Clinical implications of persistent beta cell function in long duration type 1 diabetes

Shannon Mary Marren

Masters by Research, Medical Studies

Clinical implications of persistent beta cell function in long duration type 1 diabetes

Submitted by Shannon Mary Marren, to the University of Exeter as a thesis for the degree of Masters by Research in Medical Studies, in August 2018.

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature) ……

Abstract

Type 1 diabetes was thought to be a disease of absolute insulin deficiency. However, recent evidence has shown that most people with Type 1 diabetes have persistent endogenous insulin production, even those with long disease duration. Close to diagnosis preserved beta cell function is associated with reduced HbA1c, hypoglycaemia and complication rates. However, very little is known about the clinical impact of persistent endogenous insulin in long duration diabetes. This thesis aims to assess the clinical impact of preserved beta cell function in long duration type 1 diabetes.

During this analysis we identified the potential for glucagon to be used as a biomarker of hypoglycaemic risk. Intensive treatment is an integral part of diabetes management and is key to reducing the risk of both development and progression of microvascular complications. However, treatment induced hypoglycaemia poses a significant barrier to intensive treatment. Currently, prediction of those most at risk of hypoglycaemia is based on clinical information, such as diabetes duration, with no biomarkers used to assess hypoglycaemic risk. As such, this finding prompted an additional aim: to investigate the relationship between meal stimulated glucagon and hypoglycaemia in long duration type 1 diabetes.

In Chapter 1 I review current evidence on the role and importance of persistent beta cell function in type 1 diabetes.

In Chapter 2 I outline the methods of the TIGI Study, which provided the data for this project.

In Chapter 3 I demonstrate that preserved beta cell function is associated with significantly reduced reported hypoglycaemia in long duration type 1 diabetes.

In Chapter 4 I show that higher meal stimulated glucagon is associated with reduced hypoglycaemia rate in long duration type 1 diabetes, independent of HbA1c, C-peptide and disease duration.

Chapter 5 discusses the findings of Chapters 3 and 4 and highlights areas for future research.

Table of contents

List of tables, figures and appendices

Chapter 1

Chapter 3

group in orange (n=39). ***p≤0.001 for low vs preserved C-peptide. Error bars represent 95% CI.

Fig. 3 – Reduced hypoglycaemic awareness by C-peptide group. Proportion of the low (blue, n=113) or preserved (orange, n=38) C-peptide group with reduced hypoglycaemic awareness, defined as a Clarke score ≥4/7. Hypoglycaemic awareness was similar in both groups, p=0.6.

Fig. 4 – Boxplot of study visit HbA1c by C-peptide group. Study visit HbA1c was similar in the low (n=148) and preserved (n=70) C-peptide groups p=0.06. Outliers are shown in Fig. 5.

Fig. 5 – Boxplot of study visit HbA1c by C-peptide group. Study visit HbA1c was similar in the low (n=148) and preserved (n=70) C-peptide groups p=0.06. Including outliers.

Fig. 6 - Historic HbA1c mean analysis **(a)** Boxplot showing similar historic HbA1c mean in low (n=86) vs preserved (n=67) C-peptide group, p=0.4. **(b)** Scatter graph of study visit HbA1c and historic HbA1c mean (n=153). Spearman's rho = 0.72, p<0.0001. Low C-peptide group (n=86) shown in blue and preserved C-peptide group (n=67) in orange. **(c)** Boxplot showing similar study visit HbA1c (n=153) vs historic HbA1c mean (n=153), p=0.1.

Fig. 7 – Boxplot of daily insulin dose by C-peptide group. Insulin dose was lower in the preserved C-peptide group (n=70) vs the low (n=151) group, p=0.01. Outliers are shown in Fig. 8.

Fig. 8 – Boxplot of daily insulin dose by C-peptide group. Insulin dose was lower in the preserved C-peptide group (n=70) vs the low (n=151) group, p=0.01. Including outliers.

Fig. 9 – Rates of retinopathy and microalbuminuria by C-peptide group. Proportion of C-peptide group with retinopathy in blue; background in dark blue, pre-proliferative in medium blue, proliferative in light blue. Proportion with retinopathy was similar in the low (n=55/74) vs preserved C-peptide group (n=37/56), p=0.3. Proportion of C-peptide group with microalbuminuria (green); similar in low (n=16/69) and preserved (n=10/51) C-peptide groups, p=0.6.

Chapter 4

Table 1 - Cohort characteristics. Values reported as n (%) or median (IQR).

Table 2 - Poisson regression analysis of total monthly hypoglycaemia rate (n=71). Pseudo R^2=0.24

Table 3 - Comparison of those include in the hypoglycaemia analysis to the remaining cohort. Values reported as n (%) or median (IQR).

Fig. 1 – Analysis of glucagon at 90 minutes **(a)** Scatter graph of age at diagnosis and 90-minute glucagon (n=133). Spearman's rho = 0.18, p=0.04. **(b)** Scatter graph of age at recruitment and 90-minute glucagon (n=133). Spearman's rho = 0.40, p<0.0001. **(c)** Scatter graph of disease duration and 90-minute glucagon (n=133). Spearman's rho = 0.31, p=0.0002. **(d)** Scatter graph of daily insulin dose and 90-minute glucagon (n=133). Spearman's rho = -0.21, p=0.02.

Fig. 2 - Scatter graph showing total monthly hypoglycaemia rate and glucagon at 90 minutes (n=72). Glucagon 90 minutes post-meal explains 11% of variation in total hypoglycaemia rate and a 1pmol/l increase reduces rate by 23% (IRR 0.77, CI 0.72-0.81, p<0.0001, pseudo R^2=0.11).

Appendix

Appendix 1: Clarke/Edinburgh Hypoglycaemia Questionnaire

Acknowledgements

I am hugely grateful to all who have supervised me this year, in particular Richard Oram, Angus Jones, Tim McDonald, Beverley Shields and Andrew Hattersley. I would like to thank them very much for their reliable mentorship, time and guidance.

I thank everyone in the Exeter Clinical Diabetes Research Group and in the NIHR Exeter Clinical Research Facility. I couldn't have asked for a more welcoming, approachable and supportive team and feel very privileged to have worked with them this past year.

I would also like to thank my housemates and fellow Masters Students for an excellent year and making me laugh when I needed to. Finally, I thank my parents, and sisters Aylish and Niamh for their dependable patience and encouragement, to whom this thesis is dedicated.

Abbreviations

α	Alpha
ACEi	Angiotensin Converting Enzyme inhibitor
ACR	Albumin:Creatinine Ratio
AST	Arginine Stimulation Test
β	Beta
BMI	Body Mass Index
CFR	Clinical Research Facility
CGM	Continuous Glucose Monitoring
DCCT	Diabetes Control and Complications Trial
DKA	Diabetic Ketoacidosis
eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme-Linked Immunosorbent Assay
GAD	Glutamic Acid Decarboxylase
GST	Glucagon Stimulation Test
HbA1c	HaemoglobinA1c/Glycosylated Haemoglobin
IA2	Islet Antigen 2
ICT	Islet Cell Transplantation
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IQR	Interquartile Range
IRR	Incidence Rate Ratio
IV	Intravenous
MMTT	Mixed Meal Tolerance Test
n	number

NHS	National Health Service
NIHR	National Institute for Health Research
r	Correlation coefficient
R^2	r squared
RAS	Renin-Angiotensin System
TIGI	Type 1 diabetes, Immunology, Genetics and endogenous Insulin production
U	Units
UCPCR	Urine C-peptide:Creatinine Ratio
UNITED	Using pharmacogeNetics to Improve Treatment in Early onset Diabetes

Chapter 1: Introduction

Structure of introduction

This chapter is divided into three sections. Part one addresses the structure and aims of the thesis. Part two discusses the current evidence on the importance of persistent endogenous insulin production in type one diabetes. Finally, part 3 focuses on glucagon dysregulation in type 1 diabetes.

Introduction part 1: Structure and aims of thesis

Structure and aims of thesis

The aim of this thesis is to investigate the clinical implications of persistent beta cell function in long-duration type 1 diabetes. Whilst completing this analysis we identified the potential for glucagon to be used as a biomarker of hypoglycaemic risk. This led to the development of an additional aim: to investigate the relationship between stimulated glucagon and hypoglycaemia in long duration type 1 diabetes.

Chapter 2

Chapter 2 provides details on aspects of methodology relevant to both Chapters 3 and 4, such as the design of the TIGI Study. More specific methods for each chapter, including statistical analysis, are outlined in the relevant chapters.

Chapter 3

Very little is known about the clinical impact of preserved beta cell function in long duration diabetes. This chapter aims to investigate the clinical impact of preserved beta cell function on hypoglycaemia, HbA1c and complications in a long duration type 1 diabetes cohort.

Chapter 4

During analysis for Chapter 3 we found there to be an association between mixed-meal tolerance test stimulated glucagon and hypoglycaemia rate. Ordinarily glucagon is secreted in response to hypoglycaemia and supressed following a meal. However, glucagon is known to be dysregulated in type 1 diabetes. This chapter investigates the association between mixed-meal tolerance test stimulated glucagon and hypoglycaemia in long duration type 1 diabetes.

Chapter 5

In this chapter I will summarise the findings of Chapters 3 and 4 and discuss their limitations, implications and potential directions for future research.

Introduction part 2: There is very limited evidence of the effect of C-peptide on complications and hypoglycaemia in long duration Type 1 Diabetes

2.1 Type 1 Diabetes

2.1.1 Autoimmune destruction

Type 1 Diabetes is an autoimmune condition resulting in the progressive destruction of pancreatic beta cells, leading to insulin deficiency. Disease progression follows a sequence first proposed by Eisenbarth in 1986 which has since been modified, see Fig. 1. (1,8) The pathway begins with a genetically determined background risk, followed by triggering of autoimmune activation and development of measurable autoimmunity; currently the triggers for this are unknown but possibilities include environmental exposures and early life infections. (9) Individuals develop autoantibodies directed at the insulin-producing beta cells of the pancreas, these antibodies can be present for a number of years prior to clinical onset. (10) Ordinarily beta cells regulate blood glucose levels to maintain homeostasis, secreting insulin in response to hyperglycaemia. In pre-clinical type 1 diabetes, beta cell mass gradually depletes without impacting glucose homeostasis, and individuals suffer no symptoms of diabetes. Eventually a critical beta cell mass is reached, at which point homeostasis can no longer be maintained. Dysregulation leads to increasingly elevated blood glucose levels and eventually symptoms such as polydipsia, polyphagia and polyuria become apparent and insulin treatment is required. Post-diagnosis it is common to experience a honeymoon period where remaining beta cell mass protects from insulin induced hypoglycaemia and good blood glucose control is easily achieved. (1)

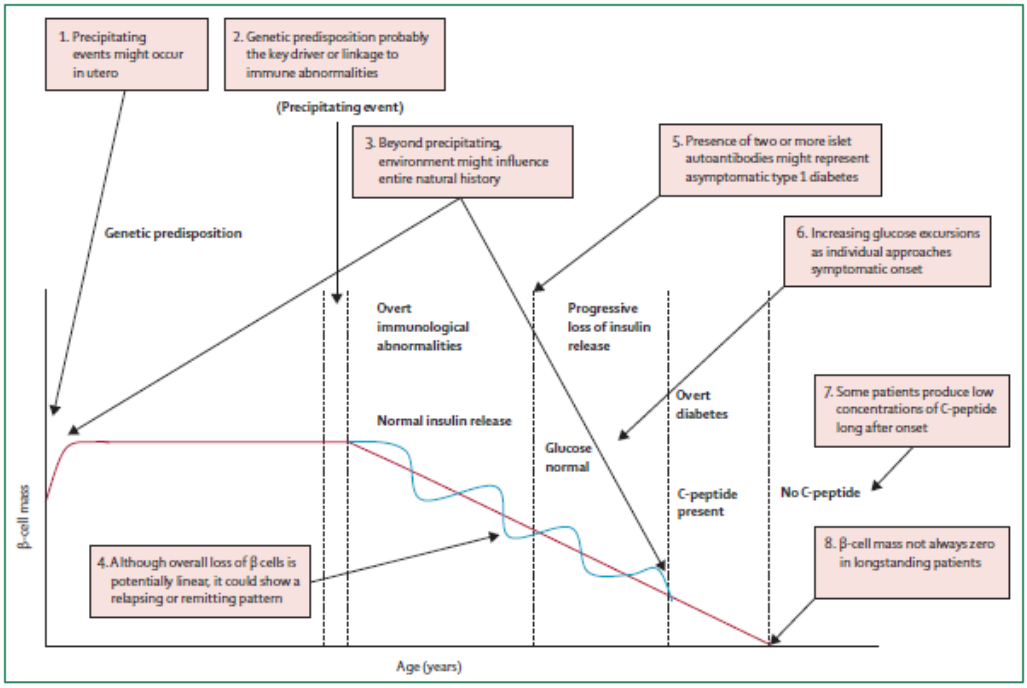


Fig. 1: "The natural history of type 1 diabetes", modified from Eisenbarth's original 1986 model. Taken from Atkinson et al. (1)

Traditionally it was thought that this depletion progressed to complete beta cell loss resulting in total insulin deficiency. Recent evidence suggests a two stage decline in beta cell function. Initially there is a substantial decline in function for approximately 7 years, this is followed by a plateau in which there is minimal further loss of beta cell function, see Fig. 2. This suggests that at approximately 7 years there is a change in the disease process. Perhaps the immune process changes or there are a remaining cohort of beta cells that have evaded autoimmune destruction. (2)

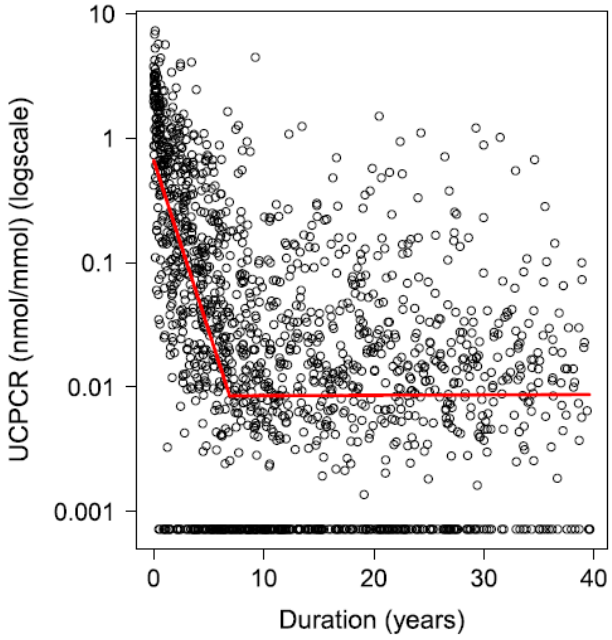


Fig.2: Decline of beta cell function in type 1 diabetes, as measured by Urinary C-peptide-Creatinine ratio (UCPCR) –described later in text. Taken from Shields et al. (2)

2.1.2 Genetic influence

Genetic susceptibility is a key component of the development of type 1 diabetes, with an identical twin concordance of up to 70% and 10% of cases having an affected first degree relative. (11) Type 1 diabetes is a polygenic disease with over 50 predominantly immune loci identified so far. (12) The major genetic loci driving genetic risk are the antigen presentation genes of the immune system in the HLA region. In type 1 diabetes the HLA class 2 DR-DQ haplotypes DR3-DQ2 and DR4-DQ8 are the strongest risk haplotypes. Environmental influences impact this genetic background risk and could have an effect as early as in utero. A popular explanation is the hygiene hypothesis, in which a lack of childhood infections is thought to increase susceptibility to autoimmune conditions. (9,11,13,14)

2.1.3 Epidemiology

Type 1 diabetes is one of the most common chronic diseases of childhood. Worldwide incidence is increasing by 2-5% and in the UK prevalence in those under 15 years is predicted to increase from 19,000 in 2005 to 33,000 in 2020. (15,16) In the past type 1 diabetes had been viewed as a childhood disease, commonly referred to as juvenile onset diabetes. However, whilst diagnosis is classically made in childhood the incidence may be much higher in adults than previously thought. (17) Unlike most autoimmune conditions type 1 diabetes affects slightly more males than females. Type 1 diabetes diagnostic criteria are: a fasting blood glucose >7.7mmol/l or any blood glucose >11.1mmol/l accompanied with symptoms of hyperglycaemia. (1) Due to severe insulin deficiency once the diagnosis of type 1 diabetes is made patients are started on injected insulin treatment.

