35], however this first-generation SU is not commonly used and is no longer available in intravenous form. Gliclazide is a second-generation SU which is commonly prescribed in the UK in the form of tablets. It also exists as an oral suspension, however this formulation is an unlicensed medicinal product (according to MHRA classification) and therefore cannot be used in clinical trials. Glibenclamide, also known as glyburide, is another second-generation SU which is less commonly used in the UK due to its prolonged hypoglycaemic action [149] and is only licensed in tablet form. It is however more widely available worldwide (unlike gliclazide, it is licensed for use in the USA).

Originally the decision was made to formulate a low-dose oral suspension of gliclazide to use in the LEGEND-A clinical trial. GMP (good manufacturing practice)-accredited facilities from around the UK were contacted and eventually arrangements were made with the University of Nottingham to supply the medication as an investigational medicinal product (IMP). However, after multiple attempts it was not possible to locate an appropriate supplier of the active pharmaceutical ingredient and so the arrangements fell through.

At the same time, data were presented on a novel oral suspension of glibenclamide produced by a group in France [105]. This new formulation (GlibenTek) had been developed in two strengths (0.6mg/ml and 6mg/ml), which were suitable for the dose ranges being tested in the LEGEND-A protocol. The group and the manufacturer (AmmTek) were contacted, and a materials transfer agreement was arranged to supply the oral suspension for use in the clinical trial. This involved a confidentiality agreement prior to the release of the Investigator's Brochure, and a manufacturing agreement with the companies AmmTek and Pharma Services (both in Paris, France).

5.2. Glibenclamide pharmacokinetics

Glibenclamide is well absorbed from the gut, and this is not affected by food intake [150]. It is extensively bound to plasma proteins and has a volume of distribution of 9-10 litres. Compared to other sulfonylureas it has a relatively prolonged half-life at 10 hours and reaches peak plasma concentration at 3 hours. It is almost entirely metabolised in the liver and plasma levels fall to less than 5% of peak value within 24hours. Excretion is 50% in the urine and 50% in the bile. The metabolites of glibenclamide also exert a hypoglycaemic effect, which may contribute to its prolonged clinical action [151, 152].

The Investigator's Brochure for GlibenTek provides information from the manufacturer on the pharmacokinetic profiles of the two oral suspension strengths, and this was also presented at the 54th annual meeting of the European Society of Paediatric Endocrinology [105]. It demonstrated that the novel oral suspension had comparable pharmacokinetic properties to the licensed glibenclamide tablet (Table 7).

Table 7 The two difference strengths of GlibenTek (6mg/ml and 0.6mg/ml) were compared to crushed tablets of the licensed tablet Daonil [105]

	0.83ml GlibenTek 6mg/ml	**8.33ml GlibenTek 0.6mg/ml**	**5mg Daonil**
t_{max}	2.5h	2.5h	3h
C_{max}	201.71± 71.43 ng/ml	206.93± 67.33 ng/ml	148.34± 46.74 ng/ml
$AUC_{0-\infty}$	1120.9± 400.5 ng.h/ml	1172.3± 422.0 ng.h/ml	
Bioavailability	114.1% (relative to Daonil)	121.6% (relative to Daonil)	100%
$t_{1/2}$	8h	8h	10.45h

5.3. Patient involvement project

As described in Section 2.3.1, a patient involvement project (PIP) was carried out to receive feedback on the structure of the proposed trial and identify methods that could potentially improve participants' ease of participation and adherence to the protocol. Invitation letters and questionnaires were sent to 68 patients with T2DM who were identified through the CRU database. Twenty-two (32%) responded, and of those sixteen (67%) indicated that they would be interested in participating in the trial. A breakdown of the answers can be found in Table 8.

Table 8 Responses to PIP questionnaire

1. If continuous glucose monitoring (CGM) was not compulsory I would be:		
	More likely to participate	32%
	Less likely to participate	4%
	No difference	64%
2. What sorts of meal / exercise diaries do you think would be easier for you to fill out over a 4 week period:		
	Paper diary	21%
	Computer document	12%
	Daily email	21%
	Daily email sent by CRU which I reply to	18%
	Text message	14%
	Mobile phone app	14%
3. In addition to written information, what other methods do you think might be helpful to remind participants of the correct dose to take and their future appointments:		
	Daily email	34%
	Daily text message	33%
	Mobile phone calendar / reminder	19%
	Mobile phone app	14%

The responses from the PIP, along with individual comments, were reviewed and the clinical trial protocol was modified in line with the feedback provided. Specifically, the CGM component was made non-compulsory (as it was not a primary objective), the meal / exercise diary was removed from the protocol (due to high variability in the responses and likely problems with its implementation), and technology-based reminders for the dosing schedule were abandoned in favour of a personalised paper calendar with clear instructions for each day (as was suggested in the comments).

5.4. Trial set-up and approvals

The research protocol and supplementary documents (including patient information sheet, consent form, GP letter, case report forms and sample dosing schedule) were submitted online via the integrated research application system to the relevant authorities (IRAS project ID 179317). The trial was given a favourable ethical opinion by the South Central – Berkshire B Research Ethics Committee (REC reference 16/SC/0202), and approved by the MHRA (Medicines and Healthcare products Regulatory Agency) and by the Health Research Authority (HRA). The trial was registered on the European Clinical Trials Database (EudraCT number 2015-002837-23) and on ClinicalTrials.gov (NCT02830048). Local approval was also obtained from the Oxford University Hospitals NHS Foundation trust.

The trial was monitored by The Oxford University Hospitals Trust / University of Oxford Trials Safety Group (TSG), which conducted a review of all adverse events (AE) and checked for adherence to the trial protocol.

5.5. Results

The results from the trial were analysed according to the predefined statistical plan (see Section 2.3.4). Data from a total of 16 participants was included (Figure 20), as glucagon measurements could not be obtained from three time points (three different participants) and therefore an extra participant was recruited to avoid loss of statistical power (as predefined in the sample size calculation, Section 2.3.3).

5.5.1. Participant characteristics

In total 19 patients attended screening visits, of which 3 did not meet the inclusion / exclusion criteria (HbA1c < 42mmol/mol, 6.0%) and 1 had to defer entry to the trial due to concomitant medication use (Figure 20). Details of the participants' baseline characteristics can be found in Table 9. Overall, there was a wide range of diabetes duration (0.5 - 18 years) and HbA1c values (43 – 68 mmol/mol). Six participants had HbA1c values less than 47 mmol/mol (6.5%), of whom 5 had a duration of diabetes ≤ 1 year, and 1 had had diabetes for 6 years.

Figure 20 Flow diagram of LEGEND-A trial

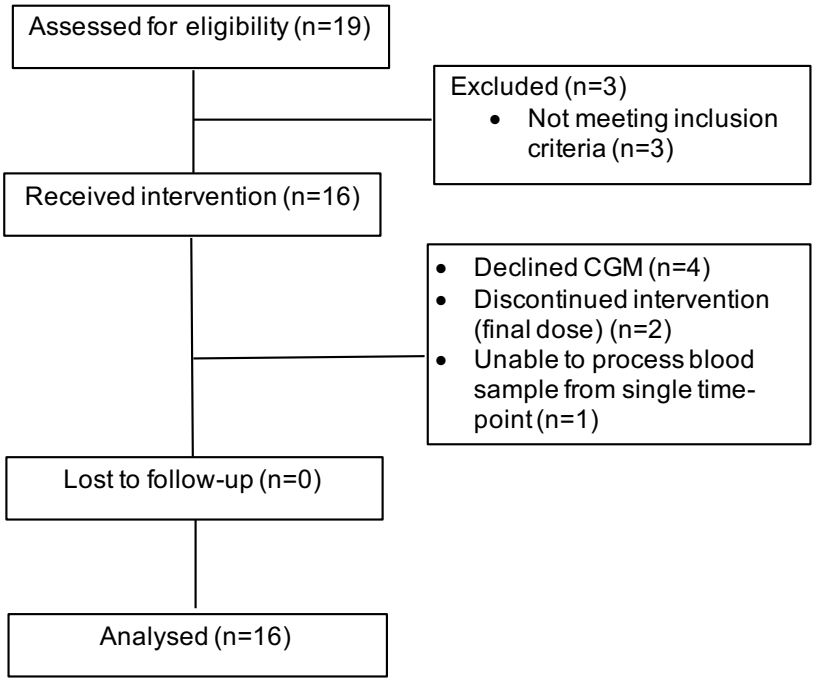


Table 9 Participant baseline characteristics. Data are shown as median (minimum – maximum).

Age (years)	66 (43-82)
Sex	7 males, 9 females
BMI (kg/m^2)	29.7 (24.7-38.3)
Duration of diabetes (years)	4 (0.5-18)
HbA1c (mmol/mol)	51 (43-68)
HbA1c (%)	6.8 (6.1-8.4)
Taking metformin	9 participants
Metformin dose (g)	1 (0.5-2)

5.5.2. Glucagon

Plasma glucagon levels after an overnight fast in individuals without diabetes generally range between 6 – 12 pmol/L and fall to 3 – 5 pmol/L after an oral glucose tolerance test (OGTT) [95, 96, 102]. However, in patients with T2DM fasting values can be 50 – 100% higher, and there can be impaired suppression of glucagon secretion or even stimulation after an OGTT [69].

For the purposes of analysis at the end of the trial, participants were grouped into 'Normal' and 'High' baseline fasting plasma glucagon (which was defined in this study as >15pmol/L). This grouping was pre-defined in the statistical plan and accounted for during sample size calculations (see Section 2.3.4). While the majority (twelve out of sixteen participants) had levels in the 'Normal' range (visit 3 mean 6.2 pmol/L, ±0.45 SEM), four participants (25%) had fasting levels that were 4 times higher (mean 25.6 pmol/L, ±6.3 SEM; $p<0.001$) (Figure 21).

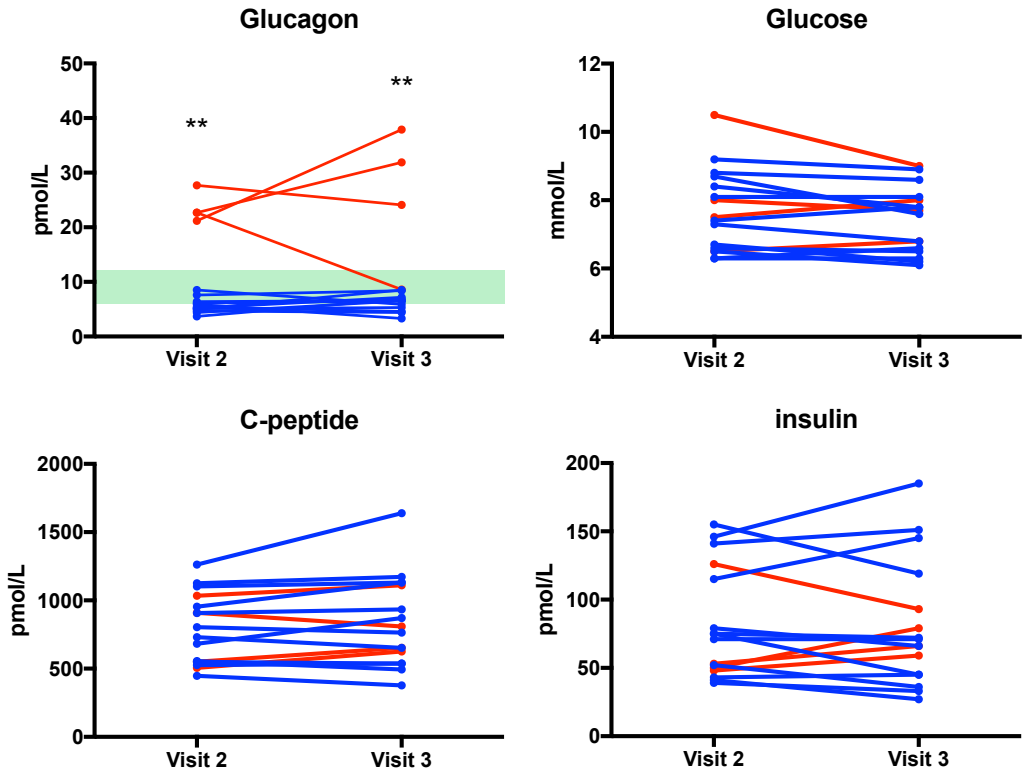
5.5.2.1. Baseline variation

Two baseline blood samples were taken on separate occasions (visits 2 and 3, Appendix A.3). This was done to determine the baseline variation in levels of fasting glucagon, glucose, c-peptide and insulin within our study population. As specified in the statistical plan (Section 2.3.4.1), mean baseline fasting glucagon measurements were used to determine subgroup inclusion for analysis.

While levels remained relatively stable between visits 2 and 3 for most participants within the 'Normal' and 'High' groups (Figure 21), fasting glucagon values for one participant in the 'High' group changed from the 'High' range to the 'Normal' range (visit 2: 22.7

pmol/L, visit 3: 8.6 pmol/L). This occurred despite a lack of change in fasting glucose values (visit 2: 6.5 mmol/L, visit 3: 6.8 mmol/L). As the mean fasting glucagon value for this participant exceeded 15 pmol/L, they were included in the 'High' group.

When looking at the baseline characteristics of participants grouped into either 'Normal' or 'High', there were no distinguishing features that separated the two groups other than the difference in fasting glucagon levels (Table 10). This included estimation of their steady state beta-cell function (HOMA2-B) and insulin sensitivity (HOMA2-S) using the Homeostasis Model Assessment [153]. There was a tendency for participants in the 'Normal' group to be older, however this did not reach statistical significance (p=0.05)



	V2 mean (SEM)	V3 mean (SEM)	adjusted p value
Glucagon pmol/L			
Normal	5.7 (0.38)	6.2 (0.45)	0.79
High	23.6 (1.4)	25.6 (6.3)	0.54
adjusted p value	<0.001	<0.001	
Glucose mmol/L			
Normal	7.5 (0.3)	7.3 (0.3)	0.14
High	8.1 (0.8)	7.9 (0.4)	0.14
adjusted p value	0.34	0.34	
C-peptide pmol/L			
Normal	804 (77)	854 (106)	0.20
High	750 (131)	799 (111)	0.20
adjusted p value	0.76	0.76	
Insulin pmol/L			
Normal	86 (12)	83 (15)	0.89
High	69 (19)	74 (7)	0.89
adjusted p value	0.62	0.62	

Figure 21 Variation in baseline blood tests between CRU visit 2 (V2) and visit 3 (V3), grouped by fasting glucagon values at V2. There was a statistically significant difference (two-way RM ANOVA with Bonferroni correction) between glucagon values in the 'Normal' and 'High' group for both V2 and V3 ($p<0.001$), and despite the degree of variation displayed at V3 (note one participant whose glucagon values appear to change group), there was no statistically significant difference between visits within the groups ($p=0.96$ and $p=0.79$ for 'Normal' and 'High' respectively). There was no statistically significant difference between visits or between groups for any of the other blood tests.