2.2 Complications of diabetes

This section reviews complications of diabetes with particular focus on hypoglycaemia, retinopathy and nephropathy as they are the major focus of the research chapters in this thesis.

The discovery of insulin in 1921-22 transformed a type 1 diabetes diagnosis from being a death sentence to a chronic condition. (1) However, living with diabetes leaves individuals at risk of acute and chronic complications, with high mortality and morbidity. A recent Scottish type 1 diabetes cohort study compared individuals with and without type 1 diabetes at 20 years old, estimating an 11-13 year loss of subsequent life expectancy for those with type 1 diabetes. (18) Management of complications is extremely costly and is the mainstay of NHS spending on diabetes. Currently diabetes is thought to use up to 10% of the NHS budget, costing approximately £1million per hour. (19) Prevention and effective management of complications is very important for people with diabetes and for funding of the NHS.

Whilst persistent hyperglycaemia results in both macrovascular and microvascular complications, treatment induced hypoglycaemia can be equally damaging. It is recommended that individuals with type 1 diabetes self-monitor their blood glucose levels at least 4 times a day, before all meals and before sleep, so that they can adjust insulin dose accordingly. On waking a fasted level of 5-7mmol/l is recommended and before meals and during the day the aim is 4-7mmol/l. Alternatively, for individuals who like to self-monitor after meals they must do so at least 90 minutes post-meal with a target of 5-9mmol/l. Clinicians also monitor blood glucose control using HbA1c. Circulating glucose binds to haemoglobin throughout the life of a red blood cell (approximately 120 days) and forms glycated haemoglobin (A1c), as such HbA1c provides an estimate of blood glucose over the last 3 months. This should be routinely measured every 3-6 months with a target of <48mmol/l (6.5%) and insulin regimes adjusted accordingly. (20) Management consists of a balance between using insulin to avoid chronic complications and preventing life-threatening insulin induced hypoglycaemia.

2.2.1 Acute complications

Acute complications include diabetic ketoacidosis (DKA) and hypoglycaemia.

Diabetic ketoacidosis

DKA typically occurs in unmanaged type 1 diabetes and is a way in which type 1 diabetes may first present. Almost 4% of people with type 1 diabetes experience DKA each year. Common precipitants include intercurrent illness or lack of insulin administration. (21) Severe lack of insulin causes intracellular glucose deficit and results in the breakdown of fatty acids producing acidic ketone bodies. Metabolic acidosis occurs requiring immediate hospitalisation and fluid resuscitation. Diagnosis is made through confirmation of hyperglycaemia, ketosis and acidosis. (22) DKA is a medical emergency with a mortality rate of 3-5% and is responsible for a 54-76% of type 1 diabetes deaths in people with type 1 diabetes <30 years old. (21,23)

Hypoglycaemia

Hypoglycaemia is a serious and common problem in type 1 diabetes that occurs as a result of administration of exogenous insulin. It is biochemically defined as a blood glucose ≤3.9mmol/l and carries significant morbidity and mortality. Typically, individuals experience both autonomic and neuroglycopenic symptoms during hypoglycaemia. Common symptoms include sweating, weakness, drowsiness, loss of balance, visual disturbance and cognitive impairment. (24,25) These symptoms can have a substantial impact on quality of life; including increasing the risk of accidents and falls and causing driving licence restrictions. More serious symptoms include arrhythmias, myocardial ischaemia, seizure or coma. Beyond its immediate effects, hypoglycaemia can have long-term cardiac and neurological implications. (25) It has been reported that hypoglycaemia causes the death of 4-10% of patients with type 1 diabetes. (26)

Commonly hypoglycaemia is categorised as mild or severe depending on whether the individual can self-treat. Mild hypoglycaemia is common, with adults experiencing an average of 1-2 episodes per week. However, quantification can be difficult due to reliance on patient recognition and recall. Prevalence of severe hypoglycaemia is approximately 30% per year. (25) Self-

reported severe hypoglycaemia is associated with a 3.4 times increased risk of death at 5 years compared to those with mild or no hypoglycaemia. (27)

Hypoglycaemic symptoms reduce with increased time spent at low blood glucose levels, resulting in reduced hypoglycaemic awareness. Hypoglycaemic awareness is a vital protector from severe hypoglycaemia. For this reason, hypoglycaemia typically occurs in those with long duration diabetes, with disease duration being an important predictor of hypoglycaemic risk. Additional risk factors for severe hypoglycaemia include strict glucose control, extremes of age, and sleep (approximately 50% of episodes occur during sleep). (25)

Hypoglycaemia is the most serious limiting factor in achieving good glycaemic control in type 1 diabetes. (28,29) As such, patient education is key to maintaining tight control whilst preventing hypoglycaemia. It is important that patients frequently monitor their blood glucose levels and subsequently appropriately adjust their diet and insulin dose. New technologies including continuous glucose monitoring and modified insulin pumps are been developed and refined to help combat hypoglycaemia. (25) We still have an incomplete understanding as to why some individuals have more severe episodes of hypoglycaemia than others, this is likely due to both physiological and psychosocial factors.

2.2.2 Chronic complications

Chronic complications result from persistent hyperglycaemia damaging vasculature, with damage dependent on hyperglycaemic duration and severity. They are classified as microvascular and macrovascular. Microvascular complications include diabetic retinopathy, nephropathy and neuropathy.

Microvascular

Retinopathy

Diabetic retinopathy is the most common microvascular complication of diabetes. (30) Amongst people with type 1 diabetes in the UK, prevalence is estimated to be approximately 55% (predominantly background retinopathy) with the vast majority developing retinopathy within 20 years of diagnosis. (31,32) Duration is a key risk factor for the development of retinopathy; each 5 year increase in disease duration increases risk of diabetic retinopathy by 10% and severe diabetic retinopathy by 26%. (31)

Hyperglycaemia and hypertension damage retinal blood vessels. This can result in reduced blood supply damaging the retinal tissues. Vessels also develop microaneurysms and leak; causing macular oedema. This progresses to the development of new vessels on the retina. Although the early stages of diabetic retinopathy are asymptomatic, progression can result in significant visual impairment and without treatment will lead to blindness. (33) Currently nearly 3 million people worldwide are blind as a result of diabetic retinopathy and it is the leading cause of blindness in people of working age in the western world. (33,34) In the UK alone there are 1280 new cases of blindness each year resulting from diabetic retinopathy. (35)

A national diabetic eye screening programme was founded in the UK in 2004. From the age of 12 people with diabetes are invited to attend annual visits for digital retinal photography. Reports of the findings are sent to their GP to inform ongoing management or individuals are escalated to treatment if there have been sight-threatening changes. A report evaluating the service from 2004-14 found that 79.3% of eligible individuals attended screening. Furthermore, over this time age-standardised prevalence of diabetic retinopathy remained stable (approximately 55%), and the prevalence of severe diabetic retinopathy halved to 10.35%. (31)

Retinopathy is classified as background, pre-proliferative and proliferative, all with strict diagnostic criteria. Individuals progress through each stage, with proliferative retinopathy requiring treatment. Background retinopathy is defined as microaneurysms, retinal haemorrhages with or without exudate. Pre-proliferative retinopathy is characterised by venous bleeding, venous loops or reduplication, intra-retinal microvascular abnormality, multiple deep, round or blot haemorrhages, and cotton wool spots. Proliferative retinopathy is diagnosed when there are new vessels, pre-retinal or vitreous haemorrhage, pre-retinal fibrosis with or without tractional retinal detachment. (35–38) A recent cohort study of 5000 people using the Welsh national screening service showed that 44% of people with type 1 diabetes had no diabetic eye changes, 40% had background retinopathy, 8% had pre-proliferative retinopathy and 4% had proliferative retinopathy; the remaining 4% had maculopathy. (39)

Laser photocoagulation is used for the treatment of proliferative retinopathy and maculopathy. (34) Laser light energy is directed at the retina which is absorbed

by the retinal pigments, heating the retina and resulting in thermal damage. It is used to treat sight-threatening retinopathy and has been shown to reduce the risk of both retinopathy progression and severe visual loss 12 months post-treatment by approximately 50%. (33)

Nephropathy

Diabetic nephropathy is a common complication of type 1 diabetes. It is estimated to affect 40% of people with type 1 diabetes and substantially increases the risk of cardiovascular morbidity and mortality. (40) Chronic hyperglycaemia leads to damage and scaring of the glomerular vasculature. This damage is termed glomerulosclerosis and encompasses several defining features including; thickening of the glomerular basement membrane, microaneurysms, mesangial sclerosis, hyaline arteriosclerosis and mesangial node formation (Kimmelstiel-Wilson nodules). (32,40) These changes result in impaired blood flow to the kidney, hypertension, proteinuria and renal impairment. (41) Early recognition and intervention are key to reducing both morbidity and mortality.

An early sign of diabetic nephropathy is microalbuminuria, with 7% of people diagnosed with type 1 diabetes already having microalbuminuria at the time of diagnosis**.** (40) Without intervention microalbuminuria progresses to macroalbuminuria, with progression reported in 30-45% of people with microalbuminuria. (40,42) Proteinuria (microalbuminuria or macroalbuminuria) is estimated to affect up to 40% of people with type 1 diabetes and has a peak incidence at 15-20 years disease duration. Eventually individuals develop renal failure and become reliant on renal replacement therapy or kidney transplant. Diabetic nephropathy is the leading cause of renal failure in developed countries. (32,40,43,44)

Diagnosis of microalbuminuria and macroalbuminuria is made on the basis of 2 out of 3 abnormal test results in a 3 to 6-month window. Traditionally microalbuminuria is defined as an albuminuria of 30-299mg/g on a spot urine sample and macroalbuminuria as albuminuria >300mg/g. (40) However, in order to allow for the dilution effect of urine, albuminuria can also be defined by albumin-creatinine ratio (ACR). Microalbuminuria is defined as an ACR >2.5 mg/mmol in men and >3.5 mg/mmol in women, with macroalbuminuria >30 mg/mmol. (45)

New guidance on diagnosis of diabetic nephropathy combines estimated glomerular filtration rate (eGFR) with ACR (Fig. 3). (3) eGFR should be routinely measured to monitor nephropathy progression as it provides the best estimate of overall measure of kidney function. Individuals with an eGFR <30 should be referred to a nephrologist. (40)

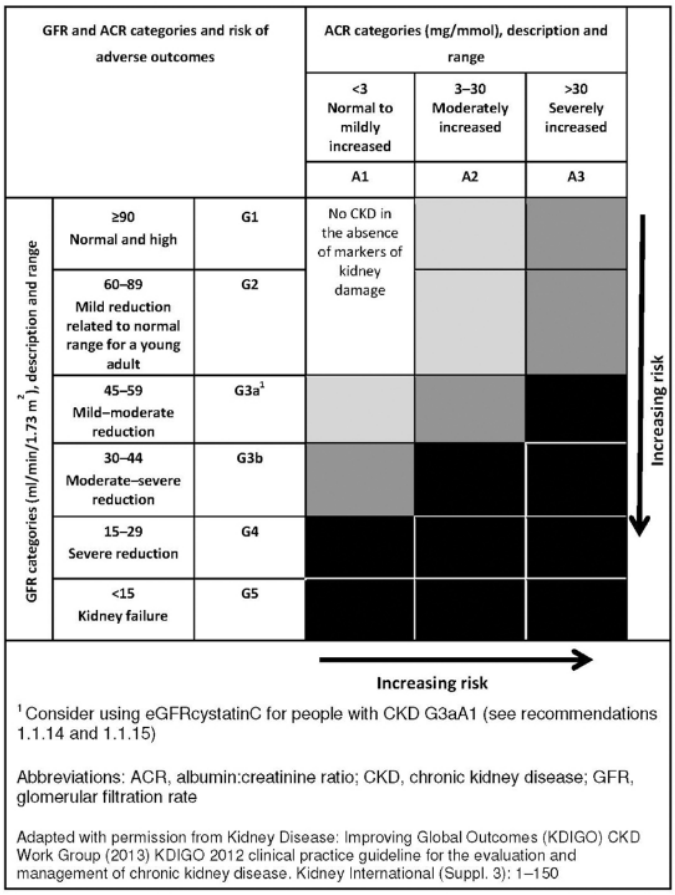


Fig 3: NICE Classification of chronic kidney disease. Taken from Chronic kidney disease in adults: assessment and management guidance [CG182] (3)

The mainstay of management for diabetic nephropathy is management of modifiable risk factors such as hyperglycaemia, hypertension and dyslipidaemia, alongside smoking cessation**.** (40) Renin-angiotensin system (RAS) blockade is also a key part of diabetic nephropathy management. RAS blockade reduces the risk of both nephropathy and cardiovascular disease through reducing blood pressure. This lowers intraglomerular pressure and reduces passage of proteins into the proximal tubule, resulting in a reduction in

albuminuria. Angiotensin-converting enzyme inhibitors (ACEi) have not only been shown to reduce the risk of microalbuminuria progressing to macroalbuminuria by 60%, but also make reversion to normoalbuminuric more likely. However, they should be prescribed with caution as use is contraindicated in patients with bilateral renal artery stenosis and during pregnancy, due to associated severe birth defects. (40,46)

To minimise risk of nephropathy it is recommended that all people with type 1 diabetes have an annual ACR recorded, aim to have a blood pressure <130/80mmHg, avoid a high-protein diet, and if they develop microalbuminuria be prescribed a RAS blockade. (20) In addition, those with nephropathy should be routinely assessed for coronary heart disease due to their vastly increased risk. (40)

Neuropathy

Diabetic neuropathy is a further microvascular complication. 10% of people with diabetes have neuropathy at diagnosis, this rises to 40-50% after 10 years. (47) The mechanism by which peripheral nerves are damaged is not currently understood but thought to be due to damage to small blood vessels. There are multiple manifestations including sensory /motor, focal/multifocal and autonomic. A distal symmetrical sensorimotor polyneuropathy is the most common diabetic neuropathy. This involves loss of sensation to light touch, vibration and temperature. This leaves individuals at risk of injury particularly to the foot and subsequent ulceration, with the potential to progress to amputation. (32)

Macrovascular

Chronic macrovascular complications of diabetes manifest as cardiovascular disease. This is the leading cause of death in people with diabetes and the largest expense in their care. Hypertension and hyperglycaemia lead to chronic injury and inflammation of both peripheral and coronary arterial walls. The resultant atherosclerosis increases risk of myocardial infarction along with elevating mortality rates from both ischaemic heart disease and cerebrovascular disease. (32) Macrovascular complications of diabetes are extensively reviewed elsewhere and were not studied during this Masters research.

2.2.3 Preventing development of complications

Diabetic complication rates have improved, however, complication management remains the focus of diabetes care. (11,48,49) Maintaining good glycaemic control is consistently shown to be the most effective way of reducing incidence and progression of diabetic complications. (48,50) However, the risk of hypoglycaemia due to insulin therapy remains a barrier to tight control. (28,29)

Whilst type 1 diabetes has historically been described as a disease that leads to total insulin deficiency, there is increasing evidence of variable beta cell loss following diagnosis. (2,8) Furthermore, studies have shown persistent low levels of endogenous insulin production in many people with type 1 diabetes, including those with long disease durations. (51–53) This is important as preserved endogenous insulin has been associated with reduced complication rates. (6,54,55) This has led to preservation of endogenous insulin production becoming the focus of immunotherapy trials. (56)

2.3 C-peptide

2.3.1 What is C-peptide?

C-peptide is produced in a 1:1 ratio with insulin during the cleavage of proinsulin. It was first discovered in 1967 and is the molecule adjoining insulin's alpha and beta chains. (57) Processing of preproinsulin to proinsulin, followed by proinsulin to insulin and C-peptide, occurs within the beta cells of the islets of Langerhans in the pancreas. Preproinsulin is cleaved in the endoplasmic reticulum by signal peptidase to form proinsulin. Proinsulin folds to form 3 disulphide bonds. Once folded proinsulin is transported through the Golgi complex into immature secretory granules, here cleavage of proinsulin occurs. (58,59) Cleavage is mediated by two endopeptidases working at separate sites, prohormone convertases 2 and 3. Intermediate molecules are produced due to loss of basic amino acids at each site via carboxypeptidase H. These intermediate molecules are termed des forms, des 31-32 split and des 64-65 split, see Fig. 4. Following completion of cleavage at both sites, a single molecule of both insulin and C-peptide is produced. (4) The two molecules are stored in the secretory granules in beta cells ready for exocytosis, releasing insulin and C-peptide into systemic circulation. In healthy individuals plasma C-peptide level is variable, reference ranges include 300-600pmol/l fasted and 1000-3000pmol/l in the post-prandial state. (60,61)

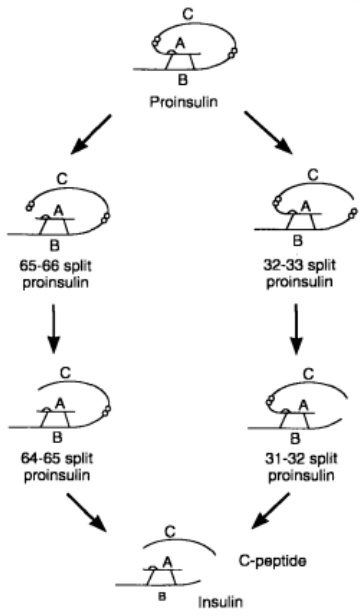


Fig. 4: Processing proinsulin to insulin. Taken from Temple et al. (4,5)

2.3.2 A measure of endogenous insulin production

C-peptide is the best measure of endogenous insulin production in insulin treated patients. C-peptide measurement allows measurement of endogenous insulin production whilst overcoming the barriers of measuring insulin directly. (62) All endogenous insulin produced initially travels through the portal venous system, with the liver responsible for regulating peripherally circulating levels. (63) First pass hepatic metabolism has been shown to extract 60-70% of endogenous insulin. (64) Moreover, hepatic clearance varies with physiological state, thus peripheral insulin does not necessarily reliably reflect endogenous insulin production. (65) In contrast, hepatic metabolism of C-peptide has been shown to be negligible. (66) C-peptide has a half-life of 20-30 minutes compared to insulin's 3-5 minutes and slower metabolic clearance than insulin, 4.5 vs 15 ml/min/kg. (66,67) With circulating levels 5 times greater than insulin and relatively low cross reactivity; it is a reliable, reproducible, practical alternative to determining endogenous insulin production. (66,68) Furthermore, it's ultimate utility lies in its ability to differentiate between endogenous and exogenous insulin levels in those requiring exogenous insulin therapy. (66) This is because exogenous insulin treatment does not contain C-peptide.

2.3.3 Measuring C-peptide

C-peptide can be assessed in both in blood and urine. In the past there have been concerns regarding stability, however, provided it is collected correctly, C-peptide is stable in EDTA plasma for 24-hours and urine in boric acid for 72-hours at room temperature. Measurements can be taken fasting, randomly or following stimulation. C-peptide levels in blood are commonly reported in both nmol/l and pmol/l (1nmol/l = 1000pmol/l). In this thesis, where studies have reported in nmol/l levels have been converted to pmol/l to provide direct comparison with the results of our studies. Absolute deficiency is estimated as <80pmol/l when fasted in blood, <200pmol/l following stimulation in blood, and <0.2nmol/mmol following stimulation in urine. Stimulated serum C-peptide is commonly used as a reliable reproducible measure of C-peptide production. (66)

Stimulated and fasting measurements

Stimulated measurements are the most precise, but fasting C-peptide does correlate well with stimulated C-peptide. (69) Clinical trials typically use a stimulated C-peptide measurement. (68) Beta cells can be stimulated to produce insulin using a number of tests, these include a mixed-meal tolerance test (MMTT), glucagon stimulation test (GST), oral glucose tolerance test, intravenous (IV) glucose tolerance test and arginine stimulation test (AST). MMTT's and GST's are commonly used in trial settings, with AST's also being particularly sensitive. (70) Participants are fasted overnight for both the MMTT and GST. During the MMTT a liquid meal is ingested, and serum C-peptide measured at varying intervals for 2-4 hours. In comparison, the GST involves an IV glucagon injection with multiple serum C-peptide measurements for 10 minutes. C-peptide classically peaks at 90 and 6 minutes in the tests respectively. (62,66) GST is associated with a higher number of adverse effects, the most common being nausea which Greenbaum et al. showed was experienced in 95% of 8-12 year olds. Patients generally prefer the better tolerated MMTT despite it being more time consuming. Both are highly reproducible but the MMTT is more sensitive at 90 minutes than the GST at 6 minutes. Thus, stimulated C-peptide measurement following a MMTT is established as the gold standard in type 1 diabetes trials. (62)

A single C-peptide measurement 90-minutes following a MMTT provides a reliable estimate of beta cell function. (69) MMTT results are commonly analysed by assessing area under the curve at 120 minutes. (66) However, recently a single measurement at 90 minutes has been found to be both sensitive and specific for peak insulin secretion. Having a single sample taken is beneficial for both the patient and clinician, reducing the number of samples and duration of the test also reduces cost. This provides a practical alternative to a full MMTT. It was also shown that as a single measurement 90 minute C-peptide following a MMTT was more reliable than a single fasting serum C-peptide. (69)

Measuring C-peptide in urine

C-peptide undergoes renal clearance, this occurs through catabolism and glomerular filtration. (60,71) Approximately 5% of C-peptide is excreted unchanged in urine. (66) C-peptide can be measured following a 24-hour urine collection. However, by utilising urinary C-peptide creatinine ratio (UCPCR) a single urine sample can be used. (66) UCPCR provides both a reliable and reproducible measurement and has the benefit of being efficient and non-invasive. (72) However, as it is renally excreted urinary C-peptide measurement is not validated in those with renal impairment. (73) Stimulated UCPCR 2 hours after a meal is highly correlated with MMTT 90 minute C-peptide. (53,66) The test can be carried out at home and is stable for 72 hours allowing time for transport and analysis. UCPCR comes with a number of cautions. It is a less precise test and has a different normal range to serum C-peptide making cross comparison difficult. Whilst not as accurate, stimulated UCPCR provides a very practical, non-invasive, inexpensive alternative to MMTT 90 minute serum C-peptide. (73)

2.3.4 New information - C-peptide assay development

The development of ultra-sensitive assays has allowed for detection of C-peptide at very low and previously undetectable levels. Until recently, C-peptide assays have been restricted by lower limits of detection around 30pmol/l. (52) However, newly developed isotopic assays utilising monoclonal antibodies have been found to be both sensitive and specific. Roche's electrochemiluminescence immunoassay offers a limit of detection of 3.3pmol/l with Mercodia's ultrasensitive ELISA pushing the limit of detection to 1.5 pmol/l. (51,52) Due to a variety of assays and units used, lack of standardisation makes results potentially incomparable and they should be interpreted with caution. (66) However, both assays have demonstrated the ability to identify significant numbers of individuals with persistent C-peptide that would have been previously undetectable.(51,53) Currently there is evidence to suggest that despite the ELISA having a lower limit Roche offer a more sensitive test. (51,52)

2.3.5 C-peptide as an Outcome Measure

Research has looked to the potential benefit of prolonging C-peptide production. A number of immunotherapy trials have looked to prevent, delay or even reverse diabetes development. Trials typically focus on groups at high risk of developing type 1 diabetes or those with newly diagnosed diabetes. While there are no current studies that have been able to demonstrate a prolonged impact, there have been a number showing some degree of short term success. This provides both hope and direction for future research. C-peptide is a key primary outcome in immunotherapy trials. (56)

C-peptide has been selected as a primary outcome in intervention trials because it reflects underlying beta cell function. Declining beta cell function is the key pathophysiological process in the development of diabetes. Thus, preservation of beta cells is intrinsic to disease prevention and is the focus of many immune therapy and intervention trials. (68) In contrast to autoantibodies, which can be present for a number of years prior to disease development, C-peptide is a good measure of an individual's current disease status. (66) It is a direct measure of endogenous insulin production with reproducible results that can be reliably monitored over time. Therefore, C-peptide is the most appropriate primary outcome in intervention studies focusing on beta cell function. (68) Evidence regarding the utility of C-peptide comes from two key areas, The Diabetes Control and Complications Trial and islet cell transplantation studies. (54,74)

2.4 Clinical benefits associated with preserved C-peptide

2.4.1 Diabetes Control and Complications Trial (DCCT)

The DCCT, a landmark trial in type 1 diabetes, revealed that intensive treatment was paramount to reducing complications. This was a large scale randomised control trial across 29 centres comparing intensive and conventional therapy in type 1 diabetes. It assessed primary and secondary prevention of micro and macrovascular complications and risk of severe hypoglycaemia. The 1441 participants with diabetes duration 1-15 years were followed for an average of 6.5 years. The trial concluded that intensive insulin therapy was significantly superior in both preventing and delaying progression of microvascular complications when compared to conventional treatment. (50,54,75) Furthermore, approximately 30 year follow up showed that those who had received intensive treatment had a lower risk of mortality compared to the people who had received conventional therapy (absolute risk reduction 1/1000 patient years). (76) The lessons learnt from the DCCT have greatly influenced treatment standards today.

Intensive therapy aimed to maintain normal glycaemic levels. It involved 3-4 insulin injections per day or use of an insulin pump. Blood glucose was checked at least four times each day and insulin dose adjusted accordingly. Individuals checked their 3am blood glucose weekly and focused on both diet and exercise. Comprehensive supervision and support was given through monthly clinic visits and telephone contact for troubleshooting. In contrast, conventional treatment involved 1-2 insulin injections each day and once daily blood or urine glucose checks. Initial dietary advice was provided and participants attended clinics every three months. (54)

The role of intensive therapy in prolonging endogenous insulin production was also investigated in a restricted cohort with short disease duration. Screening of over 3000 individuals identified 855 people with short duration type 1 diabetes (<5 years) aged 13-39 with a stimulated C-peptide ≤500pmol/l. The cohort was divided into C-peptide responders (200-500pmol/l) and non-responders (<200pmol/l). Individuals were then randomly allocated intensive or

conventional therapy. There were 303 responders (138 intensive treatment, 165 conventional treatment) and 552 non-responders (274 intensive treatment, 278 conventional treatment). Stimulated C-peptide was assessed annually for up to 6 years or to the point C-peptide fell below 200pmol/l. (54) A limitation of the DCCT was the selective exclusion of people with higher levels of endogenous insulin (>500pmol/l within the first 5 years of diagnosis, and >200pmol/l after this point). This was by design to excluded non-type 1 diabetes cases, but also limited the ability to study the full impact of preserved C-peptide.

Initial analysis of this group of participants showed intensive treatment resulted in improved outcomes regardless of C-peptide response, in line with the findings of the main study. The intensively treated achieved better glycaemic control with a consistently lower HbA1c. However, this came at the cost of higher rates of severe hypoglycaemia. Among responders, retinopathy and microalbuminuria were less likely to occur when intensively treated, and when present, were slower to deteriorate. This was attributed to their lower HbA1c. Intensive treatment also significantly slowed loss of beta cell function, with responders maintaining higher C-peptide levels for longer. These findings illustrated intensive treatment was integral to good diabetes management and reducing complications. (54)

C-peptide responders benefited from reduced complications when intensively treated, but not with conventional treatment. Within the intensively treated group, responders had a lower HbA1C, risk of retinopathy and microalbuminuria progression and 65% reduction in risk of severe hypoglycaemia compared to non-responders. However, these differences were not reflected in the conventionally treated group, where responders and non-responders had similar HbA1c, risk of retinopathy progression and microalbuminuria development. This suggested that the benefit of C-peptide could only be utilized when intensively treated. (54)

Further analysis showed that higher levels of C-peptide reduced the incidence of retinopathy, nephropathy. Steffes et al. divided C-peptide response into four groups; undetectable (≤30pmol/l), minimal (40-200pmol/l), baseline only (>200pmol/l at baseline but subsequently <200pmol/l) and sustained (210-500pmol/l at baseline and at least the first annual visit). In the intensively treated group the rate of deterioration in retinopathy and nephropathy was

significantly lower in those with detectable C-peptide compared to those with undetectable levels. However, a key finding was that rates of both complications decreased according to C-peptide group, favouring C-peptide production. Similar results were seen in the conventionally treated group. (6)

Sustained levels of C-peptide reduced hypoglycaemia rates despite intensive treatment. Overall the intensive group were found to have higher rates of hypoglycaemia than the conventionally treated group. However, those with sustained levels of C-peptide had a lower rate and prevalence of hypoglycaemia than the other three intensively treated groups. Interestingly, their hypoglycaemia rate was considerably more similar to those in the conventionally treated group, see Fig. 5. This suggests the possibility that in those with sustained C-peptide levels intensive therapy may be utilised to minimise microvascular complications whilst endogenous insulin protects from hypoglycaemia. In addition, these findings propose that a C-peptide of 200pmol/l is a clinically significant value. (6)

	Stimulated C-peptide group				
	Undetectable	Minimal	Baseline-only	Sustained	All
All	13 ± 1^{a}	10 ± 1^{b}	7 ± 1^{c}	6 ± 1^{c}	12 ± 0.5
Intensive	21 ± 1^{a}	16 ± 1^{b}	17 ± 3^{ab}	7 ± 1^{c}	16 ± 0.8
Conventional	8 ± 1^{a}	6 ± 1^{b}	3 ± 1^{c}	5 ± 1^{bc}	6 ± 0.4

Data are rates ± SE per 100 participant-years. Rates were compared among stimulated C-peptide groups (horizontally) with each treatment group. For each comparison, rates with different letters were significantly different ($P < 0.05$), while rates sharing the same letter were indistinguishable.

Fig. 5: "First occurrence of hypoglycaemia during the first 6 years of the DCCT, by stimulated C-peptide and treatment group". Taken from Steffes et al. (6)

A subsequent analysis of the intensive group by Lachin et al. echoed these findings. Using C-peptide as a continuous variable they demonstrated an approximately linear association with HbA1c, insulin dose and rate of hypoglycaemia. There was a strong association between decreasing C-peptide and retinopathy deterioration. This study further strengthened the evidence of the positive impact of persistent C-peptide with intensive treatment and highlighted the potential importance of even small differences in C-peptide. (55)

2.4.2 Islet Cell Transplant

The DCCT provided evidence on the impact of C-peptide in a cohort close to diagnosis (<5 years duration). In contrast, islet cell transplantation (ICT) studies can be used to assess the impact of C-peptide in individuals with advanced diabetes and absolute insulin deficiency. ICT provides a unique opportunity to investigate the impact of restoring C-peptide production in an individual.