Table 10 Baseline characteristics of participants, divided into 'Normal' or 'High' group according to baseline fasting plasma glucagon concentrations. Data are mean ± SEM. T-tests with Holm-Sidak correction for multiple comparisons were used.

	'Normal' (n=12)	'High' (n=4)	Adjusted p-value
Age (years)	68 ± 2	52 ± 5	p=0.05
Sex	4 males, 8 females	3 males, 1 female	
BMI (kg/m^2)	30 ± 1	31 ± 3	p>0.99
Duration of diabetes (years)	5.1 ± 1.8	6.1 ± 2	p>0.99
HOMA2-B	74 ± 9	63 ± 10	p=0.98
HOMA2-S	56 ± 7	52 ± 6	p>0.99
HOMA2-IR	2.1 ± 0.3	2.0 ± 0.3	p>0.99
Taking metformin	6 participants	3 participants	
Metformin dose (g)	0.5 ± 0.2	1.2 ± 0.4	p=0.75
Baseline blood tests			
Glucagon (pmol/L)	6.2 ± 0.4	25.6 ± 6	p<0.001
Glucose			
mmol/L	7.3 ± 0.3	7.9 ± 0.4	
mg/dL	131 ± 5	142 ± 8	p=0.95
HbA1c			
mmol/mol	50 ± 2	54 ± 2	
%	6.7 ± 0.2	7.0 ± 0.2	p=0.95
C-peptide (pmol/L)	854 ± 106	799 ± 111	p>0.99
Insulin (pmol/L)	83 ± 15	74 ±7	p>0.99

5.5.2.2. Effect of glibenclamide on glucagon levels

As shown in Figure 22, glibenclamide at a dose of 0.3 mg/day resulted in a 32% reduction in plasma glucagon concentration in the 'High' group (17.4 pmol/L vs 25.6 pmol/L at baseline; $p<0.05$). This reduction was not seen with the higher doses, and glibenclamide had no effect on glucagon levels in the 'Normal' group.

5.5.3. Glucose

Mean fasting glucose values in both the 'Normal' and 'High' group were > 7.0 mmol/L at baseline (7.3 mmol/L ± 0.2 and 7.9 mmol/L ± 0.3 respectively), and 0.3mg/day of glibenclamide had no statistically significant effect on this (7.3 mmol/L ± 0.2 and 7.6 mmol/L ± 0.3) (Figure 22). In the 'Normal' group, doses > 1.8mg/day resulted in a statistically significant decrease in fasting glucose (1.8mg: 6.2 mmol/L ± 0.2, 3mg: 5.9 mmol/L ± 0.2, 6mg: 5.6 mmol/L ± 0.2, $p<0.001$), however in the 'High' group this only occurred at the highest dose of 6mg/day (6.4 mmol/L ± 0.3, $p<0.05$).

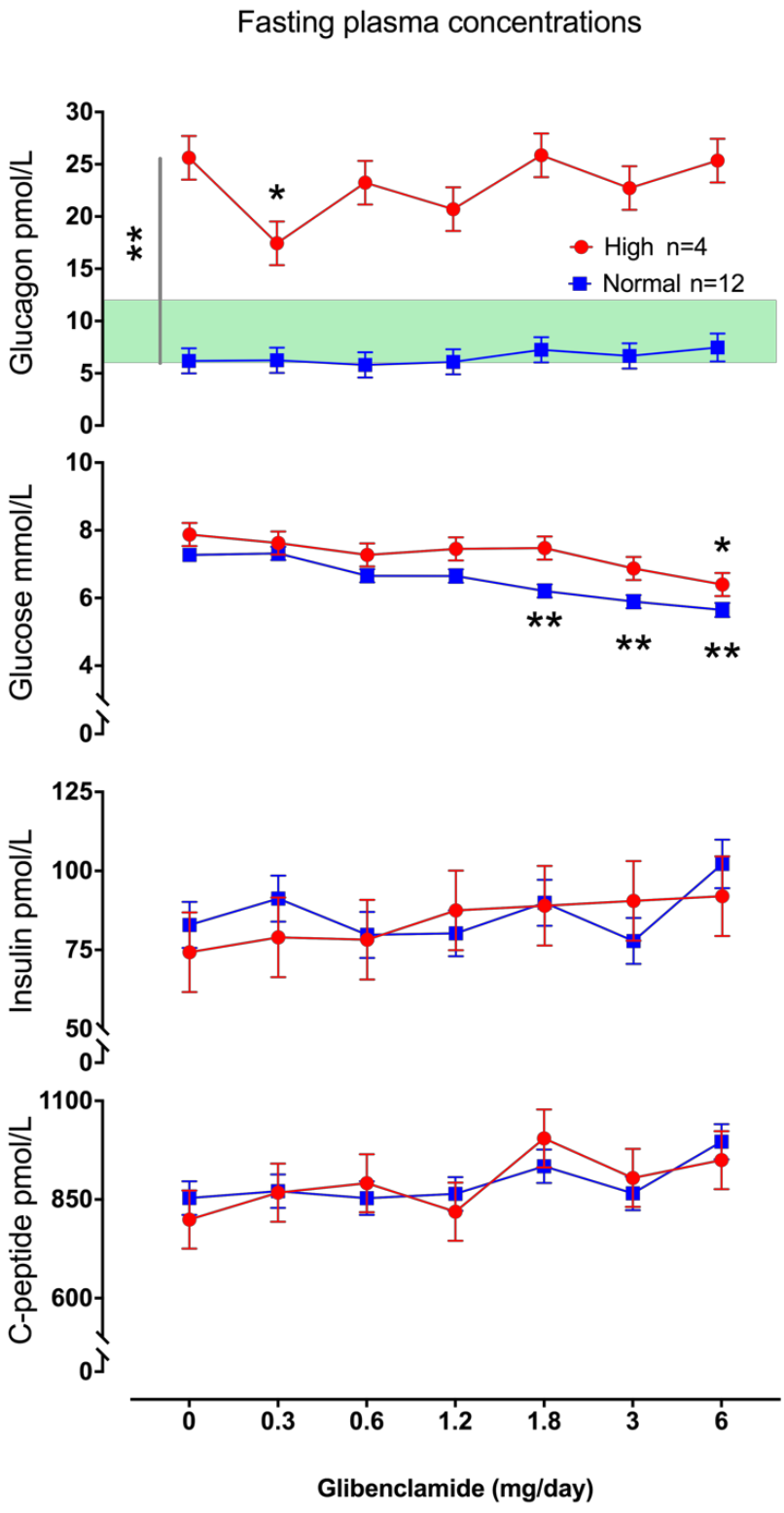


Figure 22 Fasting plasma concentrations of glucagon, glucose, insulin and c-peptide over the course of the trial. The green bar indicates normal range of glucagon [96]. Data were analysed using two-way repeated measures ANOVA (comparison made with values at baseline 0mg in each group), and the graphs show mean and SEM for each glibenclamide dose (*$p<0.05$, **$p<0.001$).

5.5.4. Insulin / C-peptide

Both insulin and C-peptide were measured in order to investigate whether glibenclamide had any effect on insulin secretion after an overnight fast, which could be appreciated either before or after hepatic clearance of insulin. Baseline levels were similar in both the 'Normal' (insulin: 82.9 pmol/L ± 7.3, C-peptide: 853.6 pmol/L ± 42.5) and 'High' group (insulin: 74.2 pmol/L ± 12.6, C-peptide 799.2 pmol/L ± 73.6), and glibenclamide had no effect on fasting levels (Figure 22).

5.5.5. Continuous glucose monitoring (CGM)

CGM was used in a subset of 12 participants who agreed to this optional component (9 in 'Normal' group, 3 in 'High' group). Changes in diet and activity levels over the course of the trial were not accounted for in the CGM data, nor were any treatments of hypoglycaemia episodes. In addition, this analysis was not accounted for during the sample size calculations. As such, the data presented is exploratory only (Figure 23).

Overall, there was a significant difference between the 'Normal' and 'High' group with regards to mean blood glucose ($p=0.044$) and time spent in hypoglycaemia (glucose < 4.0 mmol/L, $p=0.009$). This was then further explored using post-hoc multiple comparisons tests (Dunnett).

While there was a trend towards a reduction in mean glucose in the 'High' group with the lower doses of glibenclamide (baseline: 7.8 mmol/L ± 0.3, 0.3mg: 7.1 mmol/L ± 0.2, 0.6mg: 6.4 mmol/L ± 0.3) this was not statistically significant. There was no effect of glibenclamide on mean glucose ($p=0.08$) or the percentage of time spent in hyperglycaemia (glucose >10.0 mmol/L) in either the 'Normal' or 'High' group ($p=0.46$). However, the highest doses caused

a 10 – 15-fold increase in the percentage of time spent in hypoglycaemia in the ‘Normal’ group (baseline: 2.1% ± 1.1, 3mg: 23.7% ± 7.7 p=0.016, 6mg: 30.8% ± 8.1 p=0.001) (Figure 24).

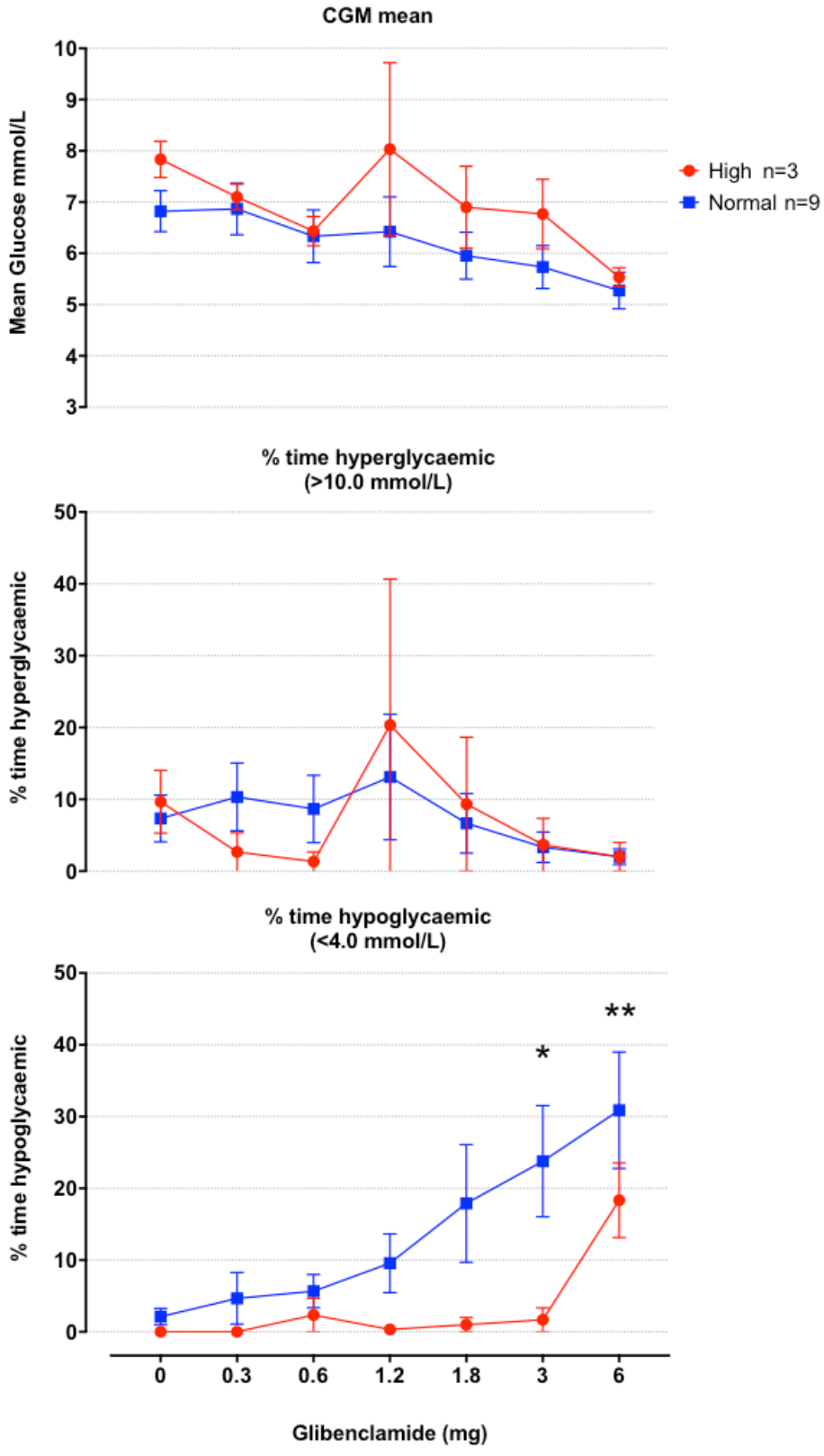


Figure 23 Continuous glucose monitoring data. Data were analysed using two-way repeated measures ANOVA (comparison made with values at 0mg in each group), and the graphs show mean and SEM for each glibenclamide dose (*p<0.05, **p<0.001).