C-peptide is used as a key outcome measure in islet cell transplantation. ICT is used when individuals experience recurrent life-threatening hypoglycaemia. An NHS funded national ICT service was commissioned in 2008 with aims to reduce insulin dose, HbA1c and rate of hypoglycaemia. Numbers of successful outcomes continue to rise but it is a complex and costly service. C-peptide is used to define graft survival. (77,78)

Data from ICT patients showed that increased levels of C-peptide reduced hypoglycaemia and improved glycaemic control. Brooks et al. analysed data from ICT recipients from the first 3 years of the service. A stimulated C-peptide >50pmol/l was used to define a functioning graft post-transplant, with all recipients having been C-peptide negative prior to transplant. At an average of 24 (13.5-36) months post-transplant, 80% of recipients had preserved graft function. Hypoglycaemia rates had fallen from 20 to 0.3 episodes per patient year ($p<0.001$) and 70% maintained an HbA1c <7.0%. This study showed that an increase in C-peptide in those that were previously C-peptide negative results in reduction of hypoglycaemia and HbA1c, along with reducing insulin dose and hypoglycaemic awareness. (77)

Higher levels of C-peptide in ICT recipients result in greater reductions of hypoglycaemic risk and improvements in glycaemic variability. 12 ICT recipients were monitored throughout their first 18 months post-transplant. Stimulated C-peptide response was categorised as low (<200pmol/l), moderate (200-500pmol/l), good (500-1000pmol/l) and excellent (>1000pmol/l). Analysis was stratified by C-peptide group. Higher C-peptide response was shown to decrease HbA1c and insulin dose. It also reduced the risk and duration of both hyperglycaemia and hypoglycaemia, as measured by CGM. CGM also evidenced a strong continuous association between increasing C-peptide and

reduction in glucose variability. This further supports the evidence that minute changes in C-peptide have the potential to be advantageous. (79)

Additional islet transplant research suggests that achieving higher C-peptide levels slows deterioration of microvascular complications. A crossover study assessing progression of microvascular complications compared intensive insulin treatment to ICT. It involved 45 participants with type 1 diabetes and undetectable C-peptide. Initially participants received intensive insulin therapy and then progressed to ICT when a donor became available. Both retinopathy and GFR were found to progress significantly slower following ICT and there was no statistically significant difference in progression of neuropathy. It is proposed that ICT is associated with higher C-peptide response than intensive treatment and thus better glycaemic control. This supports the relationship between higher C-peptide and slower complication progression. (80)

Increased beta cell function is associated with improved mean blood glucose. ICT recipients underwent 72-hour CGM prior to and at 3, 6, 9, 12, 24 and 36 months following transplantation. Graft function was assessed by beta-score which incorporates C-peptide, HbA1c, blood glucose and current treatment. A key finding of the study was that better graft function was correlated with lower mean blood glucose. Mean glucose itself is an independent predictor of long-term cardiovascular risk. As such, increasing C-peptide may have the ability to improve blood glucose and in turn reduce mortality resulting from cardiovascular disease. (81)

Sustained C-peptide production following whole pancreas transplant has been shown to reverse diabetic complications. Selected pancreatic transplant recipients with lesions of diabetic nephropathy, who remained insulin independent at 10 years post-transplant, were seen to have substantial reversal of nephropathy 10 years post-transplant. This was ascribed to a significant duration of euglycaemia. Renal biopsies were taken before transplant and at 5 and 10 years post-transplant. Biopsies at 10 years showed significantly reduced thickness of both the glomerular and tubular basement membranes from baseline ($p<0.001$ and $p=0.004$ respectively), among other improved biopsy measures. (82) A further study assessing neuropathy five years post-ICT showed that increased C-peptide was associated with improvement in sensory nerve conduction velocity and action potentials. However, motor nerve

conduction velocity and cardiac autonomic neuropathy showed no significant change. (83) This suggests that persistent C-peptide production not only reduces risk of complication progression, but it is potentially hugely beneficial in reversal of complications.

ICT studies have highlighted the potential for improvement of hypoglycaemia with C-peptide restoration showing that restoring C-peptide production improves hypoglycaemic awareness and rate alongside reducing glucose variability. (77,79) In addition, some studies have hinted at the improvement of microvascular complications with increasing C-peptide. (80,82,83) However, evidence from ICT is limited to studies of relatively short duration. There would be great benefit in future studies assessing the long-term effect of C-peptide restoration on hypoglycaemia and microvascular complications.

2.4.3 Long duration evidence

C-peptide preservation in long duration type 1 diabetes

As many as 80% of people with long duration type 1 diabetes have detectable C-peptide. Lower limits of detection have enabled identification of C-peptide preservation in those with long duration type 1 diabetes. Evidence from stimulated C-peptide data showed that 73% of people with duration >5years had detectable serum C-peptide following a MMTT, with 20% showing levels >200pmol/l. Absolute C-peptide response was shown to decline with increasing duration. Despite this 68% of those with a type 1 diabetes duration >30years had detectable C-peptide. This suggests that C-peptide decline occurs over a number of decades rather than rapidly like previously thought. Furthermore, C-peptide positive participants had durations comparable to their C-peptide negative counterparts, suggesting additional influencing factors. Findings were replicated within the cohort using stimulated UCPCR which showed that 69% had preserved C-peptide production. This provides irrefutable evidence that C-peptide production can be preserved in long duration type 1 diabetes with large numbers proven to be C-peptide microsecretors. (52)

Multiple studies have supported the finding of preserved C-peptide production in long duration diabetes. Wang et al. used fasting C-peptide to show that 43% of those with mean duration of 19.4 years have detectable C-peptide. (51) Furthermore, Keenan et al. focused on extreme diabetes duration in 411 Joslin

Medallist's (all with duration >50years). 67.4% had detectable random C-peptide, with 64.4% having C-peptide 30-200pmol/l and 2.6% ≥200pmol/l. The higher C-peptide group were significantly older at diagnosis and had an associated lower HbA1c. (84) Supporting evidence was provided by a large population based study (n=924) that assessed C-peptide response using stimulated UCPCR following a meal at home in people with duration >5years. They showed that 52% had detectable C-peptide, with 8% >0.2nmol/mmol. Interestingly, they found that the likelihood of having a result >0.2nmol/mmol increased by 8% for each year older an individual was at diagnosis and decreased by 4% for each additional year of duration. (53)

Functioning beta cells in long duration type 1 diabetes

In the majority of people with type 1 diabetes, C-peptide levels respond to a stimulus, indicating functioning beta cells. This has been shown through a comparison between fasting and stimulated C-peptide in 74 participants with type 1 diabetes duration >5years. 54 individuals were shown to have detectable C-peptide (>3.3pmol/l) with 80% showing an increase in C-peptide response following a MMTT, 11% showing no change and no participants showing a C-peptide reduction. This shows that in long duration diabetes beta cell function is preserved. (52)

Beta cell function is preserved in those with diabetes duration >50 years. Research assessing extreme diabetes duration showed that 42% (n=13) of Joslin Medallists with random C-peptide >100pmol/l were able to at least double their fasting C-peptide in response to a MMTT. Moreover, a random C-peptide ≥200pmol/l significantly increased the chance of doubling C-peptide in response to the MMTT. This supports evidence that beta cell function remains in extreme diabetes duration, and suggests that preserved function may play a protective role. (84)

Further evidence shows that hyperglycaemia is associated with a higher C-peptide levels in long duration diabetes. Analysis of weekly fasting serum C-peptide in 4 individuals for 14 weeks showed that hyperglycaemic samples (>150mg/dl) were associated with higher levels of C-peptide than euglycaemic (<150mg/dl). Additional analysis of 3 of these individuals including non-fasting C-peptide results showed a linear association between glycaemic level and serum C-peptide. Throughout the wider cohort (n=182, mean duration 19.4

years) results from a single fasting C-peptide were stratified into well-defined C-peptide ranges. Where C-peptide was detectable (>1.5pmol/l) hyperglycaemic individuals had significantly higher levels. This further strengthens the evidence that even exceptionally low C-peptide levels (median 1.5pmol/l) have favourable effects and indicates functioning beta cells in long duration type 1 diabetes. (51)

Histological evidence

Evidence from pancreatic histology further supports beta cell function in those with long duration type 1 diabetes. Historically it was thought that type 1 diabetes resulted in total beta cell destruction. However, a number of studies have shown that insulin containing cells can be found in the pancreas of those with long duration type 1 diabetes. (84–87) Evidence from a 2005 study analysed pancreatic sections from 42 individuals with type 1 diabetes. It confirmed presence of beta cells in 88% of individuals regardless of duration (range 7-67 years). (85) Gianani et al. also studied the pancreases of those with childhood onset type 1 diabetes (mean duration 14 years) but found the majority of pancreases to be insulin deficient. Despite this they identified two distinct patterns of beta cell survival. The first exhibited lobular areas of abnormally functioning beta cells and the second showed beta cells present in all islets but with numbers consistently reduced throughout. (86)

Functioning beta cells were identified in pancreases of those with extreme duration diabetes. Keenan et al. studied 9 Joslin Medallist's pancreases and made comparisons with data collected during life. All pancreases studied showed insulin positive cells. However, in those diagnosed <8 years old (n=7) the majority of islets were atrophic with no insulin staining. In contrast, the remaining two medallists (onset aged 23 and 30) had a mix of insulin deficient and insulin positive islets. They also had both at least doubled their fasting C-peptide in response to a MMTT, indicating the potential advantage of older age of onset in maintaining endogenous insulin production. This study also demonstrated a notable correlation between C-peptide at MMTT and number of beta cells found post-mortem. When comparing Medallists with a random C-peptide ≥200pmol/l to individuals without diabetes, the Medallists had MMTT stimulated C-peptide 80-90% lower than individuals without diabetes. This difference was reflected in the proportion of functional beta cells found post-mortem when comparing the two groups. A similar relationship was seen in

those that had been unable to double their fasting C-peptide following MMTT, both their MMTT C-peptide level and number of functional beta cells were approximately 2-3% of controls without diabetes. The unique information provided by this cohort with extreme diabetes duration supports evidence that functioning beta cells can be preserved in long duration type 1 diabetes. (84)

2.5 There remains a deficiency in evidence of the clinical impact of preserved C-peptide in long duration type 1 diabetes

Evidence regarding the relationship between C-peptide and diabetes complications is limited by being almost exclusively from the DCCT and based on C-peptide relatively close to diagnosis. Lessons learnt from the DCCT undoubtedly reshaped the management of type 1 diabetes, definitively showing that intensive treatment was superior. However, the majority of evidence for the benefit of persistent C-peptide production comes from three analyses of the same data with a focus on short duration diabetes. Furthermore, the DCCT took place more than 30 years ago and since then management of diabetes, and particularly its complications, has improved drastically. Improvements in C-peptide measurement could allow replication of these findings in long duration diabetes or at even lower levels of C-peptide.

It has been established that persistent low levels of C-peptide are common in long duration type 1 diabetes. Currently there is very little evidence regarding the benefit of persistent C-peptide on complications in long-duration diabetes. This is despite long-term preservation of C-peptide being a key goal in intervention trials. This highlights a paucity of evidence and an area in need of future research.

Introduction part 3: Glucagon dysregulation in type 1 diabetes

3.1 Glucagon dysregulation in type 1 diabetes

Ordinarily alpha cells (α) secrete glucagon to work in conjunction with beta cells (β) to maintain glucose homeostasis and prevent hypoglycaemia. However, in type 1 diabetes there is evidence of glucagon dysregulation. Hypoglycaemic clamp studies show inadequate glucagon secretion during hypoglycaemia. (88) Whilst studies assessing glucagon following a meal have demonstrated inappropriate production. (89–92)

One possible explanation is that endogenous insulin regulates glucagon production, with falling endogenous insulin levels triggering glucagon release. This regulatory effect is lost in those with type 1 diabetes. (7) Glucagon production becomes reliant on glucose, with inappropriate secretion of glucagon in response to a rise in glucose (Fig. 6). Evidence supporting the regulatory role of endogenous insulin comes from declining C-peptide levels being associated with increasingly dysregulated glucagon. (89)

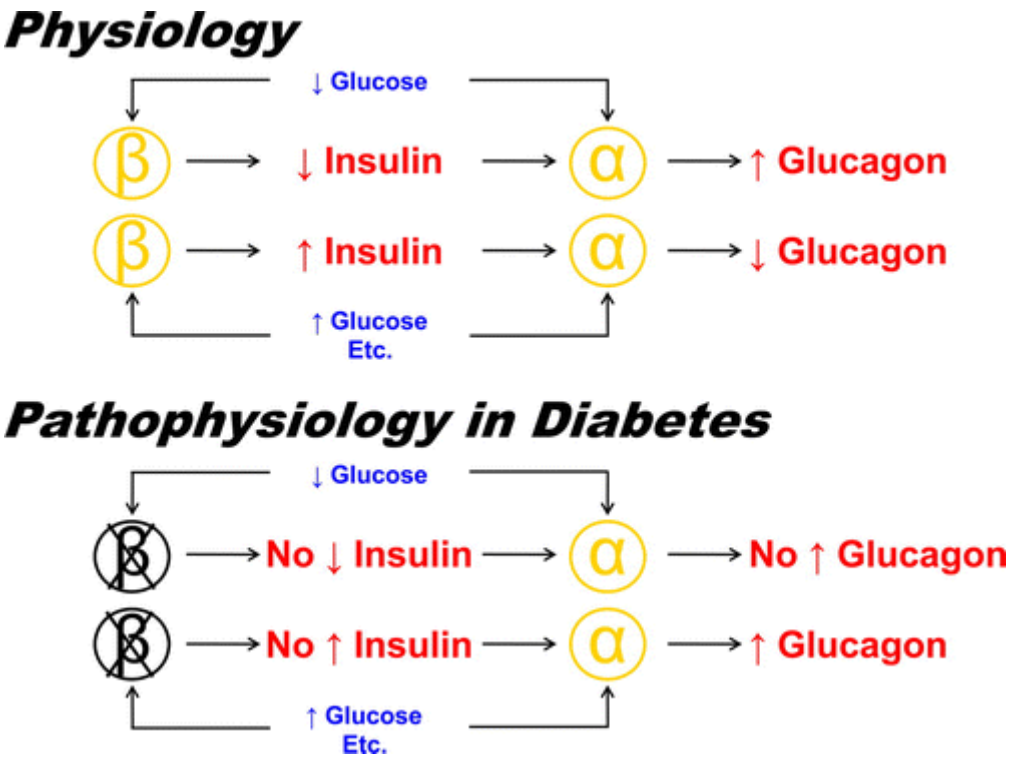


Fig.6: Physiology and pathophysiology in type 1 diabetes of alpha and beta cell function. Taken from Cryer 2011. (7)

Further evidence comes from islet cell transplantation studies using hypoglycaemic clamps. Prior to transplant glucagon levels during hypoglycaemia were comparable with those at euglycaemia. However, at both 6 and 18 months post-transplant, glucagon levels moderately increased in response to hypoglycaemia; although not to the level of controls without diabetes. This demonstrates that restoration of endogenous insulin production is associated with a striking improvement in glucagon regulation. However, these results must be interpreted with caution as transplanted α-cells are likely to influence glucagon levels. (93,94)

Currently research into the regulatory role of endogenous insulin is restricted to relatively few studies. Until recently the lack of a reliable glucagon assay remained a limiting factor. (95) Additionally, evidence for the relationship between glucagon and hypoglycaemia in type 1 diabetes has been limited to relatively few hypoglycaemic clamp studies, with nobody robustly investigating the relationship between meal stimulated glucagon and hypoglycaemia in long duration diabetes.

Chapter 2: Methods

The TIGI study

The TIGI (**T**ype 1 Diabetes, **I**mmunology, **G**enetics and endogenous **I**nsulin production) study is a cross-sectional case-control study carried out in the South West of England (NCT03490773). It aims to assess the genetic and immunological predictors of endogenous insulin production in a long duration type 1 diabetes cohort. All participants completed a MMTT and hypoglycaemia questionnaires were collected as a secondary outcome. While immune analyses continue, hypoglycaemia outcomes are able to be reported.

Study participants

Potential TIGI participants were identified from the existing UNITED (Using pharmacogeNetics to Improve Treatment in Early onset Diabetes) study cohort (NCT01238380). The UNITED study was a population-based study recruiting participants from the Tayside and Exeter areas. The UNITED study recruited individuals with type 1 diabetes diagnosed before the age of 30 who were under the age of 50 at recruitment. They recorded a meal stimulated urine C-peptide-creatinine ratio (UCPCR) in all participants. (53)

TIGI participants were selected from the UNITED study with the aim of forming high and low C-peptide groups. Potential participants had to have >5 years disease duration and be in the top or bottom quintile of UCPCR adjusted for their duration. Additional inclusion criteria were a clinical diagnosis of type 1 diabetes, insulin treatment from diagnosis, 5-65 years old at recruitment (inclusive) and ability to provide consent. Exclusion criteria included pregnancy or lactation, history of infectious illness within the previous 2 weeks, use of steroids or immunosuppression medications, having immunoglobulin treatments or blood products in the previous 3 months, any medical condition that would impact patient safety, recreational drug or alcohol abuse and severe diabetes complications.

Young onset type 2 diabetes and monogenic diabetes were robustly excluded from the TIGI cohort. All participants with UCPCR >0.2nmol/mmol underwent autoantibody testing (GAD and IA2). Those that were antibody negative with a BMI >30 kgm^{-2} were excluded to avoid inclusion of young onset type 2 diabetes.

Participants with monogenic diabetes were excluded by genetically testing potential participants with UCPCR >0.2nmol/mmol and negative antibodies, as previously described. (2,53,96)

All remaining 221 participants attended a single study visit where C-peptide status was confirmed using a MMTT. All TIGI participants provided informed consent and the National Research Ethics Service Committee South West approved the study (13/SW/0312).