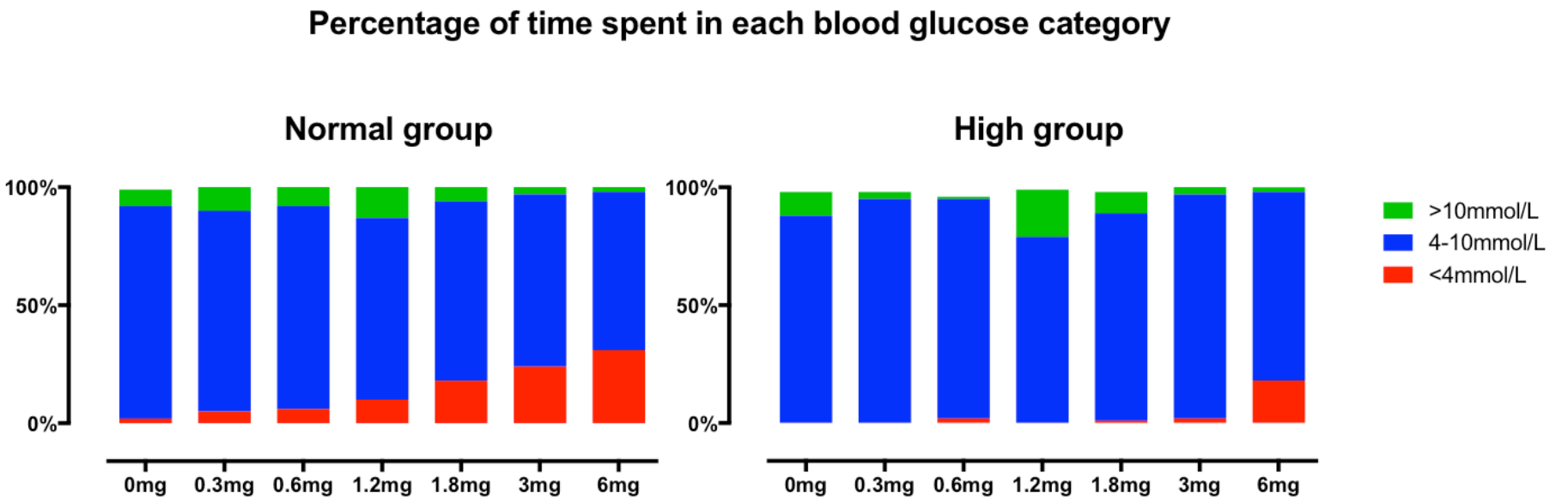


Figure 24 Percentage of time spent in euglycaemia (blue), hyperglycaemia (green) and hypoglycaemia (red), separated by group and dose-step. Participants in the 'Normal' group experienced an overall 10 – 15-fold increase in time spent hypoglycaemic at the highest doses (3mg and 6mg / day).

5.5.6. Glibenclamide

Plasma levels of glibenclamide were measured using a liquid chromatography tandem-mass spectrometry assay, which was optimised for use with human plasma. As described in Section 2.3.6, the total daily dose of glibenclamide was split into morning and evening, and blood samples were taken approximately 12 hours after the evening dose.

The calibration curve (Figure 25) was constructed using human plasma samples spiked with known concentrations of glibenclamide and deuterated glibenclamide internal standard (see Section 2.2.2). The resulting correlation coefficient (R=0.97) was satisfactory for the analysis, and the minimum detection limit was around 10 ng/ml.

At doses $\leq$ 1.2mg/day, plasma glibenclamide levels could not be detected in the pre-dose blood samples (Figure 26). These rose to 15.3, 17.2 and 29ng/mL at 1.8, 3 and 6mg/day respectively.

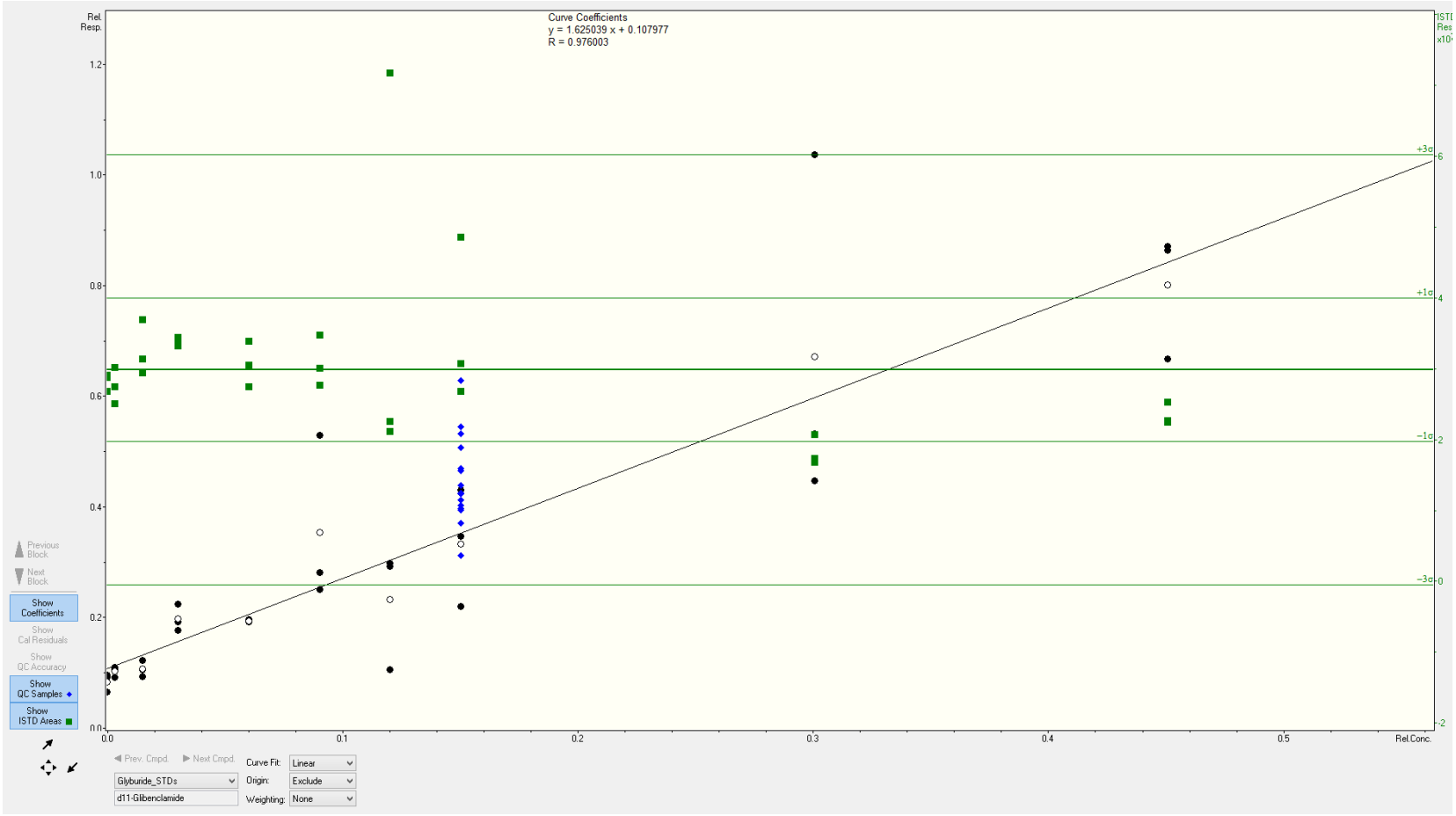
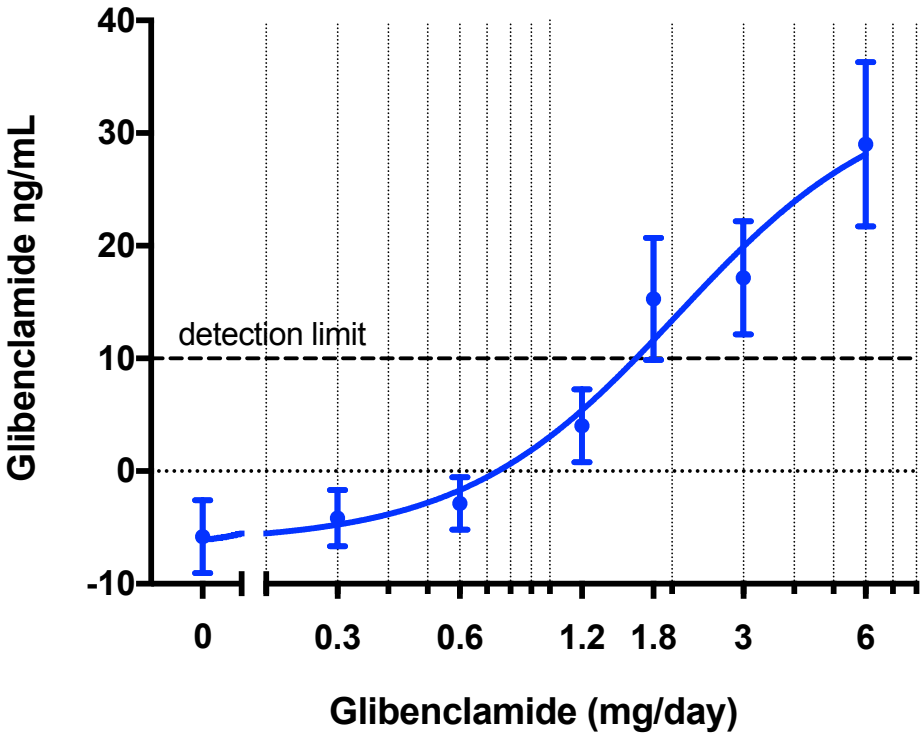


Figure 25 Calibration curve for glibenclamide assay (screenshot from QuantAnalysis 2.0 software). Black circles = glibenclamide standards (no drug, 1 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml, 30 ng/ml, 40 ng/ml, 50 ng/ml, 100 ng/ml 150 ng/ml), white circles = mean of standard, blue diamonds = quality control samples (50 ng/ml), green squares = deuterated glibenclamide internal standard (333 ng/ml). R = 0.97. Data courtesy of Dr. Rod Chalk, Structural Genomics Consortium.



Glibenclamide (mg/day)	glibenclamide (ng/mL)		
X	Mean	SEM	N
0.000	-5.825	3.234	16
0.300	-4.181	2.519	16
0.600	-2.863	2.320	16
1.200	4.025	3.247	16
1.800	15.288	5.424	16
3.000	17.169	5.042	16
6.000	29.000	7.303	14

Figure 26 Plasma glibenclamide levels at each dose-step, as determined by mass spectrometry (graph shows mean and SEM). Dashed line indicates detection limit, as determined by calibration curve data. Note that only 14 samples were available at 6mg/day, as two participants discontinued the medication due to adverse events (hypoglycaemia).

5.5.7. Adverse events

All adverse events (AE), defined as any untoward medical occurrence in a participant to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product) that occurred during the trial were recorded in the case report form (CRF). AEs were documented as "related to IMP" if in the opinion of the chief investigator (CI) there was a reasonable temporal sequence from trial medication administration and it could not be attributed to any other causes. All participants who experienced AEs were followed up until their symptoms had resolved.

Severity of the AEs was defined as mild (self-limiting, participant able to treat themselves), moderate (requiring third party assistance) or severe (life-threatening, requiring hospitalisation, or other important medical events as determined by the CI). An "expected" AE was defined as one which was listed in the Summary of Medicinal Product Characteristics (SmPC) for glibenclamide [154].

A list of the AEs reported during the trial can be found in Table 11. All AEs which were related to the IMP were deemed "expected", and there were no serious adverse events. Of note, the majority of hypoglycaemia episodes occurred in the 'Normal' group and were recorded in the final two dose steps. Five hypoglycaemia episodes were reported in the 'High' group at the highest dose of glibenclamide.

Table 11 Adverse events during LEGEND-A trial.

Adverse event	Related to IMP	Dose of IMP	Frequency	Severity
Headache	no	1.2mg	1	mild
Tired	no	3mg	1	mild
Urinary tract infection	no	6mg	1	mild
Dizzy	yes	1.2mg	1	mild
Hypoglycaemia	yes	0.3mg	1	mild
		0.6mg	0	mild
		1.2mg	1	mild
		1.8mg	1	mild
		3mg	6	mild
		6mg	11	mild

5.5.1. Withdrawals

Two participants from the 'Normal' group discontinued the IMP at the highest dose on the advice of the CI due to hypoglycaemia episodes (Figure 20). They were followed up and their symptoms quickly resolved after they stopped taking glibenclamide.

5.6. Discussion

This is the first clinical trial to demonstrate that sulfonylureas may be able to influence the secretion of glucagon without affecting insulin secretion in patients with T2DM. In participants who had inappropriately high (for their plasma glucose) fasting glucagon levels, oral glibenclamide at 0.3mg/day (<10% the normal starting dose) was able to reduce fasting glucagon by ~1/3 after 3-4 days with no adverse events and without increasing insulin secretion. No impact on overall glycemic control could be shown using CGM, however does ≥3mg/day significantly increased the risk of hypoglycemia.

5.6.1. Prevalence of hyperglucagonaemia

Fasting hyperglucagonaemia was not an entry requirement in the LEGEND-A trial, as its prevalence in this population of "early" (i.e. not on multiple medications) T2DM patients is not well defined. In order to minimise the variability that exists when measuring glucagon (see Section 1.6.2), it was decided to send all plasma samples to the Royal Devon and Exeter NHS Foundation Trust biochemistry laboratory for assaying (see Sections 1.6.2 and 2.3.8), as their department has produced high quality and consistent results [155].

The impact of low-dose glibenclamide on normal-range fasting glucagon levels was also unknown. In this pilot study, 25% of participants had fasting hyperglucagonaemia. However, their baseline characteristics were not significantly different from the rest of study population (Figure 23). This included metformin treatment, duration of diabetes and HbA1c, as well as insulin resistance and beta-cell function (HOMA2). Interestingly, a recent study in women with pre-diabetes and newly diagnosed T2DM found that 21% had elevated fasting glucagon and delayed glucagon suppression after oral glucose tolerance test [98].

5.6.2. Plasma glibenclamide levels

While treatment duration in this trial was limited to 3-4 days at each dose-step, pre-dose plasma levels of glibenclamide (taken at least 12 hours after the previous evening's dose) were comparable to those detected by radioimmunoassay in patients with T2DM treated with glibenclamide for >1 month [156]. This suggests that steady state was achieved in the trial participants, although there are no formal pharmacokinetic data in the literature for doses <1.25mg/day.

The degradation products of glibenclamide are known to be pharmacologically active [152], as such they could potentially have an effect on alpha-cell K_{ATP} channels. Previous studies which evaluated the plasma concentrations of glibenclamide metabolites in patients with T2DM who were prescribed relatively low doses of glibenclamide (1.75 – 7.0 mg/day) showed that levels were largely undetectable at doses ≤5.25 mg/day [151]. However, the same studies also showed that even doses <3.5 mg/day were able to exert a glucose-lowering effect (by stimulating insulin release), which would be consistent with achieving plasma levels between 25–50 ng/ml for a significant period of time. As the plasma glucose data in the LEGEND-A trial did not suggest a significant decrease in fasting or mean (CGM) levels in doses <1.8mg/day, this suggests that the majority of plasma glibenclamide levels in the 0.3 – 1.2mg/day dose-range remained <25ng/ml (50nM) throughout the day.

5.6.3. Effect of low-dose glibenclamide on fasting glucagon

In our study population, the effect of glibenclamide differed depending on whether fasting glucagon levels were in the normal or high range (section 5.5.2). Specifically, 0.3mg/day significantly reduced high levels but had no effect on normal-range levels. In order to

understand this, we must revisit the concept of a bell-shaped response of glucagon secretion in relation to K_{ATP} channel activity (see Section 1.5).

Data from studies in mouse and human islets (isolated from donors with and without T2DM) indicate that alpha-cell K_{ATP} channel conductance is increased in T2DM, possibly due to intracellular metabolic dysregulation or impaired mitochondrial function [38]. This leads to an increase in glucagon secretion such that the starting point shifts to the (pathological) right side of the bell-shaped curve (Figure 27).

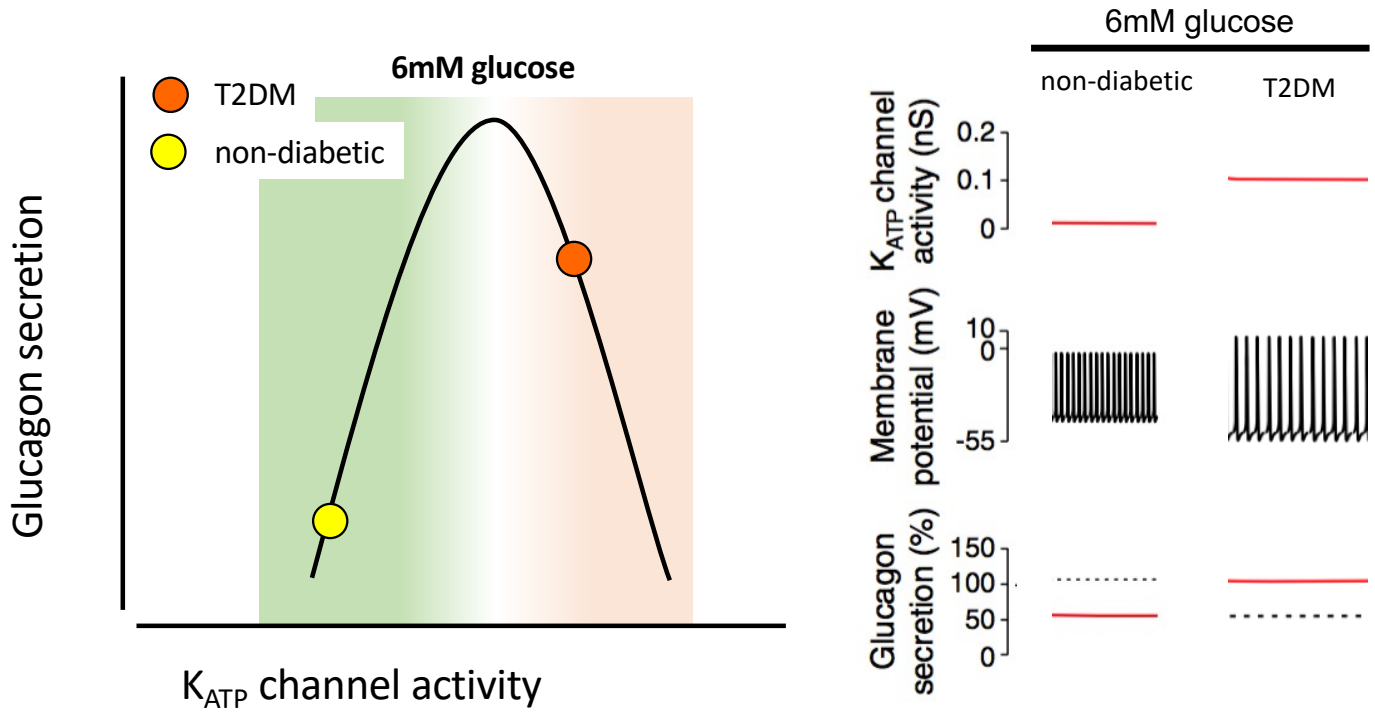


Figure 27 Schematic representation of glucagon secretion at 6mM glucose in islets from donors with and without diabetes. Metabolic disturbances within the alpha-cell cause an increase in K_{ATP} channel activity, which leads to lower frequency / higher amplitude action potential firing (as indicated by the membrane potential), resulting in higher levels of glucagon secretion. This shifts the starting point to the (pathological) right side of the bell-shaped curve. Modified from [38].

The results of the LEGEND-A trial indicate that the T2DM population in our study may be metabolically heterogeneous, despite having similar baseline characteristics (Table 10). On the one hand, the 'High' group displayed elevated glucagon secretion despite higher mean glucose (~8mmol/L), suggesting their starting point on the K_{ATP} channel activity / Glucagon secretion graph was near the peak of the bell-shaped curve (Figure 28). Treatment with 0.3mg/day of glibenclamide resulted in a decrease in K_{ATP} channel conductance (horizontal black line), and a shift from the right (pathological) side of the curve to the left (physiological) side. This also led to a reciprocal change in glucagon secretion (vertical black line) equal to ~30% reduction. On the other hand, the 'Normal' group had fasting glucagon levels similar to those expected in a non-diabetic population, indicating that they had a lesser degree of metabolic dysfunction and their starting point was already on the left (physiological) side of the curve. As such, a decrease in K_{ATP} channel conductance (horizontal black line) possibly elicited a much smaller change in glucagon secretion (vertical black line) which the study was not powered to detect. Taken together, these data also suggest the need to re-evaluate the shape of the bell-shaped curve of glucagon response: instead of a simple parabola the graph may be more complex (Figure 28).

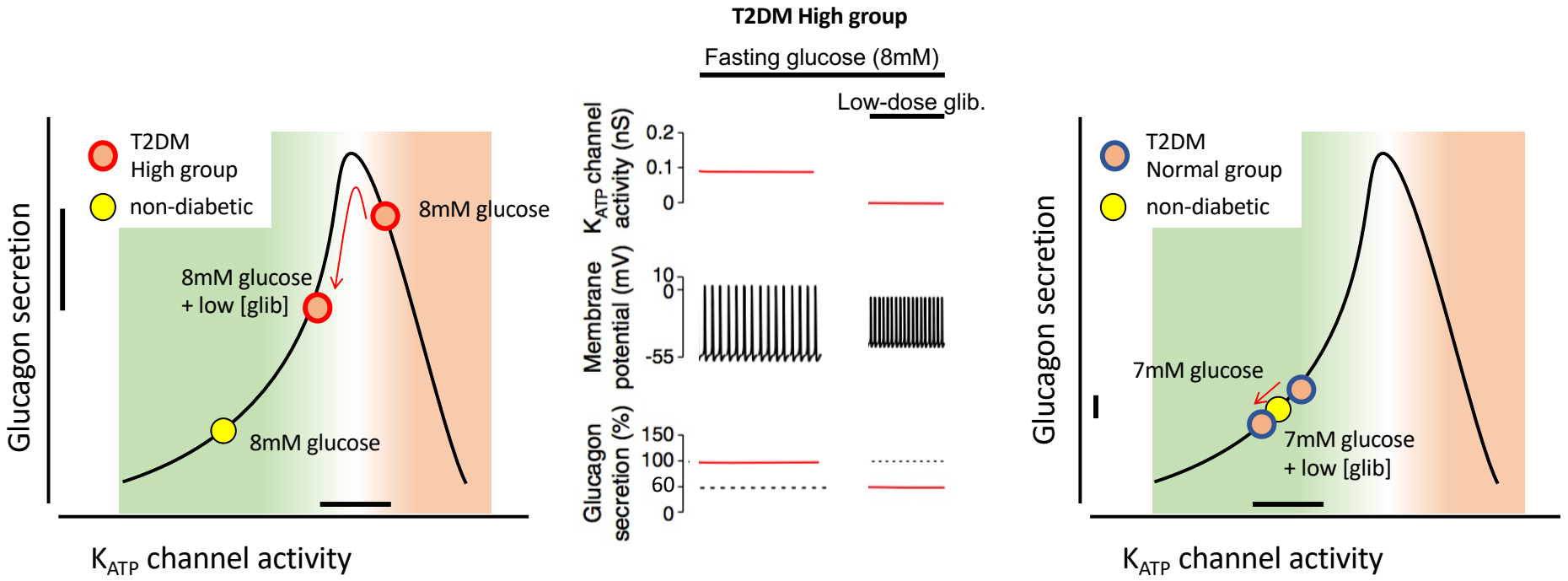


Figure 28 Schematic representation of the effect low-dose glibenclamide [glib] exerts on glucagon secretion in the 'High' (red circle) or 'Normal' (blue circle) group of participants with T2DM. Note the revised shape of the bell-shaped curve compared to Figure 27, and the slightly different theoretical glucagon values in a non-diabetic population (yellow circle) at 8mM (left) and 7mM (right) glucose, as K_{ATP} channel activity decreases with increasing glucose concentrations. Modified from [38].

5.6.4. Effect of increasing doses of glibenclamide

Another finding of the trial was that the modulation of glucagon secretion by glibenclamide appears to occur over a narrow dose-range. As the participants increased their dose of glibenclamide beyond 0.3mg/day, fasting plasma glucagon in the 'High' group returned to pre-treatment levels while those in the 'Normal' group remained unchanged throughout. At the same time, fasting glucose levels decrease significantly at doses ≥1.8mg/day with a corresponding increase in the percentage of time spent in hypoglycaemia in the 'Normal' group (Figure 22 & Figure 23). While there was no difference in fasting plasma insulin or C-peptide levels, these results indicate that insulin secretion from beta-cells throughout the day was significantly stimulated at doses >1.2mg/day.

Given the above observations, it may be that the net effect of glibenclamide at 0.3mg/day is almost exclusively on alpha-cells, whereas increasing doses produce a mixed picture with changes in the membrane potential of alpha-, beta-, and possibly delta-cells, until finally the insulin secretagogue effects of glibenclamide predominate (Figure 29). While the affinity of the K_{ATP} channel for glibenclamide is the same in all three cell types (as they contain the same subunits, see Section 1.5), its relative contribution to the cell's resting membrane potential differs [38]. In alpha-cells, K_{ATP} channel activity is low (~0.1 nS, equivalent to approximately 8 active channels) and therefore very small changes in current (1-2pA, equivalent to the opening of a single ion channel) can results in large changes in membrane potential. Conversely, K_{ATP} channel activity in beta-cells is relatively high (3 nS; equivalent to ~300 active channels) and represents the main contributor to the cells' resting K^+ permeability at low glucose. However, closure of K_{ATP} channels alone does not result in membrane depolarisation - the activity of other channels is also required [157]. Delta-cells

share similar electrophysiological properties with beta-cell [11], and are also electrically coupled to them [42].

As discussed in Section 1.3.2, paracrine regulation of glucagon secretion occurs via the action of somatostatin on alpha-cells. It is therefore possible that at glibenclamide doses ≥0.6mg/day closure of delta- and beta-cell K_{ATP} channels leads to release of somatostatin, which would then act via SSTR2 to activate GIRK channels (Figure 4). This outward flow of K^+ would oppose the effect that closure of K_{ATP} channels would have on alpha-cell membrane potential, leading to a rightward shift of glucagon secretion back to the pathological side of the curve (Figure 28). This hypothesis is supported by the *in vitro* results in islets from a donor with T2DM, in which a rise in somatostatin secretion at 10nM glibenclamide resulted in glucagon secretion returning to baseline values after an initial decrease (see Section 4.5.2).

While the possible involvement of somatostatin secretion in the outcomes of the LEGEND-A trial had not been anticipated beforehand, separate frozen aliquots of the participants' plasma samples have been kept as specified in the trial consent form (see Appendix A.1). Somatostatin exists as two main forms in the circulation: somatostatin-14 which is secreted by islet delta-cells, and somatostatin-28 which is largely gut-derived [11]. It would be very interesting to test the LEGEND-A plasma samples for somatostatin-14 concentrations, however at present antibody-based assays cannot differentiate between the two forms. Work is currently underway to develop a mass spectrometry-based assay, using similar techniques to those employed in the glibenclamide assay (Section 2.2.2).

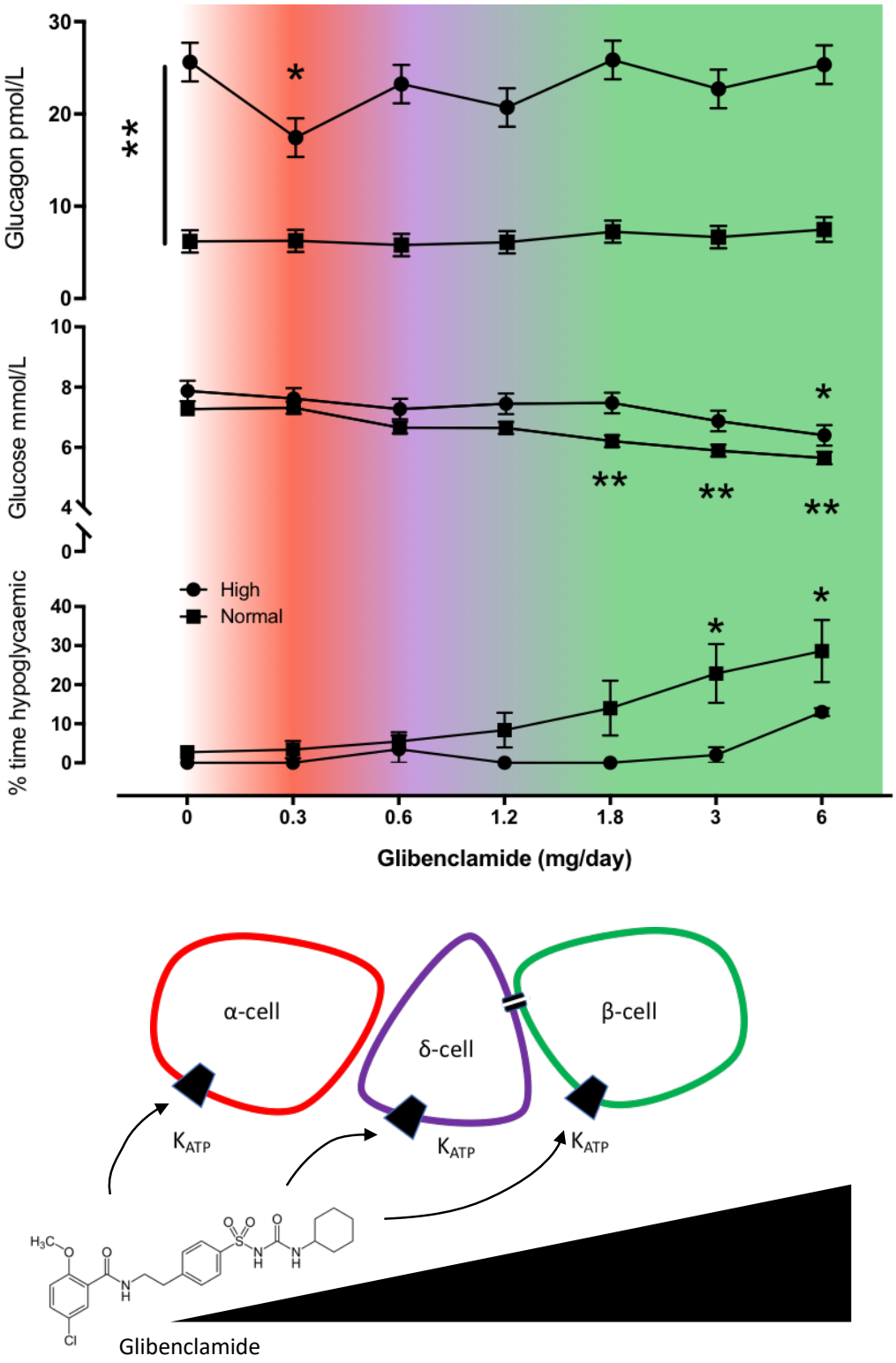


Figure 29 Effect of increasing doses of glibenclamide. At lower glibenclamide doses the action on alpha-cells (red) predominates, while at higher doses the effect on delta- (purple) and beta-cells (green) predominates.