Study visit

Most participants visited the National Institute for Health Research (NIHR) Exeter Clinical Research Facility (CRF). A study nurse visited participants at home if they could not attend the CRF. Baseline data was collected on all participants, this included height, weight, age of diagnosis, previous and current insulin treatment and family history of diabetes. Participants were also asked to complete a modified Clarke/Edinburgh hypoglycaemia questionnaire comprised of both the Clarke and Gold hypoglycaemia questionnaires. (97,98)

All participants completed a standard MMTT. (62) Participants ingested 160ml of Fortisip Compact, Nutricia, UK. This consists of 240kcal, 9.6g protein, 9.3g fat and 29.7g carbohydrate per 100ml. Participants attending the CRF completed a multiple time point MMTT with blood samples taken at 0, 30, 60, 90 and 120 minutes. Those who were visited at home and/or under the age of 16 had an abbreviated MMTT where samples were taken at 0 and 90 minutes.

Blood samples taken were analysed for peripheral blood mononuclear cells, DNA extraction, and serum C-peptide among other routine biomarkers including HbA1c. C-peptide and glucose were measured at all time points. Proinsulin and glucagon were measured both fasting and at 90 minutes.

C-peptide at 90 minutes following MMTT was used as the C-peptide measure for analysis. (62,69) For grouped analysis 90-minute C-peptide was used to confirm C-peptide group as either preserved (>20pmol/l) or low (<10pmol/l), to ensure participants remained in the pre-defined groups.

Key assay's

Both C-peptide and glucagon analyses were carried out at the Clinical Chemistry Department at the Royal Devon and Exeter NHS Foundation Trust, Exeter, UK.

C-peptide assay

Serum C-peptide was analysed on the Roche E170 analyser (Roche Diagnostics, Mannheim, Germany). The assay used a direct electrochemiluminescence immunoassay utilising mouse monoclonal anti-C-peptide antibody labelled with ruthenium and a second mouse monoclonal anti-C-peptide antibody coupled to paramagnetic particles. The limit of detection of the assay is 3.3pmol/l. C-peptide assays are standardised against The World Health Organisation International Standards 84/510. (99)

Glucagon assay

Glucagon was measured using the Mercodia (Mercodia, Uppsala, Sweden) Glucagon ELISA, measured on the Dynex D2 ELISA (Dynex, Lincoln, UK). The limit of detection for this assay is 1.5pmol/l.

Modified Clarke/Edinburgh hypoglycaemia questionnaire

Participants were asked to complete a modified Clarke/Edinburgh hypoglycaemia questionnaire, see appendix. This is comprised of Clarke's hypoglycaemia questionnaire with the addition of the Gold score for impaired hypoglycaemia awareness. (97,98,100) This was added to the TIGI study protocol after the initial recruitment phase, as such it is a secondary outcome available in a subset of TIGI study participants.

Clarke's hypoglycaemia questionnaire

Clarke's hypoglycaemia questionnaire can be used to assess both impaired hypoglycaemic awareness and rate of hypoglycaemia. It is a multiple choice questionnaire made up of 8 questions with a maximum score of 7. It asks about both moderate and severe hypoglycaemia, along with frequency of hypoglycaemia and perceived hypoglycaemic awareness. Each multiple choice answer is defined as either reduced awareness (1 point) or aware (0 points). Selection of a reduced awareness answer for questions 1-4 and 7-8 scores the participant a point. An additional point is gained when the answer to question 5 is less than the answer to question 6. A score ≥4 indicates reduced hypoglycaemic awareness and ≤2 indicates awareness, with a score of 3 a middle ground. (97,100)

Self-reported hypoglycaemia rate can also be calculated using Clarke's hypoglycaemia questionnaire using responses to questions 5 and 6. These questions ask how many times in the last month participants have had a blood glucose <3.5mmol/l with and without symptoms respectively. Monthly symptomatic and asymptomatic hypoglycaemia rates can then be estimated from the participants answer. "Never" is recorded as 0; "1-3 times" as 2; "1 time per week" as 4 (1x4); "2-3 times per week" as 10 (2.5x4); "4-5 times per week" as 18 (4.5x4); and "almost daily" as 25. Symptomatic and asymptomatic rates can be combined to provide an overall monthly rate of hypoglycaemia. (101)

The Clarke score estimated impaired hypoglycaemic awareness to be 26% in a type 1 diabetes cohort. This is consistent with other studies on prevalence of

impaired hypoglycaemic awareness. (102) Furthermore, its use has been validated using hypoglycaemic clamps, with 66.7% sensitivity and 85.7% specificity for impaired hypoglycaemia awareness. (103)

Gold score for impaired awareness of hypoglycaemia

The Gold score assesses hypoglycaemic awareness using a Likert scale (1-7). Asking participants score the extent to which they feel particular hypoglycaemic symptoms during a day time episode of hypoglycaemia, from “not present” to “present a great deal”. Symptoms are divided into autonomic, neuroglycopenic and non-specific. The questionnaire also asks participants to score their hypoglycaemic awareness (1-7), from “always aware” to “never aware”. Reduced awareness is defined as a total score ≥4. Reduced hypoglycaemic awareness as defined by the Gold score has been shown to be highly concordant with reduced awareness in the adult population. In addition, it correlates well with impaired hypoglycaemic awareness as defined by Clarke’s hypoglycaemia questionnaire (Spearman’s rho = 0.868, p=0.001). (98,100,102)

Chapter 3: Persistent C-peptide is associated with reduced hypoglycaemia but not HbA1c in patients with longstanding Type 1 diabetes: evidence for lack of intensive treatment in UK clinical practice?

Shannon M Marren, Suzanne Hammersley, Timothy J McDonald, Beverley M Shields, Bridget Knight, Anita Hill, Rob Bolt, Tim Tree, Bart Roep, Andrew T Hattersley, Angus G Jones, Richard A Oram, on behalf of the TIGI consortium

Acknowledgements of co-authors and contributions to paper

Richard Oram, Angus Jones, Timothy McDonald and Andrew Hattersley conceived of and designed the study with input from Bart Roep and Tim Tree. Suzanne Hammersley, Bridget Knight, Anita Hill, Rob Bolt and I collected data for the study. I carried out data analysis with assistance from Beverley Shields, Angus Jones, Andrew Hattersley and Richard Oram. I wrote the first draft of the manuscript. All authors reviewed and edited the final manuscript.

Abstract

Aims

Most people with type 1 diabetes have low levels of persistent endogenous insulin production. The Diabetes Control and Complications Trial showed that close to diagnosis preserved endogenous insulin was associated with lower HbA1c, hypoglycaemia and complication rates, when intensively treated. We aimed to assess the clinical impact of persistent C-peptide on rate of hypoglycaemia and HbA1c in those with long duration (>5years) type 1 diabetes.

Methods

We conducted a cross-sectional case-control study of 221 people with type 1 diabetes. We confirmed ongoing endogenous insulin secretion by measuring C-peptide after a mixed-meal tolerance-test. We compared self-reported hypoglycaemia, HbA1c, insulin dose and microvascular complications in those with preserved and low C-peptide.

Results

Stimulated median (IQR) C-peptide was 114pmol/l (43, 273) and <3pmol/l (<3, <3) in those with preserved and low C-peptide respectively. Participants with preserved C-peptide had lower reported monthly rates of hypoglycaemia, with 21% fewer symptomatic episodes, 5.9 vs 7.5 (incidence rate ratio (IRR) 0.79, p=0.001); and 65% fewer asymptomatic episodes, 1.0 vs 2.9 (IRR 0.35, p<0.001). Those with preserved C-peptide had a lower insulin dose (0.68 vs 0.81 units/kg, p=0.01) but similar HbA1c (preserved 69 vs low 67mmol/mol, p=0.06).

Conclusions

Patients with type 1 diabetes and preserved endogenous insulin production receiving usual care in the UK have lower daily insulin doses and fewer hypoglycaemic episodes, but no difference in HbA1c. This is consistent with non-intensive treatment in previous studies, and suggests a need for therapy intensification to gain full benefit of preserved endogenous insulin.

Introduction

Recent work has shown that many individuals with long duration type 1 diabetes continue to produce low levels of endogenous insulin, however, the clinical significance of this is uncertain. Beta cell function declines with increasing disease duration in type 1 diabetes and this was assumed to progress to total beta cell loss. (8) Recent studies have demonstrated persistent endogenous insulin in many people with long duration type 1 diabetes. (51–53,68,104) Whilst there is rapid initial decline post diagnosis, insulin secretion reaches a plateau after approximately 7 years post diagnosis. (2) Using highly sensitive C-peptide assays up to 80% of those with long duration type 1 diabetes (median duration 18 years) have been shown to have low level, detectable endogenous insulin secretion. (53) Furthermore, some people with long duration type 1 diabetes have surprisingly high levels of endogenous insulin, with 8-15% of people diagnosed in adulthood having either a serum C-peptide >200pmol/l or a urine C-peptide creatinine ratio of >0.2nmol/mmol. (53,104) The strongest clinical associations of C-peptide appear to be disease duration and age of diagnosis, with those diagnosed younger being much less likely to have persistent C-peptide. (53,68,104,105)

The Diabetes Control and Complications Trial (DCCT) and islet cell transplant studies have provided evidence for the clinical significance of persistent C-peptide. Grouped and continuous prospective analyses of the DCCT showed higher C-peptide levels were associated with lower insulin dose, improved glycaemic control, fewer microvascular complications and markedly lower rates of hypoglycaemia. (6,54,55) These findings were only seen in the intensively treated arm of the DCCT, where among intensively treated participants persistent postprandial blood C-peptide >200pmol/l was associated with a reduction in HbA1c and a 65% risk reduction in severe hypoglycaemia when compared to those with C-peptide <200pmol/l. (54) Findings from the DCCT highlight that the benefit of persistent C-peptide may arise from allowing tighter glucose control with intensive treatment through protection from hypoglycaemia. Additional data from Islet transplant recipients reveal that restoration of even partial beta cell function improves glycaemic control, variability and hypoglycaemic awareness along with reducing rates of hypoglycaemia. (77–79,81,106) The effects of improved beta cell function in islet transplantation

appear continuous and not linked to an absolute threshold, with hypoglycaemic episodes in particular often improving with minimal graft function. (79) These results are important evidence for international efforts to prevent or reverse beta cell loss. (107) While the DCCT provides clear evidence of benefit from preserved endogenous insulin secretion in an intensively treated trial setting, and studies of islet cell transplants show the clear benefit of restoring relatively large amounts of endogenous insulin secretion, the impact of preserved endogenous insulin in patients with longstanding diabetes receiving usual clinical care is unclear.

We aimed to assess the clinical impact of preserved endogenous insulin secretion, measured using C-peptide, in patients with long duration type 1 diabetes.

Methods

The TIGI (Type 1 diabetes, Immunology, Genetics and endogenous Insulin production) study is a cross sectional, observational case-control study of people with long duration type 1 diabetes in the UK. (2) We recruited participants from the cross sectional UNITED (Using pharmacogeNetics to Improve Treatment in Early onset Diabetes) study, a population based study of those diagnosed with diabetes before age 30 (and aged under 50 at recruitment). (53) Potential TIGI participants were selected on the basis of diabetes duration >5 years and being in either the top or bottom quintile of urinary C-peptide creatinine ratio (UCPCR) for their diabetes duration in UNITED(53). All patients included in TIGI had clinically defined type 1 diabetes diagnosed under the age of 30, were treated with insulin from diagnosis and lived in the South West of the UK. Those with renal impairment were excluded from the analysis as C-peptide is not a reliable measure of endogenous insulin production due to its renal excretion. (66) Potential participants with UCPCR >0.2nmol/mmol had GAD and IA2 autoantibody testing performed. If autoantibody testing was negative individuals were tested for monogenic diabetes as previously described, and were excluded if found to have monogenic diabetes. (96) To avoid inclusion of young onset type 2 diabetes, those with UCPCR >0.2nmol/mmol who were islet autoantibody negative were excluded if they had a BMI >30kg/m^2. 96% of participants were white British. All participants provided informed consent and the National Research Ethics Service Committee South West approved the study (13/SW/0312).

Confirmation of C-peptide status

C-peptide status was confirmed using a standard mixed-meal tolerance test (MMTT). This test was either performed at the Exeter National Institute for Health Research (NIHR) Clinical Research Facility (CRF), or at home where patients were visited by the study nurse. All participants fasted from midnight and did not take their morning insulin. Individuals were given a standard MMTT (Fortisip Compact, Nutricia, UK) consisting of 160ml containing per 100ml: 240kcal, 9.6g protein, 9.3g fat and 29.7g carbohydrate. Participants attending the CRF had a full multiple time point MMTT, with samples taken at 0, 30, 60, 90, and 120 minutes post meal. Participants visited at home had an abbreviated

single time point MMTT, with a blood sample taken at 90 minutes post meal. Serum C-peptide was analysed using a direct electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). We confirmed C-peptide group, preserved (>20pmol/l) or low (<10pmol/l) using serum C-peptide post MMTT to ensure participants remained in the pre-defined groups. 8 recruited participants were excluded from further analysis as a result of discordant C-peptide on mixed-meal tolerance testing. These participants all had high C-peptide in the UNITED study, but serum C-peptide <20pmol/l after MMTT.

Assessment of hypoglycaemia

Participants completed a modified Clarke's Hypoglycaemia Questionnaire to assess rate and awareness of hypoglycaemia at the time of MMTT. The questionnaire is comprised of eight multiple choice questions, with answers being scored as 0 (aware) or 1 (reduced awareness). The maximum score is 7 and a score ≥4 indicates reduced hypoglycaemic awareness. (97) Rates of hypoglycaemia were determined by response to questions 5 and 6, they record frequency of hypoglycaemic episodes in the last month (defined as blood glucose <3.5mmol/l) with and without symptoms during the episode respectively, as previously described. (101) Frequency of episodes was taken as a monthly average; those answering "1-3 episodes in the last month" were averaged to 2; "once a week" to 4 (1x4); "2-3 episodes per week" to 10 (2.5x4); "4-5 episodes per week" to 18 (4.5x4), and "almost daily" to 25.

Assessment of HbA1c and microvascular complications

HbA1c was measured at the study visit and a historic HbA1c mean calculated from a local laboratory records. With informed consent we collected historic glycaemic control data from a biochemistry laboratory download of all recorded samples over the preceding 12 years in participants from our local area. HbA1c was measured at this time using ion exchange chromatography HPLC on the TOSOH G8 Analyser (TOSOH Diagnostics, Tokyo, Japan) and standardised to IFCC. Historic HbA1c mean was calculated for each participant from all available results prior to recruitment (median (IQR) of 18 (12, 26) observations over 8 (5, 10) years).

For participants from our local area clinical data on microvascular complications was obtained from hospital laboratory and retinal screening records. These records were not available for participants whose general practice used the laboratory & retinal screening service of other regional hospitals. Retinopathy status was obtained from participant's most recent retinal screening record. The worst grade of retinopathy identified at the retinal screening visit prior to recruitment was recorded. Nephropathy status was defined according to whether an individual had ever had clinically defined microalbuminuria, as based on their biochemistry records. Microalbuminuria was defined as having 2 of 3 consecutive albumin-creatinine ratios high (>2.5mg/mmol for men and >3.5mg/mmol for women).

Statistical analysis

Analysis was carried out using *Stata Statistical Software: Release 14* (StataCorp, Tx, USA). Non-parametric statistical tests were used for analysis if on visual examination key continuous outcome variables, were non-normally distributed. Differences in clinical parameters were assessed using the Mann Whitney U Test. Differences in the severity of retinopathy (characterised as none, background, pre-proliferative or proliferative) were compared using a Kruskal-Wallis test. Hypoglycaemia rates were considered to follow a Poisson distribution. Therefore, results are displayed as rates and incidence rate ratios, with confidence intervals also in the Poisson distribution. Statistical significance was defined as $p<0.05$ for all statistical tests.

Results

70 participants with preserved C-peptide and 151 with low C-peptide were included in this analysis (characteristics presented in Table 1). Median (IQR) C-peptide was 114 pmol/l (43, 273) in the preserved C-peptide group, and <3 pmol/l (<3, <3) in the low C-peptide group. Whilst duration of diabetes was similar between the two groups (median 13 years in both groups, p=0.2), the preserved C-peptide group were diagnosed at an older age, 15 vs 6 years (p<0.0001).