5.6.5. Fasting hyperglucagonaemia and the liver

While the impact of fasting hyperglucagonaemia on hepatic glucose output has been known since the mid 1980s [73], it remains unclear whether this is a cause or effect of T2DM. More recently, studies have cast doubt on whether T2DM is a contributing factor at all [158] and could instead be a bystander to a liver – alpha-cell feedback loop mediated by amino acids [159]. Indeed, the culprit behind raised fasting glucagon levels may in fact be non-alcoholic fatty liver disease (NAFLD) [160].

There are some important differences between the participants included in the above studies and those who took part in the LEGEND-A trial. The findings by Knop et al. of fasting hyperglucagonaemia in obese insulin-resistant (but not diabetic) subjects, as well as in obese T2DM patients, come from a study which included 8 participants in each group (obese T2DM, obese non-diabetic, lean T2DM, lean non-diabetic). Both obese groups had significant insulin resistance (HOMA-IR 7.7 +/- 1.0 and 3.2 +/- 0.6 respectively), while the lean T2DM group was less resistant (HOMA-IR 2.2 +/- 0.6). Moreover, the T2DM groups were a mixture with regards to glycaemic control and treatment (T2DM obese: mean HbA1c 7.2%, 3 diet-controlled, 3 metformin, 1 sulfonylurea, 1 sulfonylurea + metformin; T2DM lean: mean HbA1c 8.6%, 3 diet-controlled, 5 sulfonylurea). In contrast, the LEGEND-A participants (‘Normal’ and ‘High’ groups; Table 10) were borderline obese, considerably more insulin sensitive and had better glycaemic control without the use of sulfonylureas (which was an exclusion criterion for the study). Fasting glucagon levels in the ‘Normal’ group (6.2 +/- 0.4 pmol/L) were closer to those reported in the lean non-diabetic group, while the ‘High’ group (25.6 +/- 6 pmol/L) had levels above those in the obese T2DM patients. These observations suggest there might be more underlying heterogeneity to the T2DM

population in the study by Knop and colleagues, of which fasting hyperglucagonaemia may be a marker.

In keeping with a confounding factor leading to raised fasting glucagon levels, the study by Junker and colleagues involved: 10 patients with T2DM and NAFLD, 10 with NAFLD alone, 8 with T2DM alone and 10 healthy controls. Participant characteristics were more similar to the LEGEND-A study (BMI borderline obese in all groups, HbA1c 7.0% and 6.2% in the T2DM group with and without NAFLD respectively, though anti-diabetic therapy was not reported) but again HOMA-IR was considerably higher in all groups apart from the controls. Most importantly, however, was the definition of fasting hyperglucagonaemia; this was reported as a mean of 7.5pmol/L in NAFLD patients with and without diabetes, while in participants with T2DM alone it was 4.5pmol/L, and 3.4pmol/L in controls. While the absolute numbers are considerably different to those in the Knop et al. study (obese T2DM group had fasting glucagon levels approximately 15pmol/L, while in healthy controls this was approximately 7pmol/L), there is also a large interquartile range reported in the NAFLD + T2DM group, suggesting a high degree of heterogeneity. While none of the LEGEND-A participants had a diagnosis of fatty liver disease, assessment of liver fat content was not undertaken. As it has recently been estimated that around 13% of non-insulin-treated T2DM patients also have NAFLD [161], it might be interesting to formally characterise this in the LEGEND-A population (particularly the 'High' group) as part of a future study.

5.7. Limitations

There are some important limitations of the LEGEND-A trial, which need to be taken into account when interpreting the results and planning future trials.

The sample size was small and the majority of patients had normal baseline fasting glucagon, in whom low dose glibenclamide did not affect glucagon levels. The original sample size calculation used a Bonferonni correction to account for the existance of 2 groups (High and Normal), but assumed the distribution would be 50/50 (based on the *ex-vivo* human islet data). Furthermore, the expected magnitude of effect of low-dose glibenclamide was also based on the *ex-vivo* human islet data, and therefore may have been an overestimate for the patient population. As mentioned in section 5.6.1, the baseline variability of fasting glucagon is not well documented in this particular patient population.

Another limitation of the small sample size was the degree of within-individual variability in baseline fasting glucagon values, which was particularly evident in the 'High' group. This was unexpected and led to the inclusion of a participant in the 'High' group whose baseline glucagon values changed from the 'High' to 'Normal' range on repeat measurement (see section 5.5.2.1). If their results had been included in the 'Normal' group, then the reduction in glucagon secretion between 0mg and 0.3mg glibenclamide would have been even more statistically significant ($p=0.007$ vs $p=0.041$).

Regarding the study design, this was "before and after" rather than a controlled study, as the prevelance of High and Normal fasting glucagon levels was unknown. There was also no accounting for the diffrences in the run-up to each dose change, such as diet, physical activity, alcohol, smoking etc. Given the number of factors that can influence glucagon secretion, setting a standardised evening meal prior to each dose change could have been a pragmatic solution to this. In addition, hypoglycaemia episodes and their treatment were not accounted for in the analysis of hormone levels, but were captured only as part of the continuous glucose monitoring data and the adverse event reporting.

Finally, only pre dose-change glibenclamide levels were measured (12 hours after the previous administration), and for the dose range of 0.3-1.2mg / day these were undetectable (see section 5.5.6). As no formal pharmacokinetic data exists for the dose-range used in the trial, post-dose levels (such as 1 hour after ingestion) could have been used to supplement the analysis.

5.7.1. Post-hoc power calculation

As discussed in section 2.3.3, the sample size calculation was based on *in vitro* data from T2DM islets treated with low-concentration sulfonylurea, as there were no human data available for this intervention. There is also a wide range of fasting glucagon values reported in the literature (see sections 1.6 and 5.6.5), which may reflect differences in experimental set-up, study populations and glucagon assays.

Following the completion of the LEGEND-A trial, data have become available of fasting plasma glucagon levels in patients with T2DM (treated with sulfonylureas, one week following treatment withdrawal, n=127), whose samples were collected and assayed in the same way as those in the LEGEND-A trial (unpublished data; Tim McDonald and Angus Jones, personal communication). In this population, mean fasting glucagon concentration was 10.99 +/- 0.66 pmol/L (median = 9.04 pmol/L, IQR 10). Using this information, it is possible to perform a post-hoc power calculation on the LEGEND-A "High" group values: this indicates there was 97.5% power (alpha error 0.05) to identify a difference between baseline fasting glucagon values at visit 3 (see section 5.5.2.1) and the wider population [162]. If comparison is made between visit 2 and the wider population, this still indicates statistical

power of 92.2%. By contrast, the values for the “Normal” group indicated 80% and 72.7% power for visits 2 and 3 respectively.

When re-evaluating the sample size of the LEGEND-A trial, the differences in baseline values between visit 2 and visit 3 of the “High” group significantly alter the number of participants required. If visit 3 was examined in isolation (mean 25.6 pmol/L; standard deviation = 12.67), then the sample size required to demonstrate 50% reduction in fasting glucagon levels (80% power, alpha error 0.05, as per the section 2.3.3) in the “High” group would be 15 participants. If visit 2 was examined in isolation, then a sample size of 4 participants would be adequate. It is therefore very important to further evaluate the variation in baseline glucagon levels in patients with fasting hyperglucagonaemia (as defined in the LEGEND-A trial), in order to design correctly powered future trials in this population.

5.8. Conclusions

This pilot, translational clinical trial of an investigational medicinal product was safely conducted in a small group of patients with T2DM (diet-controlled or on metformin alone) and suggested the following:

1. Fasting plasma glucagon levels are mostly stable on repeat measurement (on different days)
2. Most patients with T2DM (diet-controlled or on metformin alone) have fasting glucagon values within the range expected in people without diabetes, however a subset have values up to four-times higher
3. The clinical and demographic characteristics of patients with 'Normal' and 'High' fasting glucagon levels may be similar
4. In patients with 'High' fasting glucagon, low-dose glibenclamide (0.3mg/day) may be able to reduce glucagon secretion by approximately 30%, without affecting insulin secretion.
5. Patients with 'Normal' fasting glucagon were unaffected by 0.3mg/day glibenclamide, however doses >1.2mg/day led to an increased risk of hypoglycaemia.

Taken together, these data suggest that high fasting glucagon levels may indicate inappropriately raised alpha-cell K_{ATP} activity in certain patients with T2DM, and that there may be therapeutic benefit in manipulating this system with low-dose sulfonylureas. Longer follow-up trials are required to investigate their effect on post-prandial glucagon levels, and whether there is an overall impact on glycaemic control.

6. Chapter 6: The role of low-dose sulfonylureas in MODY

Having demonstrated in the LEGEND-A trial (see Chapter 5) that low-dose glibenclamide can decrease fasting hyperglucagonaemia by 30% in patients with T2DM, I then turned my attention to investigating the glucagon profile of other types of diabetes in which relatively low doses of sulfonylureas are used clinically.

MODY (Maturity Onset Diabetes of the Young), also known as familial diabetes, is a group of rare (1-2% of all cases) monogenic forms of diabetes caused by mutations in more than 8 genes.

The commonest form, accounting for > 50% of all cases in the UK, is MODY3 and is caused by mutations in the gene encoding the transcription factor HNF-1α (hepatic nuclear factor 1 alpha) [163]. These mutations result in loss-of-function (either due to haploinsufficiency or dominant-negative effects) [164], resulting in an autosomal dominant pattern of inheritance. While the overall effect on glycaemic control may be variable, over 60% of carriers will develop diabetes by the age of 25 and over 95% by the age of 55 [165].

Patients with HNF-1α mutations tend to have the following clinical characteristics [166]:

a) Young age of diabetes onset without insulin-dependent features (good glycaemic control on relatively low insulin doses, absence of ketoacidosis when insulin is omitted, detectable C-peptide outside the “honeymoon” period);
b) Family history of diabetes with onset in 20s, 30s or 40s, typically diagnosed as “type 1” initially;
c) A significant rise in blood glucose (>5mmol/L) during an oral glucose tolerance test, even if fasting values are in the normal range;

d) Glycosuria at relatively normal blood glucose levels; and
e) Marked sensitivity to sulfonylureas leading to hypoglycaemia episodes, despite poor glycaemic control before starting sulfonylureas.

These characteristics are shared with another rarer form known as MODY1, which is caused by mutations in gene encoding HNF-4α [167]. These patients also show a particular sensitivity to sulfonylurea therapy, but usually lack the euglycaemic glycosuria as the renal threshold for glucose reabsorption is unaffected.

While dietary changes is the initial intervention in MODY3 and MODY1, pharmacological treatment with sulfonylureas is usually required as glycaemic control progressively declines [166]. The starting dose of sulfonylurea in this group is 25% the normal adult starting dose, which results in four-fold greater glucose lowering than metformin [168, 169] and often better overall blood glucose control than can be achieved by using insulin [170]. In the UK, gliclazide is the sulfonylurea of choice and patients are normally started on 20-40mg oral tablets either once or twice daily. This compares with the usual dose of gliclazide for managing type 2 diabetes which is 80-320mg daily.

6.1. Role of HNF-1α and -4α transcription factors

The physiological basis of this increased sulfonylurea sensitivity is still unclear, as both HNF-1α and -4α control the transcription of a plethora of genes involved in a variety of metabolic and homeostatic processes.

HNF-1α and HNF-4α are expressed in a number of tissues including the liver, kidney, pancreas and the GI tract (Figure 30). Studies in which mice had the HNF1A gene knocked

out showed significant defects in the insulin secretion response to physiological stimuli (glucose and arginine) [171], suggesting a central role in glucose regulation. Moreover, experiments using a beta-cell line (INS1) expressing HNF-1α with disease-causing mutations revealed that insulin and glucose transporter (GLUT2) expression, and mitochondrial activity were all impaired, leading to reduced ATP production from both glucose and amino-acids [172]. This shift in ATP/ADP ratio leads to reduced closure of the K_{ATP} channel, thereby preventing membrane depolarisation, calcium influx and insulin granule exocytosis. The role of both HNF-1α and -4α with regards to glucagon secretion remains unclear, however both transcription factors have been identified in mouse alpha cells [173].

These findings provide mechanistic insight into the clinical observations that MODY3 patients are unable to secrete appropriate amounts of insulin when challenged with a high carbohydrate meal [174]. Furthermore, the reduced renal threshold for glucose reabsorption is likely due to changes in the expression of sodium-glucose cotransporter 2 (SGLT2) in the kidneys [175, 176], leading to glycosuria when blood glucose is relatively normal.

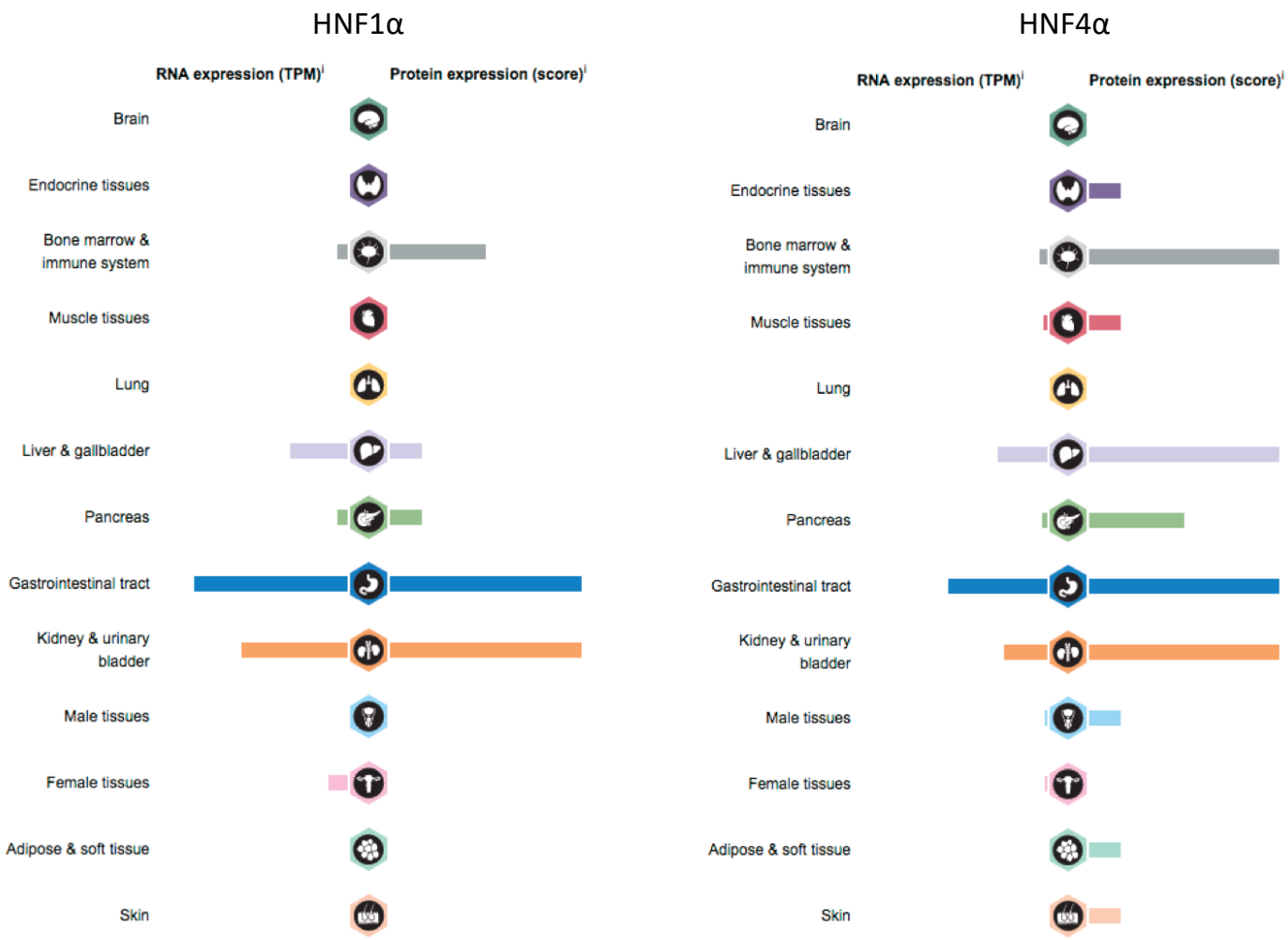


Figure 30 Tissue expression profiles of HNF1A and HNF4A genes. Data and illustration derived from Human Protein Atlas available from www.proteinatlas.org [177]. Used under creative commons license.

6.2. SGLT2 and its role in alpha-cells

The pharmacological inhibition of SGLT2 (using medications such as dapagliflozin, empagliflozin, canagliflozin etc.) has been one of the major developments in the management of type 2 diabetes over the past decade. Through their action on the proximal convoluted tubules, this class of medications reduces the glucose reabsorption capacity of the kidneys leading to increased excretion, thus improving glycaemic control without causing hypoglycaemia [178]. In this respect, patients being treated with these agents share similar characteristics with MODY3 patients.

Interestingly, clinical studies have shown that treatment with the SGLT2 inhibitor dapagliflozin leads to a rise in fasting plasma glucagon levels within 2 hours of oral administration in patients with T2DM, without inducing hypoglycaemia [179]. The implications of this raised glucagon secretion are unclear; however, it is thought that strategies to supress glucagon would improve the overall efficacy of these medications. More recently, *in vitro* studies have recapitulated this effect using isolated human islets, suggesting that it occurs via direct action on alpha-cells [37]. Therefore, it would be useful to examine the effect low-dose gliclazide might have on the secretion of glucagon in MODY3 patients, as this might shed light on a potential therapeutic adjunct to dapagliflozin treatment in T2DM.

6.3. Funding for the clinical study

An application was submitted to the Oxfordshire Health Services Research Committee detailing a pilot clinical study entitled “Investigating glucagon secretion in HNF-1α and HNF-4α MODY” (supervised by Professor Katharine Owen), which would examine the glucagon profile of MODY3 and MODY1 patients during an oral glucose tolerance test,

before and after stopping gliclazide treatment (details in section 6.5). The application was successful, and I was awarded £9,730 to perform the study.

6.4. Clinical study set-up

The research protocol and supplementary documents (including patient information sheet, consent form, GP letter, case report forms and sample dosing schedule) were submitted online via the integrated research application system to the relevant authorities (IRAS project ID 221972). The study was given a favourable ethical opinion by the South Central – Hampshire B Research Ethics Committee (REC reference 17/SC/0233), and was approved by the Health Research Authority (HRA). The study was registered on ClinicalTrials.gov (NCT03246828). Local approval was also obtained from the Oxford University Hospitals NHS Foundation trust. As of June 2018 this study is still ongoing. I will therefore present the current set-up, as well as the preliminary findings and their possible interpretation.

6.5. Glucagon in MODY study

The "Glucagon in MODY" study is a pilot interventional research study, which will involve 10 participants with either MODY3 or MODY1 omitting their gliclazide medication for 3 days. Measurements will be taken before and after this period, and as such each participant will act as their own control ("before and after" study set-up). The aim of this project is to establish whether fasting and post-prandial glucagon levels are raised in HNF-1α and HNF-4α MODY patients, and whether gliclazide supresses glucagon secretion in this population. An outline of the study can be found in Appendix B.1.

The half-life of gliclazide is 10.4 hours, therefore a washout period of 3 days (i.e. ~7 times the half-life) will ensure there is no circulating gliclazide while minimising the amount of

time off medication for the participants. A previous study which had involved taking HNF-1α MODY patients off gliclazide for 1 week resulted in symptomatic hyperglycaemia in 1 of the 18 participants, and led to their withdrawal from the trial [168]. The lead author of that trial has also suggested that a 3 day withdrawal period is less likely to result in significant hyperglycaemia (Ewan Pearson, personal communication). In addition, appropriate monitoring will be in place to check and treat symptomatic hyperglycaemia if it occurs.

In order to understand the interaction other factors have on glucagon secretion, C-peptide (as a marker of pre-hepatic insulin secretion) and non-esterified fatty acid (NEFA, as fatty acids may be important in glucagon regulation [40]) will also be measured during the OGTTs and correlated with plasma glucagon levels. This association has not previously been studied in HNF-1α-/-4α MODY patients on and off treatment, and will allow an estimation of the effect gliclazide has on beta cells vs alpha cells. Liver function tests and HbA1c will also be checked at the start of the study. Finally, participants will be offered the option to have a continuous glucose monitor (CGM; Freestyle Libre, Abbott Diabetes Care) fitted, so as to assess their blood glucose variability on and off gliclazide treatment. Further information about the study design and methodology can be found in Appendix B.

6.6. Preliminary results

At present only 3 patients have completed the study, and as such no conclusion can be drawn yet. However, some initial observations can be made which will be updated as the study progresses.

6.6.1. Baseline characteristics

Currently all participants in the study are female with mutations in HNF-1α (Table 12). The average age and BMI is 56 years and 25.3 kg/m^2 respectively, and the average HbA1c is 6.8% (51mmol/mol). The dose of gliclazide used is 40mg once or twice daily.

Fasting blood glucose values were in the normal range while on gliclazide and rose to around 9.5 mmol/L after omitting gliclazide for 3 days (Figure 31). This was well tolerated and not associated with significant adverse events. Fasting glucagon levels were generally below the normal range [96] on both occasions, and did not vary significantly.

Table 12 Characteristics of participants in the "Glucagon in MODY" study.

Study ID	Genotype	Age	Sex	BMI	Gliclazide	Metformin	Other medication	Duration of diabetes	HbA1c	Notes
001	HNF1α	51	F	24.1	40mg once daily	no		4 years	7.9% (63 mmol/mol)	Particularly prone to hypoglycaemia episodes while on medication.
002	HNF1α	62	F	23.5	40mg twice daily	500mg once daily	Ramipril 2.5mg Pravastatin 20mg	28 years	6.9% (51 mmol/mol)	
003	HNF1α	56	F	28.3	40mg twice daily	500mg twice daily	Oestradiol 2mg	53 years	5.6% (38 mmol/mol)	- Using intermittent fasting (5:2) diet to control hyperglycaemia. - Transgender

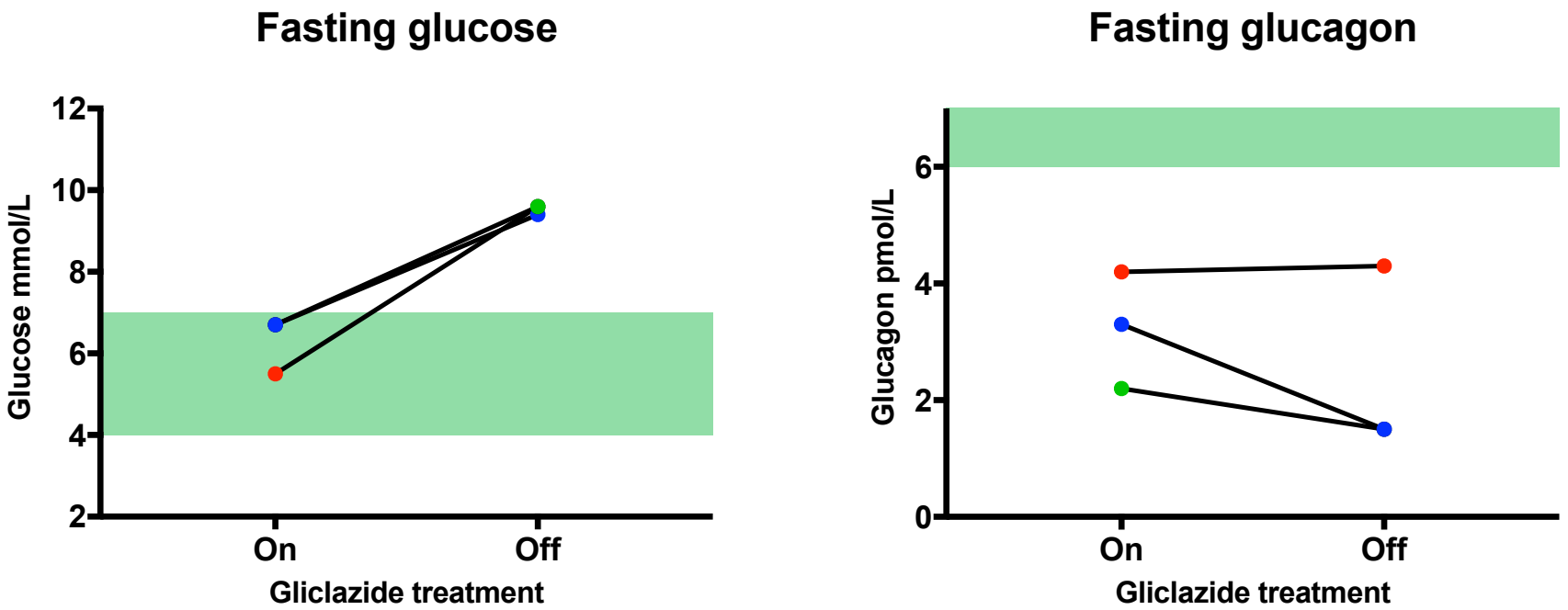


Figure 31 Fasting glucose and glucagon, on and off gliclazide treatment. Green bars indicate the normal fasting ranges.

6.6.2. Changes post-OGTT

Plasma glucose values showed a sharp increase >5mmol/L in all participants following the 75g glucose, in keeping with their genotype [166]. The shape of the glucose response curve was preserved between visits for each individual, but shifted upwards while off gliclazide (Figure 32).

Glucagon showed a higher degree of variability between individuals, with one participant's values increasing 3-fold off gliclazide despite being hyperglycaemic (Figure 33). When expressed as a change from the baseline (fasting) value, glucagon levels showed appropriate glucose-induced suppression while on gliclazide but an abnormal pattern of secretion off medication.