Table 1 – *Cohort Characteristics. Values reported as n (%) or median (IQR).*

Characteristics	**Low C-peptide**	**Preserved C-peptide**	***p* value**
No. of participants	151	70	-
No. Male (%)	86 (57)	29 (41)	0.03
Age at diagnosis (years)	6.1 (3.0, 12.5)	15.1 (12.2, 22.0)	<0.0001
Age at recruitment (years)	19.9 (14.3, 36.5)	30.9 (20.8, 42.1)	0.0001
BMI (kg/m²)	23.3 (20.2, 26.5)	25.2 (23.3, 27.3)	0.0006
BMI standard deviation score	0.8 (0.1, 1.5)	1.0 (0.5, 1.6)	0.2
Duration of diabetes (years)	13.3 (8.5, 24.5)	12.6 (7.5, 22.0)	0.2
C-peptide (pmol/l)	<3 (<3, <3)	114 (43, 273)	<0.0001
HbA1c (mmol/mol)	67 (58, 76)	69 (62, 81)	0.6
HbA1c (%)	8.3 (7.5, 9.1)	8.5 (7.8, 9.6)	0.6
Insulin dose (U/kg in 24 h)	0.81 (0.67, 0.95)	0.68 (0.54, 0.94)	0.01
No. in hypoglycaemia rate analysis (%)	121 (80)	39 (56)	-
No. in historic HbA1c analysis (%)	86 (57)	67 (96)	-

Participants with preserved C-peptide had lower rates of hypoglycaemia.

Partial questionnaire data was available on 160 participants, with complete data on 151 (Table 1 and Table 2). The preserved C-peptide group had 21% fewer symptomatic episodes per month (IRR 0.79, CI 0.68-0.91, p=0.001), 5.9 vs 7.5 episodes/month; and 65% fewer asymptomatic episodes per month (IRR 0.35, CI 0.25-0.48, p<0.001), 1.0 vs 2.9 episodes/month (Fig. 1 and Fig. 2). There was no difference in Clarke score between the two groups (p=0.3), or proportion with a reduced hypoglycaemic awareness (score ≥4 out of 7, 16% vs 19% p=0.6; Fig. 3).

Table 2 – *Hypoglycaemia analysis cohort characteristics. Values reported as n (%) or median (IQR).*

Characteristics	**Low C-peptide**	**Preserved C-peptide**	***p* value**
No. of participants	121	39	-
No. Male (%)	69 (57)	17 (44)	0.1
Age at diagnosis (years)	5.7 (2.9, 10.8)	15.0 (12.2, 24.0)	<0.0001
Age at recruitment (years)	18.4 (13.5, 34.5)	29.3 (20.4, 43.0)	0.0004
BMI (kg/m²)	23.3 (19.6, 26.2)	24.5 (22.1, 27.4)	0.02
BMI standard deviation score	0.9 (0.1, 1.4)	0.8 (0.3, 1.5)	0.8
Duration of diabetes (years)	12.6 (8.3, 20.8)	12.1 (7.5, 21.4)	0.4
C-peptide (pmol/l)	<3 (<3, <3)	108 (37, 208)	<0.0001
HbA1c (mmol/mol)	67 (57, 75)	68 (59, 80)	0.4
HbA1c (%)	8.3 (7.4, 9.0)	8.4 (7.5, 9.5)	0.4
Insulin dose in 24 h (U/kg)	0.80 (0.67, 0.95)	0.71 (0.55, 0.94)	0.09
No. in hypoglycaemia awareness analysis	113	38	-

Fig. 1 – *Total monthly rate of hypoglycaemia by C-peptide group. Rates of aware (blue) and unaware (green) episodes with blood glucose <3.5mmol/l per month; derived from Clarke's hypoglycaemia questionnaire questions 5 and 6 respectively. ****p<0.0001, for low (n=118) vs preserved (n=39) C-peptide. Error bars represent 95% CI.*

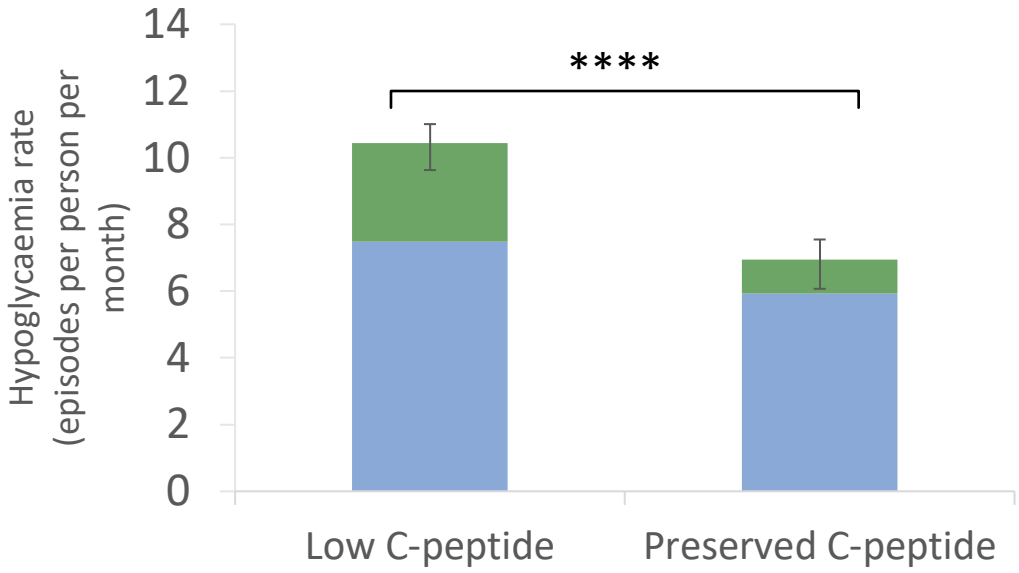


Fig. 2 – *Hypoglycaemia rates by C-peptide group. Number of hypoglycaemic (blood glucose <3.5mmol/l) episodes per month stratified by aware and unaware episodes. Rates derived from Clarkes questions 5 and 6. Low C-peptide group in blue (aware n=120, unaware n=119) and preserved C-peptide group in orange (n=39). ***p≤0.001 for low vs preserved C-peptide. Error bars represent 95% CI.*

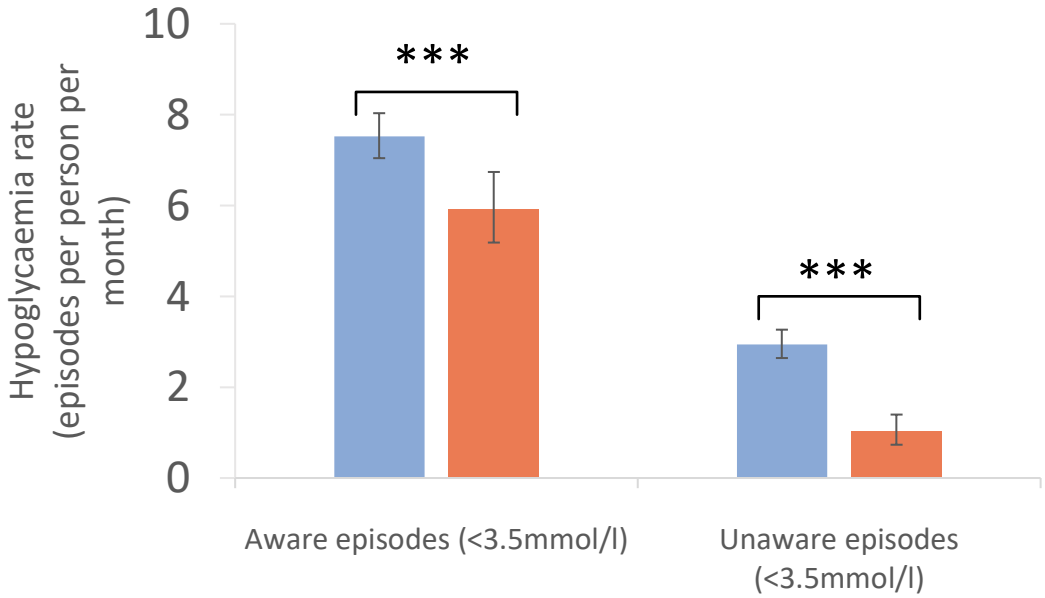
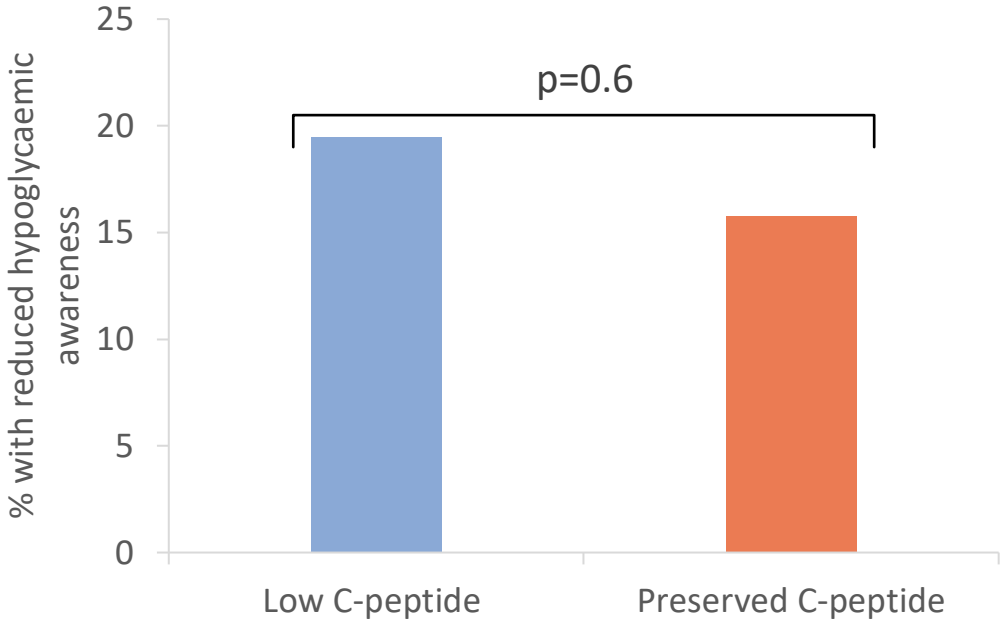


Fig. 3 – *Reduced hypoglycaemic awareness by C-peptide group. Proportion of the low (blue, n=113) or preserved (orange, n=38) C-peptide group with reduced hypoglycaemic awareness, defined as a Clarke score ≥4/7. Hypoglycaemic awareness was similar in both groups, p=0.6.*



Both the study visit HbA1c and historic HbA1c means were similar in both groups.

Participants in the preserved group had a marginal trend towards higher study visit HbA1c, 69 vs 67mmol/mol (8.5% vs 8.3%), p=0.06 (Fig. 4 and Fig. 5). Historic HbA1c mean was calculated in 153 participants (Table 1). The historic HbA1c mean was also similar in both groups, 71 vs 68mmol/mol (8.6% vs 8.4%) in those with high and low C-peptide respectively, p=0.4 (Fig. 6).

Fig. 4 – *Boxplot of study visit HbA1c by C-peptide group. Study visit HbA1c was similar in the low (n=148) and preserved (n=70) C-peptide groups p=0.06. Outliers are shown in Fig. 5.*

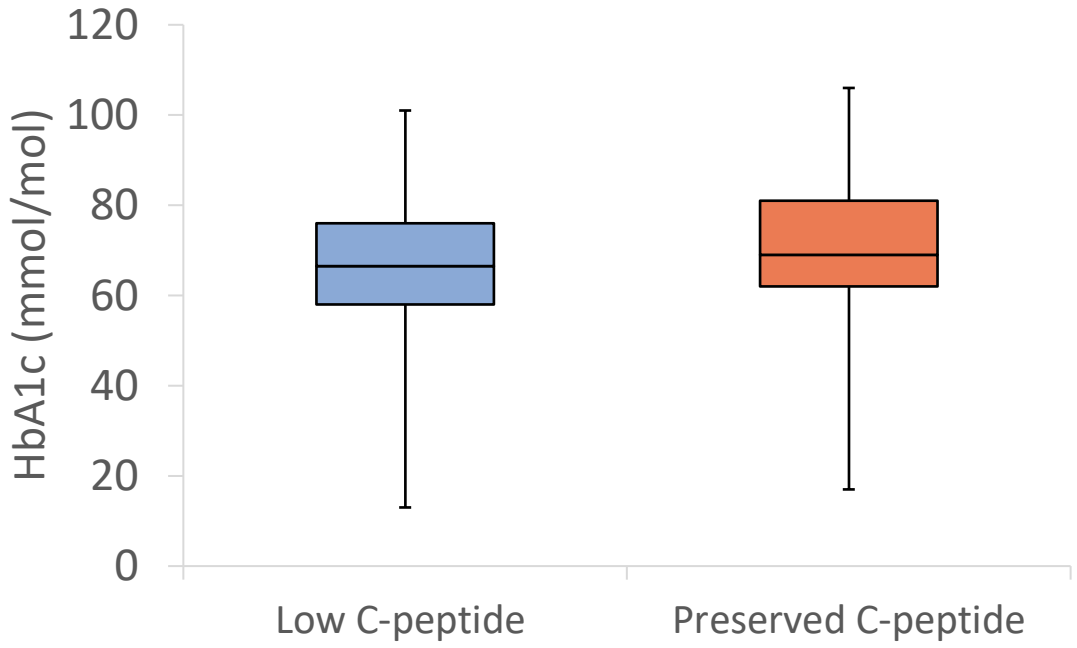


Fig. 5 – *Boxplot of study visit HbA1c by C-peptide group. Study visit HbA1c was similar in the low (n=148) and preserved (n=70) C-peptide groups p=0.06. Including outliers.*

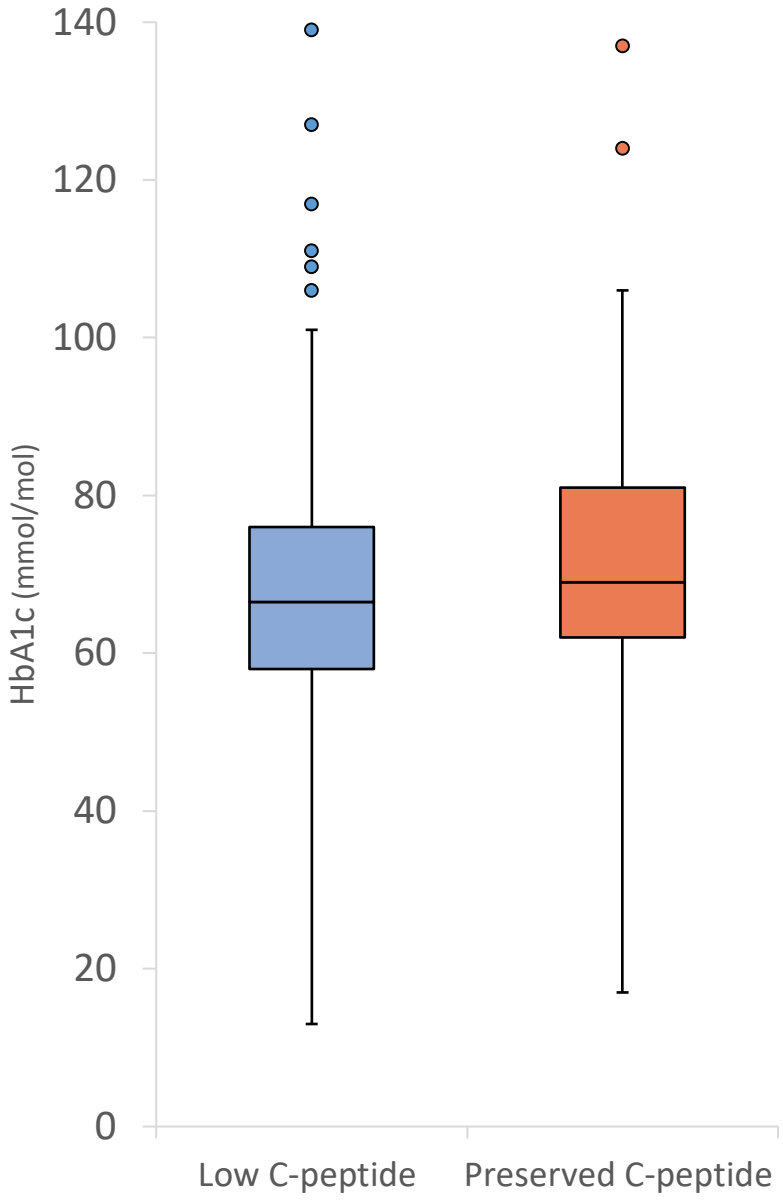
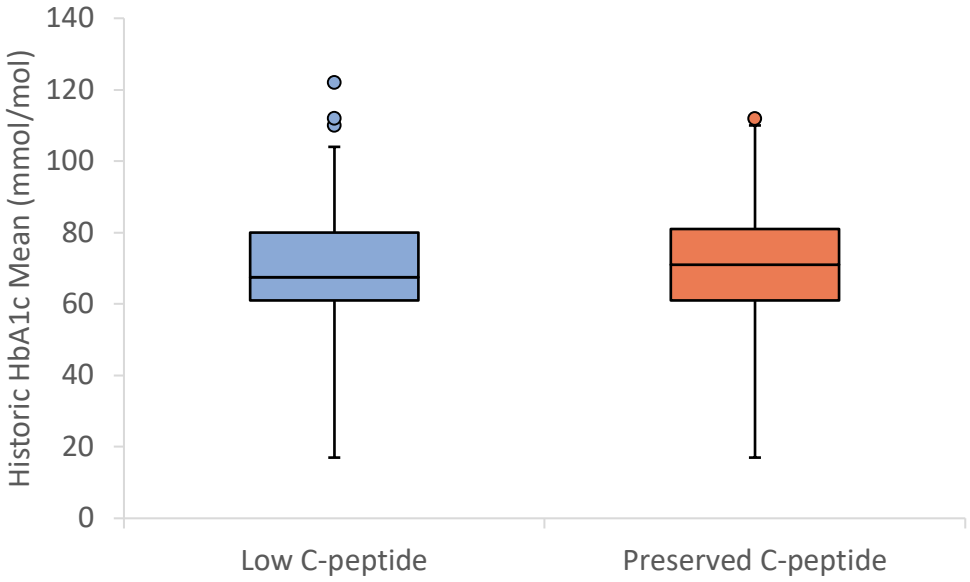
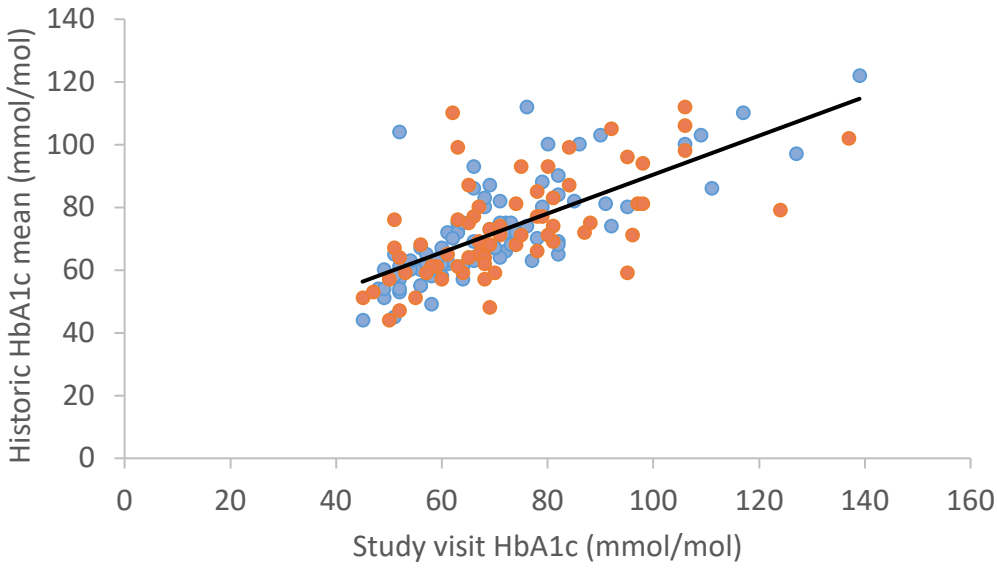


Fig. 6 - *Historic HbA1c mean analysis* ***(a)*** *Boxplot showing similar historic HbA1c mean in low (n=86) vs preserved (n=67) C-peptide group, p=0.4.* ***(b)*** *Scatter graph of study visit HbA1c and historic HbA1c mean (n=153). Spearman's rho = 0.72, p<0.0001. Low C-peptide group (n=86) shown in blue and preserved C-peptide group (n=67) in orange.* ***(c)*** *Boxplot showing similar study visit HbA1c (n=153) vs historic HbA1c mean (n=153), p=0.1.*

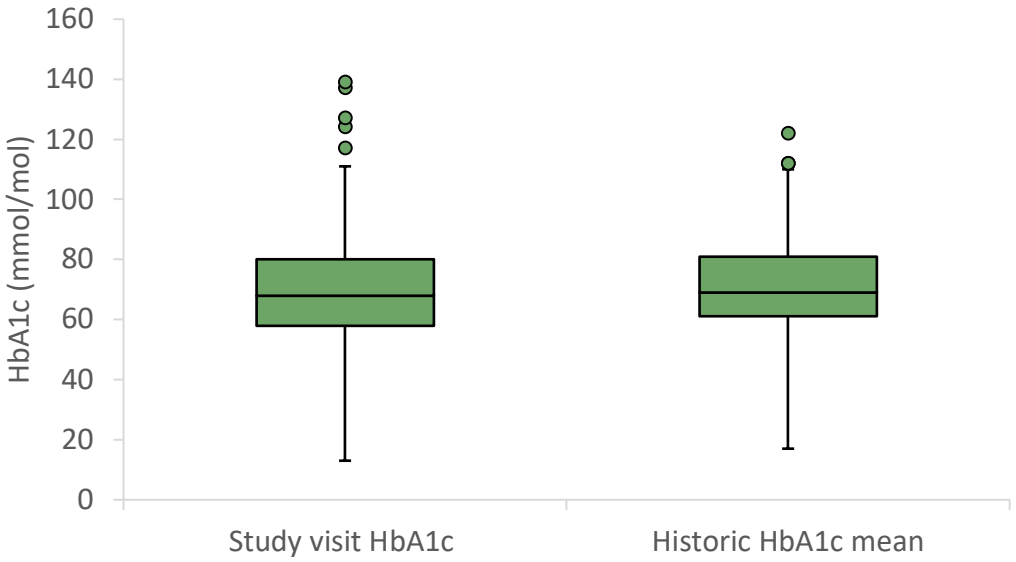
(a)



(b)



(c)



Insulin dose was substantially lower in those with preserved C-peptide production.

Participants with preserved C-peptide received a total daily dose of 0.68 units/kg (0.54, 0.94), whereas those with low C-peptide received 0.81 units/kg (0.67, 0.95), for comparison p=0.01 (Fig. 7 and Fig. 8).

Fig. 7 – *Boxplot of daily insulin dose by C-peptide group. Insulin dose was lower in the preserved C-peptide group (n=70) vs the low (n=151) group, p=0.01. Outliers are shown in Fig. 8.*

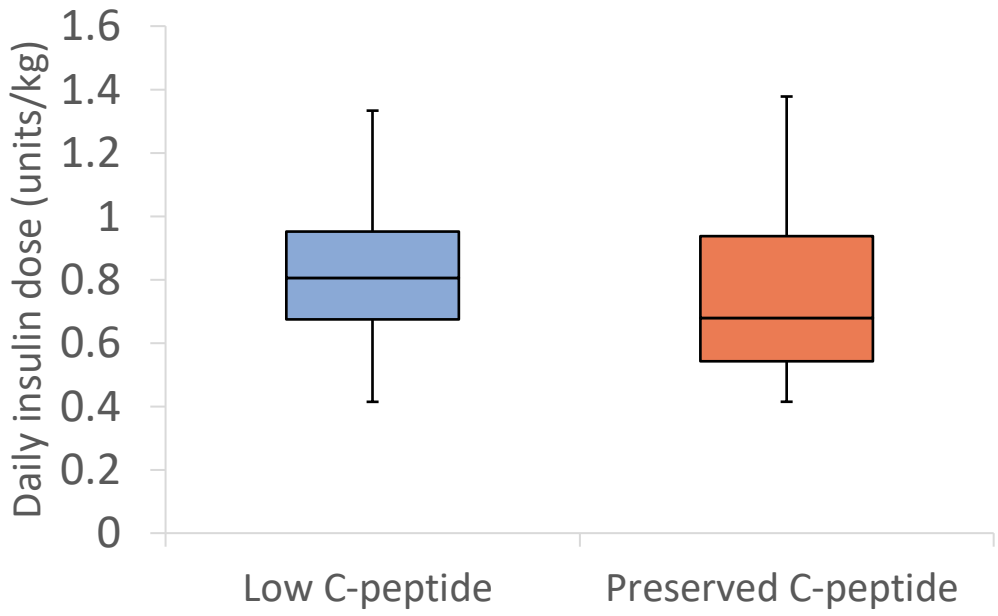
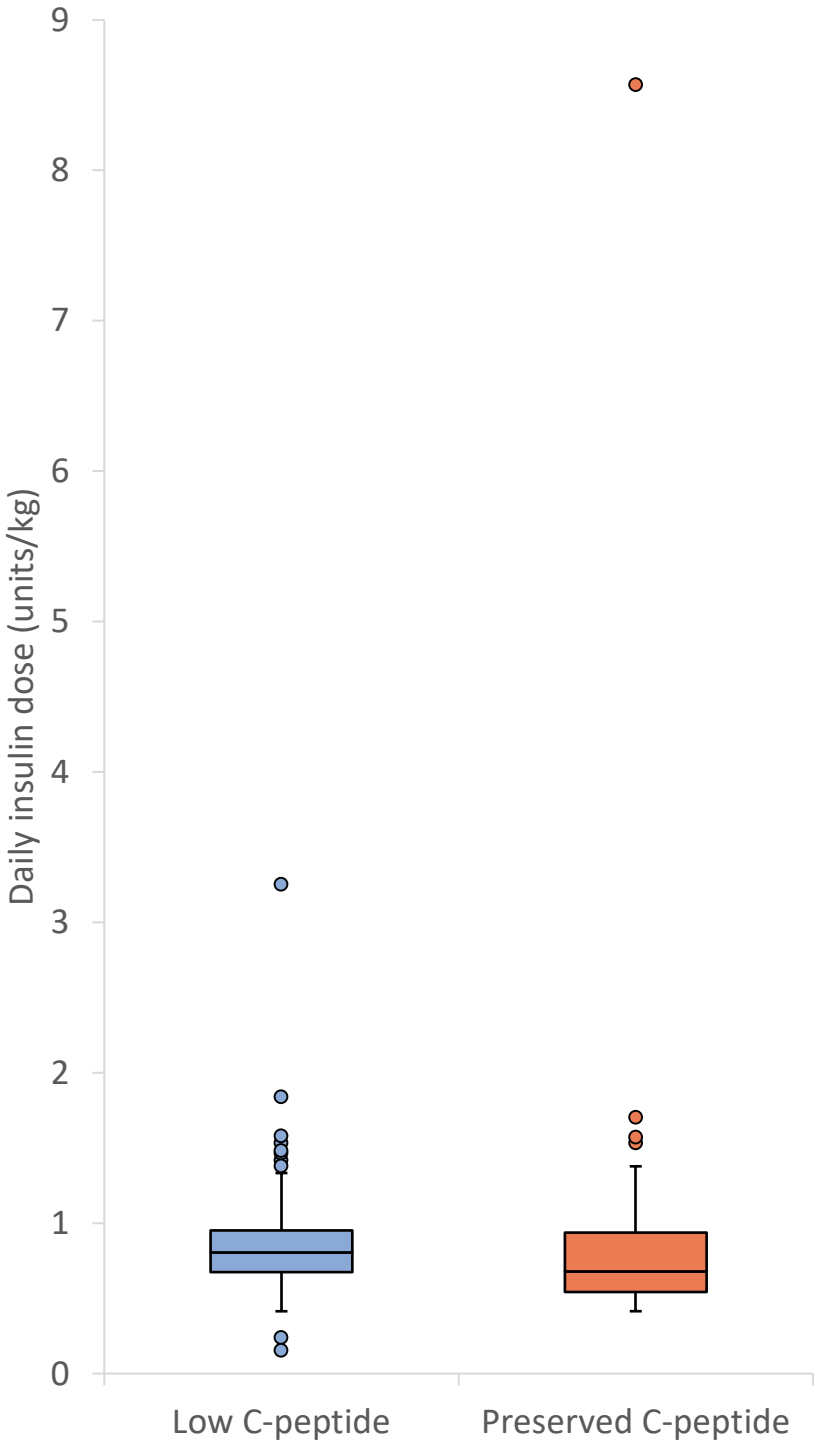


Fig. 8 – *Boxplot of daily insulin dose by C-peptide group. Insulin dose was lower in the preserved C-peptide group (n=70) vs the low (n=151) group, p=0.01. Including outliers.*



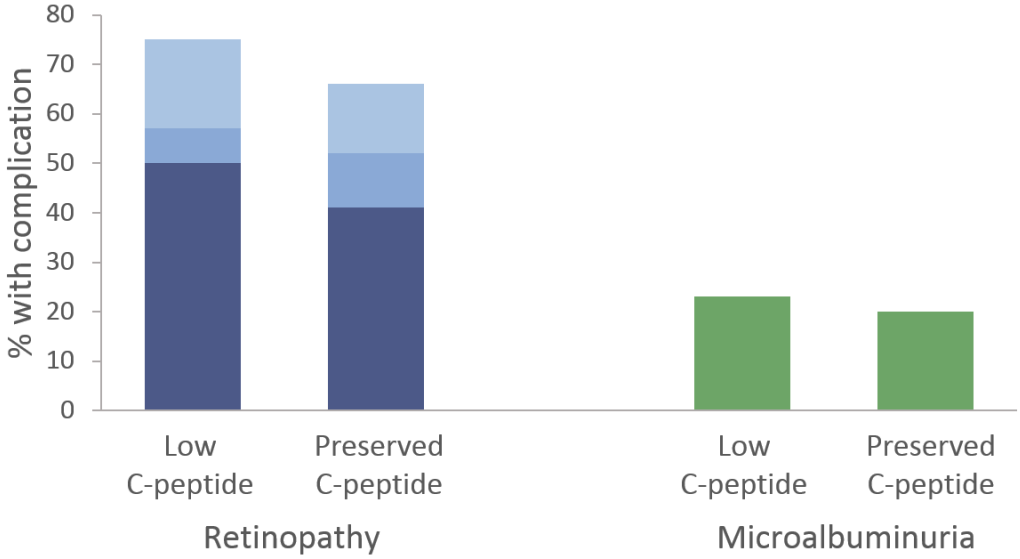
Preservation of endogenous insulin secretion was not associated with differences in retinopathy or microalbuminuria.

Retinopathy results were available on 130 participants (Table 3). Presence of retinopathy in the preserved C-peptide group was similar to the low group, 66% vs 74%, p=0.3 (Fig. 9). There was no difference in grades of retinopathy between the two groups (p=0.5).

Table 3 - *Complication analysis cohort characteristics. Values reported as n (%) or median (IQR).*

Characteristics	Low C-peptide	Preserved C-peptide	*p* value
No. of participants (%)	81	59	-
No. Male (%)	46 (57)	23 (39)	0.04
Age at diagnosis (years)	10.9 (5.2 15.2)	17.0 (12.5, 23.8)	<0.0001
Age at recruitment (years)	34.4 (23.2, 47.9)	34.1 (26.1, 43.6)	0.8
BMI (kg/m²)	24.5 (22.5, 27.2)	25.5 (22.5, 27.3)	0.6
BMI standard deviation score	1.0 (0.4, 1.5)	1.0 (0.3, 1.5)	0.9
Duration of diabetes (years)	21.3 (12.4, 37.8)	14.1 (7.8, 23.2)	0.001
C-peptide (pmol/l)	<3 (<3, 4)	135 (57, 314)	<0.0001
HbA1c (mmol/mol)	66 (58, 78)	69 (63, 81)	0.08
HbA1c (%)	8.2 (7.5, 9.3)	8.5 (7.9, 9.6)	0.08
Insulin dose in 24 h (U/kg)	0.75 (0.59, 0.88)	0.67 (0.54, 0.92)	0.2
No. in retinopathy analysis	74	56	-
No. in microalbuminuria analysis	69	51	-

Fig. 9 – *Rates of retinopathy and microalbuminuria by C-peptide group. Proportion of C-peptide group with retinopathy in blue; background in dark blue, pre-proliferative in medium blue, proliferative in light blue. Proportion with retinopathy was similar in the low (n=55/74) vs preserved C-peptide group (n=37/56), p=0.3. Proportion of C-peptide group with microalbuminuria (green); similar in low (n=16/69) and preserved (n=10/51) C-peptide groups, p=0.6.*



The prevalence of microalbuminuria was also similar in participants with high and low C-peptide. Records were available on 120 participants (Table 3). In these participants 20% and 23% of those with high and low C-peptide met study criteria for microalbuminuria, p=0.6 (Fig. 9).