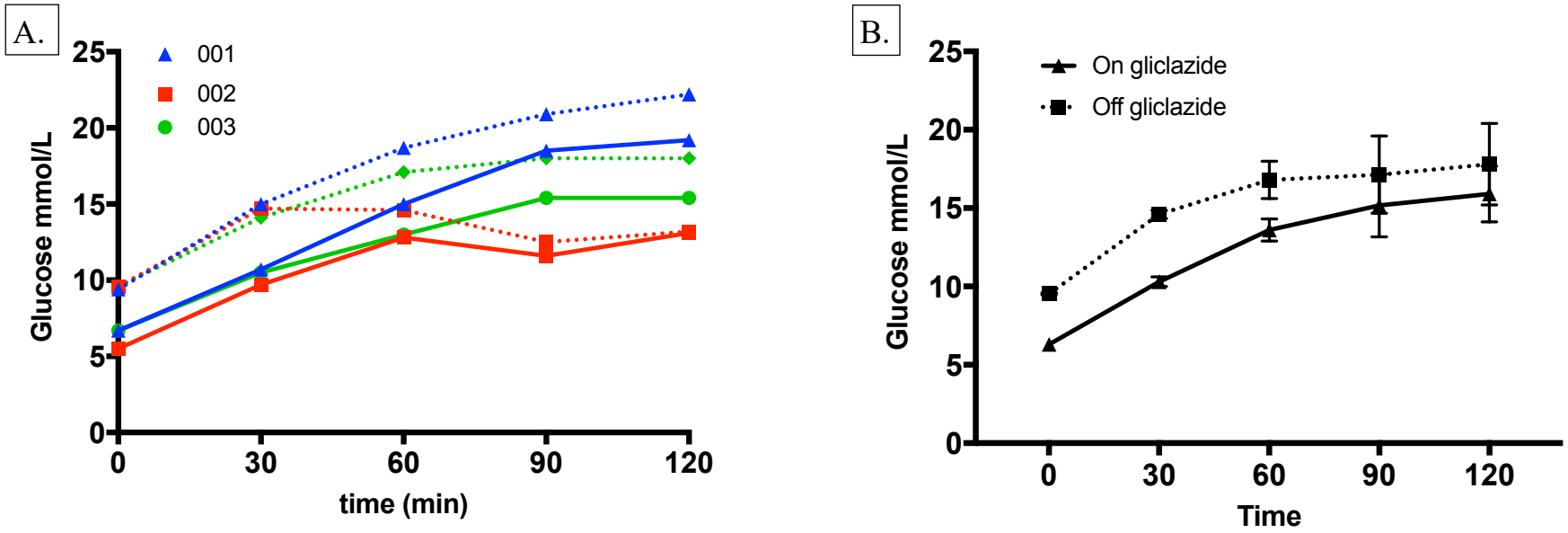


Figure 32 Plasma glucose values during the 75g oral glucose tolerance test. A: individual results for each participant, B: mean and SEM. Solid lines indicate values while on gliclazide, dotted lines indicate values off gliclazide.

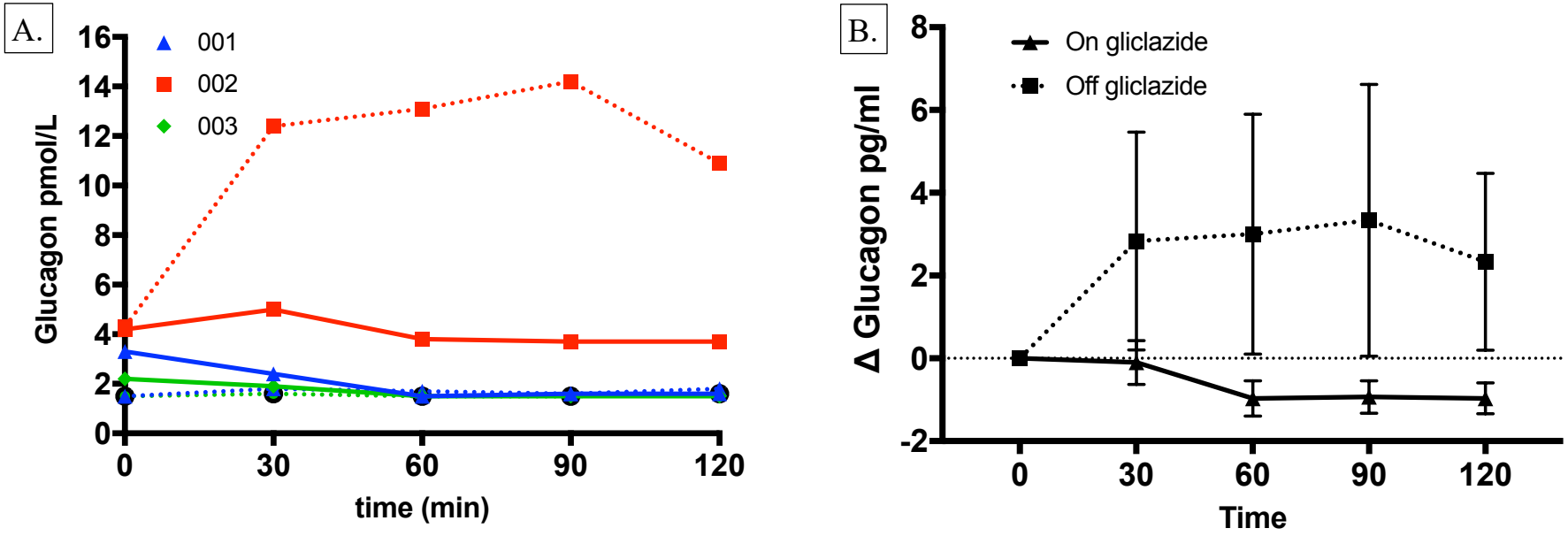


Figure 33 Plasma glucagon values during the 75g oral glucose tolerance test. A: individual results for each participant, B: change from fasting value (mean and SEM). Solid lines indicate values while on gliclazide, dotted lines indicate values off gliclazide.

6.7. Discussion

The intracellular metabolic defects described in MODY3 may be similar to those thought to contribute to the islet cell dysfunction in T2DM [38]. This is particularly relevant for glucagon secretion, as demonstrated by studies using the ATP-synthase inhibitor oligomycin in isolated islets [35].

While still preliminary, the data from the "Glucagon in MODY" study suggest that sulfonylurea treatment does have an impact on alpha-cell function, and it may be correcting aberrant glucagon secretion. Though fasting levels were relatively unchanged both on and off gliclazide, this underlying dysfunction was revealed following OGTT and led to non-suppression or stimulation of glucagon secretion off treatment despite raised plasma glucose levels (Figure 33). Furthermore, this shift in secretion pattern occurs relatively quickly (gliclazide withheld for 3 days only, with a half-life of 10.4 hours), which would be in keeping with an effect at the level of membrane potential. Finally, the study set-up appears to be both valid and safe, with a gradual rise in blood glucose over the 72h period of gliclazide omission and no instances of severe hyperglycaemia symptoms or ketoacidosis.

These results are also comparable to the (albeit limited) literature regarding glucagon secretion in MODY patients. A previous study comparing islet hormone responses following a 50g OGTT in MODY patients and controls demonstrated that glucagon values in the sample of ten MODY3 patients increased within the first 60min, followed by suppression below fasting levels [180]; in contrast, the non-diabetic control group had suppressed glucagon secretion from the outset. However, this study included a mixed MODY3 group with regards to treatment (six participants were on oral anti-diabetic medications), and fasting glucose values were considerably higher than in controls (mean ±SEM fasting plasma

glucose 8.4mmol/L ±0.8 vs. 5.1mmol/L ±0.2) indicating sub-optimal glycaemic control. In contrast, the participants so far recruited in the Glucagon in MODY study have all had fasting glucose values <7mmol/L while on gliclazide (mean 6.3mmol/L ±0.4), which increased to a mean of 9.5mmol/L ±0.06 when gliclazide was omitted. As such, the glucagon results in the study by Østoft and colleagues may have shown a difference with regards to glucagon secretion if participants had been separated into those on and off sulfonylurea treatment (which, though not reported, was most likely to have been glimepiride [181]).

6.7.1. Possible mechanism of action

In keeping with the concept of a bell-shaped relationship between glucagon secretion and K_{ATP} channel activity (see Section 5.6.3), the intrinsic metabolic defects in the islets of MODY3 patients [172] may lead to increased K_{ATP} channel activity. We speculate that MODY3 alpha-cells "sit" at the far right side of the curve under fasting conditions (Figure 34). When the alpha-cells are then challenged with raised glucose levels during the OGTT, they respond by decreasing K_{ATP} channel activity, thus stimulating glucagon secretion (to a variable degree depending on the characteristics of HNF-1α mutation).

The effect of gliclazide (given at a high enough dose to promote insulin secretion) is to shift the fasting point to the left (physiological) side of the bell-shaped curve, as it leads to closure of the K_{ATP} channel. This then restores normal glucose-dependent glucagon suppression, resulting in decreased plasma levels during the OGTT.

Results from more participants in this study will test the validity of this model, which is still a work in progress.

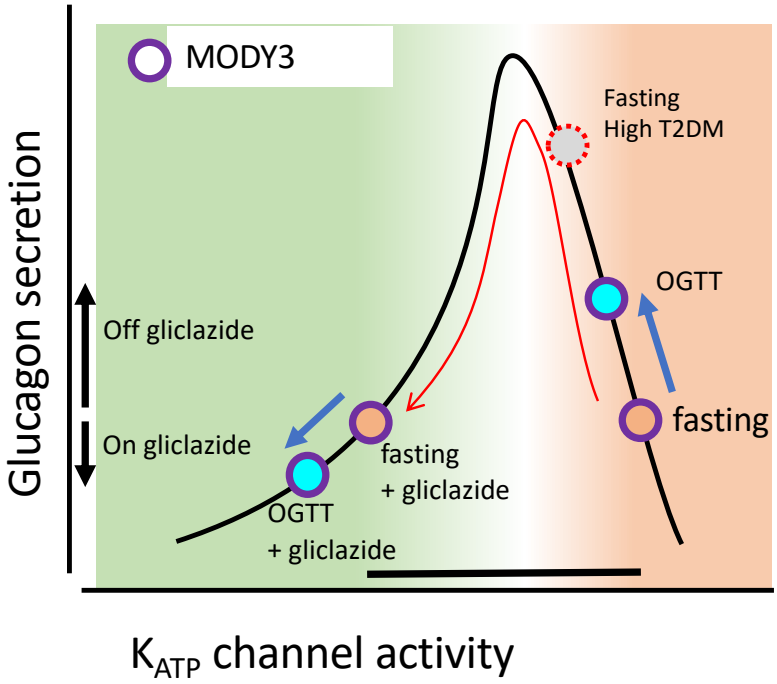


Figure 34 Schematic representation of the effect relatively low doses of gliclazide (40-80mg /day) exert on glucagon secretion in MODY3 patients (purple circles). While fasting (orange fill) plasma glucagon values remain the same on and off gliclazide treatment, they rest on opposite sides of the bell-shaped curve. This is revealed when challenged with oral glucose (OGTT, blue fill), resulting in an abnormal increase off treatment and a physiological decrease on it. For comparison, fasting glucagon levels from the High group of T2DM patients (LEGEND-A trial) are also displayed (dotted red circle). The horizonal black bar indicates the magnitude of effect these gliclazide doses have on K_{ATP} channel activity (cf. Figure 28).

7. Chapter 7: Summary and conclusions

This thesis has explored the therapeutic potential of using low-dose sulfonylureas to normalise aberrant glucagon secretion patterns in patients with diabetes, by subtly altering the activity of the alpha-cell K_{ATP} channel.

Chapter 3 described an analysis of the 11-year human islet hormone secretion dataset at OCDEM, investigating the degree of glucose-dependent insulin and glucagon secretion at hypo-, eu- and hyperglycaemic conditions (1mM, 6mM and 20mM glucose respectively). It demonstrated an overall doubling of glucose-stimulated insulin secretion between 1mM and 6mM glucose, and between 6mM and 20mM, and that glucagon secretion is maximally suppressed at 6mM glucose by 40%. It also confirmed the high degree of variability present between islet preparations, which has been reported by other large islet isolation centres. By using a statistical modelling method which accounts for this variability, I was able to show that islets from older male donors secreted twice as much insulin as those from younger ones at euglycaemic conditions, but also that islets from older donors secreted 40% less glucagon at hypoglycaemic conditions independent of gender. Neither of these findings have been previously been described, and they may help explain the susceptibility of older patients to sulfonylurea-induced hypoglycaemia.

Chapter 4 described the *in vitro* experiments that were initially performed using isolated mouse islets in order to develop and refine the method of serial incubation, which then permitted the design of larger dose-response experiments using isolated islets from human donors with and without diabetes. Though limited in number due to the nature of human islet availability, these experiments suggested that glibenclamide concentrations above 100nM (in hyperglycaemic conditions) could stimulate both insulin and glucagon secretion, while concentrations below 10nM may reduce glucagon secretion by 30% in patients with T2DM

without increasing insulin secretion. Using this information, the dose-range for the clinical trial was chosen so that it included daily glibenclamide doses of 6mg (approximately the starting dose which causes insulin secretion), 0.6mg and 0.3mg.

Chapter 5 described the set-up and execution of the "Low-dosE GlibENclamide in Diabetes – part A" (LEGEND-A) clinical trial, which involved 16 patients with T2DM (diet-controlled or on metformin alone) self-administering an oral suspension of glibenclamide (0.3 – 6 mg daily) over the course of 3-4 weeks. This was the first clinical trial to demonstrate that sulfonylureas may influence glucagon secretion without affecting insulin secretion in patients with T2DM. In four participants who had inappropriately high (for their plasma glucose) fasting glucagon levels, oral glibenclamide at 0.3mg/day ($1/20^{th}$ the normal starting dose) was able to reduce fasting glucagon by 32% after 3-4 days with no adverse events. However, 75% of participants had fasting glucagon values in the normal range (6 – 12 pmol/L), and in them glibenclamide had no effect on glucagon at any dose-step, presumably because glucagon secretion was already maximally inhibited. No impact on overall glycaemic control could be shown using CGM (though the trial was not powered to do so), however doses ≥3mg/day significantly increased the risk of hypoglycaemia. In addition, it was noted that baseline characteristics of participants with fasting hyperglucagonaemia were not significantly different from the rest of the study population.

In Chapter 6, the concept of relatively low doses of sulfonylureas affecting glucagon secretion was explored further in a small group of three patients with MODY3 (mutations in HNF-1α), in whom gliclazide treatment was withheld for 3 days. The preliminary results of the "Glucagon in MODY" clinical study suggest that, while fasting levels are relatively unaffected, gliclazide may be correcting an underlying alpha-cell dysfunction which

manifests as either non-suppression or stimulation of plasma glucagon during an OGTT. This may have wider therapeutic implications beyond the management of MODY, and hints at the possibility that the glucagon-stimulating effect of SGLT2 inhibitors in patients with T2DM could be improved with low-dose sulfonylureas.

7.1. Clinical relevance of glucagon measurement

There is mounting evidence that T2DM is a more heterogeneous disorder than previously considered [9], and that aberrant patterns of glucagon secretion may be a feature of metabolic disturbances other than diabetes [160]. However, technical limitations (see Section 1.6) have hampered efforts in the past to reliably study this important islet hormone, and as such the main clinical use of glucagon assays is in the work-up of rare pancreatic tumours [182]. Recent advances in assay technology together with thorough evaluation of the accuracy of modern platforms (see Section 1.6.2.1) have enabled the pitfalls of the past to be avoided and a new, more reproducible era to begin. It is now time to re-examine the clinical utility of glucagon measurement in the evaluation of diabetes mellitus.

It has been said that diabetologists are the only endocrinologists that don't measure their hormone (Andrew Hattersley, Albert Renold Prize lecture, EASD 2017), and this is equally as relevant for insulin (or C-peptide) as it is for glucagon. When it comes to T2DM, pharmacological management beyond metformin is largely stochastic, with little guidance as to the best choice for individual patients other than side-effect profiles. On the other hand, there have also been very impressive outcomes from dietary restriction trials such as the DiRECT study, in which 85% of participants who achieved weight loss ≥15% were able to discontinue all antidiabetic medications at 1 year (though none were on insulin) [183]. In view of these results, the type of intervention we as clinicians recommend to our patients in the future may have to be much more precise.

The collection of blood samples (fasting and post-glucose-challenge) for the measurement of plasma glucagon is relatively straightforward and has the potential to reveal an entirely new dimension of islet function (and possibly fatty liver disease). An important first step would be to establish what the “normal range” is in absolute terms in different populations, and the clinical implications that values outside this range might have. In this project I have demonstrated that glucagon secretion may be significantly altered by sulfonylureas that have been in use for decades, at doses that cause no side-effects, and that if targeted to the right subpopulation of patients (with regards to fasting glucagon) may have important therapeutic benefits. Further work is required (and planned) to better evaluate the longer-term benefits of such a treatment, and whether it could be used as an adjunct to other medications.

8. Chapter 8: Future work

8.1. Somatostatin-14 mass spectrometry assay

As discussed in Section 5.6.4, the secretion of somatostatin-14 from islet delta-cells may have contributed to the loss of glucagon suppression at glibenclamide doses ≥0.6mg/day. Elucidating this has the potential to reveal important interactions between islet hormones *in vivo*, however there is currently no antibody-based assay which can distinguish between the two main forms of somatostatin. We are setting up a mass spectrometry-based assay in collaboration with Dr. Rod Chalk, SGC, with the aim to analyse the LEGEND-A plasma samples in the next 6-8 months.

8.2. Testing LEGEND-A participants for NAFLD

The participants of the LEGEND-A trial did not have a diagnosis of fatty liver disease, however this was not formally tested. As NAFLD and hyperglucagonaemia may be linked, I will aim to propose a small follow-up study to examine whether these same participants still have fasting hyperglucagonaemia and whether they have evidence of NALFD on MRI.

8.3. Completion and extension of Glucagon in MODY study

As of June 2018 recruitment to the "Glucagon in MODY" study is ongoing and the end-date has been extended to 1/10/19, though I anticipate reaching the target of 10 participants by the end of 2018. I am currently discussing the possibility of extending the study to France with colleagues at the University of Lille, and we will be writing a joint grant application.

8.4. LEGEND-B Trial

Following on from the LEGEND-A trial, we will now aim to investigate whether the lowest doses of glibenclamide (0.3mg/day and 0.6mg/day) can improve glucagon secretion in hyperglycaemia (post-OGTT) in patients with T2DM (diet controlled or on metformin alone) who have fasting hyperglucagonaemia (>15pmol/L).

In the "Low-dosE GlibENclamide in Diabetes – part B" (LEGEND-B) trial, participants will undergo two 75g OGTTs: one before (control) and one after a 2-week course of either 0.3mg/day or 0.6mg/day glibenclamide (Figure 35). The aim with be a reduction in fasting and post-glucose-challenge hyperglucagonaemia and hyperglycaemia. A proposal for this clinical trial has been included in a grant application.

8.5. LEGEND-D Trial

Finally, it would be interesting to explore the therapeutic use of low-dose glibenclamide as an adjunct to SGLT2 inhibitors (which cause a rise in plasma glucagon levels, see Section 6.2), as this could improve their overall efficacy by reducing hyperglucagonaemia. Initial plans for a "Low-dosE GlibENclamide in Diabetes – Dapagliflozin" (LEGEND-D) clinical trial are currently underway which would examine their combined use post glucose-challenge and during hypoglycaemia, as aberrant glucagon secretion may also be associated with a vulnerability to hypoglycaemia episodes.

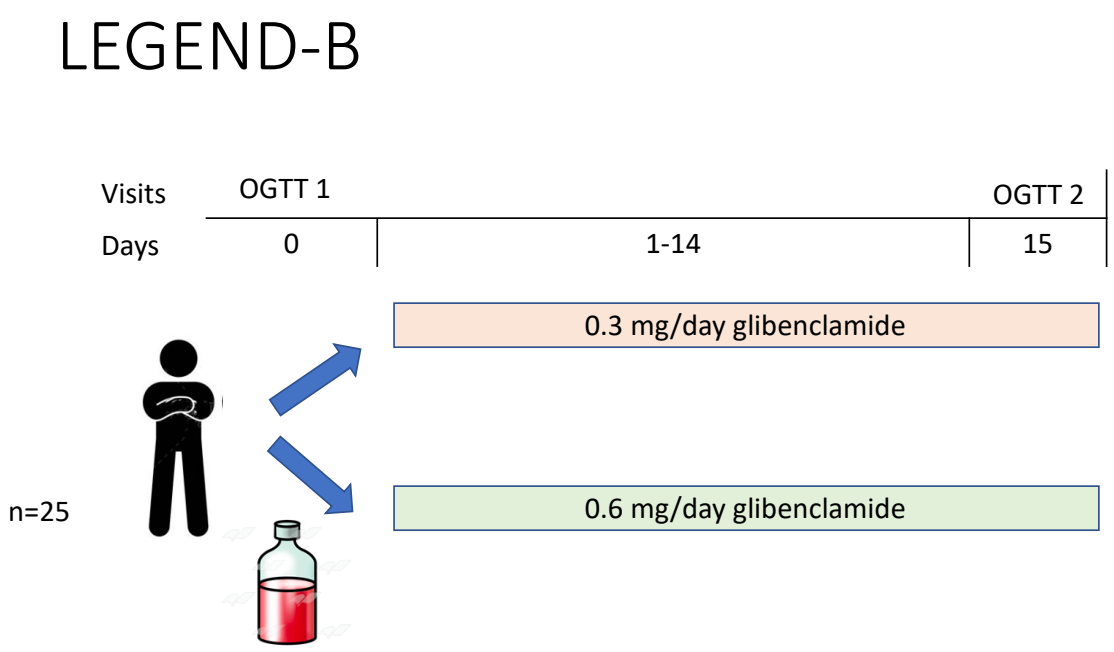


Figure 35 LEGEND-B trial outline.

A. Appendix A: LEGEND-A clinical trial paperwork

A.1. Informed consent form

Oxford University Hospitals NHS
NHS Foundation Trust

UNIVERSITY OF OXFORD

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Participant identification number:

CONSENT FORM

Low dosE GlibENclamide in Diabetes Part A (LEGEND-A)

CI: Dr Ioannis Spiliotis

If you agree, please initial box

1. I confirm that I have read the information sheet dated 22-04-16 (version 1.1) for this study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from University of Oxford, from regulatory authorities and from the Oxford University Hospitals NHS Foundation Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
4. I give my consent for blood samples to be taken during the course of this study. I consider these samples a gift to the University of Oxford and I understand I will not gain any direct personal or financial benefit from them.	
5. I agree to my General Practitioner being informed of my participation in the study.	
6. I understand that the information collected about me may be used in an anonymous form to support other research in the future. It will not be possible for me to be identified by it.	
7. I agree to take part in this study.	

8. I agree for my anonymised samples to be used in future ethically approved research by academic or commercial researchers, here or abroad, which has ethics approval (please tick either box and initial).	Yes	
	No	
9. I agree to wearing the optional continuous glucose monitor during the clinical trial (please tick either box and initial).	Yes	
	No	

_____	_____	_____
Name of Participant	*Date*	*Signature*
_____	_____	_____
Name of Person taking Consent	*Date*	*Signature*

**1 copy for participant; 1 copy for researcher site file.*

A.2. LEGEND-A Trial outline

Trial Title	Low-dose glibenclamide in type 2 diabetes mellitus – Part A		
Short title	LEGEND-A		
Clinical Phase	Phase II		
Trial Design	Open-label, non-randomised, dose-titration trial		
Trial Participants	Patients with type 2 diabetes mellitus (diet-controlled or on metformin alone)		
Planned Sample Size	15 participants		
Treatment duration	21 days		
Follow up duration	1 week		
Planned Trial Period	9 months		
	Objectives	Outcome Measures	Timepoint(s) of evaluation of this outcome measure
Primary	To identify the dose of glibenclamide that causes a significant decrease in fasting plasma glucagon concentration	Concentration of plasma glucagon using fasting blood samples prior to each dose change	Pre-dose blood sampling on study visits 3 - 9.
Secondary	1. To determine whether low doses of glibenclamide can result in an overall improvement in glycaemic control throughout the day. 2. To determine the effect low-dose glibenclamide has on fasting glucose, insulin and C-peptide levels. 3. To determine the trough concentration of glibenclamide in plasma.	1. Change in the percentage of CGM readings under 4 mmol/L, between 4-10 mmol/L and above 10 mmol/L before starting glibenclamide and prior to each change in dose. 2. Concentration of glucose, insulin and C-peptide using fasting blood samples prior to each dose change. 3. Concentration of plasma glibenclamide using fasting blood samples prior to each dose change.	1. CGM readings downloaded from the sensor on study visits 3 - 9. 2. Pre-dose blood sampling on study visits 3 - 9. 3. Pre-dose blood sampling on study visits 3 - 9.
Investigational Medicinal Product(s)	Glibenclamide oral suspension (GlibenTek)		
Formulation, Dose, Route of Administration	Oral liquid suspension at strengths of 0.6 mg/ml and 6mg/ml. Dosing schedule ranged from 0.3 – 6 mg daily, split into morning and evening (half dose in the morning, half dose in the evening).		

A.3. LEGEND-A Trial timeline

The days indicated are given as an example of a participant attending CRU visit 2 on a Friday. Blood tests are indicated by (*) and CGM sensor changes by (x). There period captured by CGM is indicated by the yellow bar.

CRU visit number	**1**		**2**			**3**			**4**				**5**			**6**				**7**			**8**				**9**	☎
Day	-17	-10	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	24/26
Dose (mg)						0.3	0.3	0.3	0.6	0.6	0.6	0.6	1.2	1.2	1.2	1.8	1.8	1.8	1.8	3	3	3	6	6	6	6		
Blood Test	*		*			*			*				*			*				*			*				*	
CGM change			x			x			x				x			x				x			x				x	

CRU Visit	Description
	Identification of potential participant
	Letter & information leaflet sent
	Reply slip received
	Invitation to screening
1 (1 h)	Screening visit: Assess eligibility criteria. Screening baseline assessment (weight, blood pressure, heart rate). Screening blood test. Informed consent obtained.
2 (1 h)	Eligible participants only. Baseline assessment (blood pressure, heart rate). Fasting blood tests. Issued with 0.6mg/ml GlibenTek dosing syringe and schedule. Educated on self-administration and given dosing schedule. CGM sensor attached.
3 (30 min)	Baseline assessment. Fasting blood tests. CGM sensor changed.
4 (30 min)	Baseline assessment. Fasting blood tests (pre-dose). Check compliance with medication. Instructed to increase dose. CGM sensor changed.
5 (30 min)	Baseline assessment. Fasting blood tests (pre-dose). Check compliance with medication. Instructed to increase dose. CGM sensor changed.
6 (30 min)	Baseline assessment Fasting blood tests (pre-dose). Check compliance with medication. Instructed to increase dose. CGM sensor changed.
7 (30 min)	Baseline assessment. Fasting blood tests (pre-dose). Check compliance with medication. Instructed to increase dose. CGM sensor changed. Issued with 6mg/ml GlibenTek and dosing schedule.
8 (30 min)	Baseline assessment. Fasting blood tests (pre-dose). Check compliance with medication. Instructed to increase dose. CGM sensor changed.
9 (30 min)	Baseline assessment. Fasting blood tests (pre-dose). Check compliance with medication. CGM sensor removed. Oral glibenclamide suspension returned. Participants informed about end of trial.
(15 min)	Telephone follow-up.

B. Appendix B: Glucagon in MODY clinical study paperwork

B.1. Glucagon in MODY study outline

Study Title	Investigating glucagon secretion in HNF-1α and HNF-4α MODY		
Short title	Glucagon in MODY		
Design	Interventional research study (pilot study)		
Participants	Patients with HNF-1α and HNF-4α MODY		
Sample Size	10 participants		
Study Period	01/05/2017-01/08/2018		
	Objectives	**Outcome Measures**	**Timepoint(s) of evaluation of this outcome measure**
Primary	To determine whether fasting and post-prandial glucagon secretion is suppressed by gliclazide in HNF-1α/-4α MODY patients.	Concentration of plasma glucagon during an oral glucose tolerance test (OGTT), while on gliclazide and after a washout period.	Fasting and at time-points 30', 60', 90', 120' during 75g oral glucose tolerance test.
Secondary	1. To determine whether changes in post-prandial glucagon levels correlate with changes in C-peptide (as a marker of pre-hepatic insulin secretion) and NEFA levels in HNF1-/4-alpha MODY patients on and off gliclazide 2. To assess the blood glucose variability on and off gliclazide treatment (only for participants who opt to take part in this optional component).	1. Concentration of plasma glucagon, C-peptide and NEFA while on gliclazide and after a washout period. 2. Change in the percentage of CGM readings under 4 mmol/L, between 4-10 mmol/L and above 10 mmol/L	1. Fasting and at time-points 30', 60', 90', 120' during 75g oral glucose tolerance test. 2. CGM readings will be downloaded from the sensor at the final study visit.

B.2. Glucagon in MODY study timeline

Note: This is a sample study flow chart. The actual timings of the study visits may differ from participant to participant.

Study visit number	**1**	**2**	**3**				**4**	☎
Day		-7	0	1	2	3	4	8/11
OGTT			x				x	
Blood Test			*				*	
CGM (optional)								
On Gliclazide								
OFF Gliclazide								

Study Visit number	Description
	Identification of potential participant
	Letter & information leaflet sent
	Reply slip received
	Invitation to screening
1 (30 min)	Screening visit: Assess eligibility criteria. Screening baseline assessment (height and weight). Informed consent obtained.
2 (30 min)	Optional: CGM sensor insertion and training.
3 (3 h)	Oral glucose tolerance test. Blood test Instruction to omit gliclazide for 3 days prior to visit 4
4 (3 h)	Oral glucose tolerance test. Blood test Optional: removal of CGM sensor. Instruction to restart gliclazide
(15 min)	Telephone follow-up.

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