Discussion

Our study showed that low levels of preserved C-peptide production in long duration type 1 diabetes in UK clinical practice were associated with reduced hypoglycaemia without improvement in HbA1c. Rates of both symptomatic and asymptomatic hypoglycaemia were reduced in the preserved C-peptide group, with similar hypoglycaemic awareness and without differences in either single measure or historic HbA1c mean. However, those with preserved C-peptide were treated with a lower exogenous insulin dose. Consistent with a lack of difference in HbA1c, levels of retinopathy and microalbuminuria were not different when examined in a subset of our study population.

These findings mirror the conventionally treated arm of the DCCT. This suggests a lack of intensive treatment in UK practice and highlights the challenges in achieving tight control outside the closely monitored clinical trial setting. The DCCT showed that where intensive diabetes treatment is given, higher levels of C-peptide are associated with markedly lower hypoglycaemia, HbA1c and microvascular complications even with low levels of secretion below 200pmol/l. However, benefits were much less marked where conventional therapy was given. (6,54,55) While this may be partly explained by more rapid loss of endogenous insulin secretion in non-intensively treated participants (55), this may also relate to reductions in hypoglycaemia risk associated with preserved C-peptide allowing intensification of treatment to a tighter level of glycaemic control. The limitation of achieving optimal glycaemic control with intensive treatment in type 1 diabetes is usually hypoglycaemia, which prevents up-titration of insulin doses. The reduced glucose variability and better hypoglycaemia counter regulation associated with preservation of endogenous insulin secretion (79,101,108,109) means that with intensive treatment a person with retained endogenous insulin secretion can obtain a lower HbA1c at an acceptable level of hypoglycaemia than would be possible where endogenous insulin is absent.

Our findings are consistent with previous research on the clinical impact of C-peptide. Hope et al. observed an approximate doubling in self-reported hypoglycaemia in those with type 1 diabetes with C-peptide <200pmol/l compared to >200pmol/l. This study focussed on patients diagnosed older with

group durations 25 vs 10 years respectively. (101) Kuhtreiber et al. also used the Clarke Score to assess hypoglycaemia. They categorised individuals as having mild, moderate or severe hypoglycaemia, showing that more severe hypoglycaemia was associated with lower levels of C-peptide. In addition, they found that higher C-peptide was associated with better glycaemic control and fewer complications. Their study had the benefit of a larger sample size and was thus better powered to assess a difference in complication rates. (105) Our data are also aligned with studies of islet cell transplant recipients. In this setting even minimal graft function, measured by C-peptide or beta score, correlates with reduced hypoglycaemia risk and improved glycaemic variability. (77–79,81,106) Vantyghem et al. showed that in islet cell transplant recipients partial beta cell function reduced rates of hypoglycaemia however improvements in glycaemic control and variability required significantly better graft function. (81) Combined these findings point toward endogenous insulin control playing a key role in preventing hypoglycaemia; perhaps directly by stopping secretion when blood glucose levels fall or indirectly through counter-regulatory hormones such as glucagon.

Strengths of our study include that we were able to utilise a highly sensitive C-peptide assay, allowing for identification and classification of C-peptide status at historically undetectable levels. We also robustly excluded individuals with both Type 2 and monogenic diabetes, ensuring that those with a high C-peptide truly had type 1 diabetes. Additionally, our recruitment process allowed the disease duration of both groups to be the same, removing a potential key confounder from this analysis. Our cohort were not part of a clinical intervention trial and received routine clinical care, making them reflective of current type 1 diabetes management in the UK, both strengthening our findings and making them relevant to routine care in the UK.

Our study was limited by self-reported hypoglycaemia and region restricted complication data reducing the power to assess a difference in complications. We used a validated hypoglycaemia questionnaire, however, this relied on participants both correctly identifying and recording hypoglycaemic episodes. It would be valuable to carry out continuous glucose monitoring on a cohort of similar patients, looking to remove participant bias and potentially validate our findings. In addition, we were only able to obtain data on complications on

participants based in the Exeter area, due to availability of medical records. This reduced our power to identify differing rates of retinopathy and microalbuminuria, so we could not rule out smaller differences that still may be clinically relevant. The sample size for our complication analysis provided 80% power (alpha 0.05), to detect a difference in proportions of 30% for both retinopathy and microalbuminuria, therefore meaningful differences in complications may not be detected with our limited sample size. A further limiting factor to this analysis was the selection criteria for our study, which excluded people with renal impairment, as C-peptide is renally excreted and therefore less reflective of endogenous beta cell function in those with impaired renal function. (66)

Notwithstanding these limitations, our study suggests a need to intensify treatment in those with persistent C-peptide. The DCCT demonstrated that the benefits of persistent C-peptide production could only be fully utilized when individuals received intensive therapy. Our study focused on those receiving routine clinical care and did not show the improvement of HbA1c associated with persistent C-peptide in the DCCT, with the benefit of maintained endogenous insulin secretion limited to lower insulin dose and less hypoglycaemia. A lack of impact on microvascular complications is therefore unsurprising considering >10 years of HbA1c records showed similar glycaemic control in both groups. We consider the most likely explanation for this finding to be a lack of intensive treatment, showing that factors other than hypoglycaemia limit achievement of HbA1c targets. Clinicians do not routinely test C-peptide and there are currently no guidelines to treat those with persistent C-peptide, who are protected from hypoglycaemia, more intensively. Therefore these patients may be considered to have acceptable glycaemic control in practice, when they would be able to achieve tighter glycaemia control and reduce risk of long term complications with more intensive treatment, without unacceptable hypoglycaemia. Targeted intensification of treatment in those with preserved C-peptide would therefore be a potential clinical strategy to improve control and an important area for future study.

Our study highlights the association of persistent high C-peptide with reduced hypoglycaemia. Additionally, it demonstrates that higher C-peptide does not always robustly associate with improved glycaemic control and reduced

complications rates. This may be a consequence of all patients being treated to the same glycaemic targets, irrespective of C-peptide production. The apparent under-treatment of those with preserved C-peptide production makes our assessment of any complication benefit difficult.

Chapter 4: Higher post meal glucagon is associated with lower hypoglycaemia risk, suggesting its potential to be used as a biomarker of hypoglycaemia risk.

Shannon M Marren, Suzanne Hammersley, Timothy J McDonald, Beverley M Shields, Bridget Knight, Anita Hill, Rob Bolt, Tim Tree, Bart Roep, Andrew T Hattersley, Angus G Jones, Richard A Oram, on behalf of the TIGI consortium

Acknowledgements of co-authors and contributions to paper

Richard Oram, Angus Jones, Timothy McDonald and Andrew Hattersley conceived of and designed the study with input from Bart Roep and Tim Tree. Suzanne Hammersley, Bridget Knight, Anita Hill, Rob Bolt and I collected data for the study. I carried out data analysis with assistance from Beverley Shields, Angus Jones, Andrew Hattersley and Richard Oram. I wrote the first draft of the manuscript with assistance from Richard Oram.

Abstract

Aims

Insulin induced hypoglycaemia remains a barrier to intensive diabetes treatment. There are currently no biomarkers used to assess hypoglycaemic risk. While glucagon protects from hypoglycaemia in health, there is significant glucagon dysregulation in type 1 diabetes. This study aims to assess the relationship between mixed-meal tolerance test (MMTT) stimulated glucagon and hypoglycaemia in long duration type 1 diabetes.

Methods

We assessed glucagon at 90 minutes following a MMTT in 133 participants with long duration (>5 years) type 1 diabetes. We investigated the relationship between stimulated glucagon and self-reported hypoglycaemia using Clarke's hypoglycaemia questionnaire in 72 participants.

Results

Median (IQR) glucagon at 90 minutes was 2.1pmol/l (<1.5, 3.9). Median (IQR) disease duration was 17.3 years (11.4, 30.2). In the majority of participants glucagon secretion increased in response to the MMTT (53%). Higher 90-minute glucagon was associated with a markedly lower reported rate of hypoglycaemia. Glucagon explained 11% of variation in hypoglycaemia rate and for a 1pmol/l increase total monthly hypoglycaemia rate was reduced by 23% (pseudo R^2=0.11, IRR 0.77, CI 0.72-0.81, p<0.0001). When used in combination stimulated glucagon, C-peptide and HbA1c explained 24% of variation in overall hypoglycaemia rate.

Conclusion

In this study MMTT stimulated glucagon was a significant predictor of hypoglycaemia rate, this was independent of HbA1c, C-peptide and disease duration. This highlights the potential for glucagon to be used as a biomarker of hypoglycaemia risk and its utility in individualising treatment strategy.

Introduction

Hypoglycaemia is a common, dangerous complication of insulin treatment in type 1 diabetes. 2-4% of deaths in people with type 1 diabetes are attributed to hypoglycaemia. (29) This potentially life threatening complication poses the largest barrier to achieving tight glycaemic control. (25,100) Management hinges on balancing hypoglycaemia risk and maintaining good glycaemic control so as to prevent the development of microvascular complications. As such, it is important to recognise individuals at high risk of hypoglycaemia. The Diabetes Control and Complications Trial (DCCT) showed the incidence of severe hypoglycaemia was three times higher in the intensively treated arm when compared to the conventionally treated. (50,74) Strict glycaemic control is a risk factor for hypoglycaemia, but the DCCT showed that HbA1c only explains 6-12% of variation in severe hypoglycaemia rate. (110,111) Other risk factors include increasing disease duration, increasing age and reduced hypoglycaemic awareness. (25,100) Reduction of hypoglycaemia risk relies upon patient education, more frequent monitoring of blood glucose with subsequent adjustment of insulin dose, and less intensive treatment with individualised blood glucose and HbA1c targets. Identification of individuals at high hypoglycaemic risk is key in improving type 1 diabetes management. (100) However, currently there are no biomarkers to predict those most at risk of hypoglycaemia.

There is very little data on the relationship between glucagon levels and hypoglycaemia in type 1 diabetes due to historic difficulties with glucagon assays. (95) Ordinarily glucose homeostasis is maintained by the opposing effects of alpha and beta cells, secreting glucagon and insulin respectively. Glucagon is supressed in response to increasing blood glucose and secreted in response to falling blood glucose so as to prevent hypoglycaemia. However, in type 1 diabetes there is significant glucagon dysregulation. (7,29)

Hypoglycaemic clamp studies have shown inadequate secretion in response to hypoglycaemia. (88) While other studies have shown inappropriate glucagon secretion in response to a meal. (89–92) In addition, glucagon response appears to become increasingly dysregulated with increasing disease duration and declining beta cell function. (88,89,112,113) However, there is no existing

literature robustly assessing meal stimulated glucagon in a cohort with long duration type 1 diabetes, a group at high risk of hypoglycaemia where glucagon regulation is key.

Our aim is to assess the relationship between glucagon secretion measured during a mixed-meal tolerance test (MMTT), and hypoglycaemia in long-duration type 1 diabetes. We will be using MMTT data from the TIGI (Type 1 diabetes Immunology, Genetics and endogenous Insulin production) study. The primary outcome of the TIGI study was a comparison of immune phenotype in people in high and low C-peptide defined groups. Hypoglycaemia questionnaire data was collected as a clinically relevant secondary outcome and whilst immune analyses are ongoing we are able to report hypoglycaemia outcomes.

Methods

Study participants

The TIGI study is a cross-sectional, observational case-control study in the UK. TIGI participants were recruited from the population study UNITED (Using pharmacogeNetics to Improve Treatment in Early onset Diabetes). In UNITED all participants had clinically diagnosed type 1 diabetes before the age of 30 and were under the age of 50 at the time of study recruitment. The UNITED study recorded a urine C-peptide-creatinine ratio (UCPCR) on all participants. (53)

Potential TIGI participants were then selected on the basis of having diabetes for >5 years and being in the top or bottom quintile of UCPCR for their disease duration. All TIGI participants were on insulin treatment from diagnosis and living in the South West of England. Young onset type 2 diabetes and monogenic diabetes were robustly excluded, as previously described. (2,53)

All participants provided informed consent and the study was approved by the National Research Ethics Service Committee South West (13/SW/0312).

Study visit

A sub-group of the TIGI cohort were visited by a study nurse at home. This analysis excludes those visited at home due to concerns regarding the stability of glucagon in transit. All other participants visited the Exeter National Institute for Health Research (NIHR) Clinical Research Facility (CRF) for a single study visit.

All participants attending the CRF completed a multiple time point MMTT, having fasted from midnight and not taken their morning insulin. Each participant was given 160ml of Fortisip Compact (Nutricia, UK), containing 240kcal, 9.6g protein, 9.3g fat and 29.7g carbohydrate per 100ml. Blood samples for C-peptide and glucose were taken at 0, 30, 60, 90 and 120 minutes. Blood samples were spun and stored at -80^{0}C prior to analysis. Glucagon was measured at 0 and 90 minutes. Serum C-peptide was analysed using a direct electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). Glucagon was analysed using the Mercodia Glucagon ELISA (Mercodia, Uppsala, Sweden) on the Dynex D2 ELISA (Dynex, Lincoln,

UK) robot platform, with a lower limit of detection on 1.5pmol/l. HbA1c was also measured, using ion exchange chromatography HPLC on the TOSOH G8 Analyser (TOSOH Diagnostics, Tokyo, Japan) and standardised to IFCC.

Hypoglycaemia analysis

Participants completed Clarke's Hypoglycaemia questionnaire at the study visit. This was a secondary outcome of the TIGI study and was added to the study protocol after some participants had been recruited. As such, questionnaires are only available on a subset of participants. Clarke's Hypoglycaemia questionnaire is a validated hypoglycaemia questionnaire used to assess both hypoglycaemic unawareness and rate of hypoglycaemia. (97,100,101,103) There is a total of 8 multiple choice questions, where answers are scored as zero for aware or one for reduced awareness, with a maximum score of 7. An individual with a score ≥4 is defined as having reduced hypoglycaemic awareness. Monthly rates of symptomatic and asymptomatic hypoglycaemia can be calculated using answers 5 and 6 respectively. Hypoglycaemia is defined as a blood glucose <3.5mmol/l. Participants are asked how many times in the last month their blood glucose has been <3.5mmol/l both with (question 5) and without (question 6) symptoms. Monthly frequency can then be estimated from the participant's answer, with "Never" being recorded as 0; "1-3 times" as 2; "1 time per week" as 4 (1x4); "2-3 times per week" as 10 (2.5x4); "4-5 times per week" as 18 (4.5x4); and "almost daily" estimated at 25. For this analysis we combined the symptomatic and asymptomatic rates to give a total monthly rate of hypoglycaemia. (97,101)

Statistical analysis

Statistical analyses were carried out using *Stata Statistical Software: Release 14* (StataCorp, Tx, USA). Statistical significance was set at $p<0.05$ for all statistical tests. Glucagon at 90 minutes post-meal was non-normally distributed both visually and using Shapiro Wilk testing ($p<0.00001$). Therefore, non-parametric tests were used throughout the analysis. Clinical variables were tested using Mann Whitney-U and Spearman's Rank tests. Hypoglycaemia rates were considered to follow a Poisson distribution; thus, results are presented as rates and incidence rate ratios (IRR) with confidence intervals also reported using a Poisson distribution.

Results

The characteristics of the 133 participants contributing to this analysis can be seen in Table 1. 94% of participants were white British. Glucagon at 90 minutes ranged from the lower limit of the assay (1.5 pmol/l) to 9.6pmol/l; with a median (IQR) of 2.1pmol/l (<1.5, 3.9). Median (IQR) disease duration was 17.3 years (11.4, 30.2).

Table 1 - *Cohort characteristics. Values reported as n (%) or median (IQR).*

	n (%) or median (IQR)
No. of participants	133
No. Male (%)	59 (44%)
Age at diagnosis (years)	13.5 (8.25, 19.75)
Age at recruitment (years)	34.14 (24.08, 45.09)
Duration of diabetes (years)	17.33 (11.44, 30.19)
BMI standard deviation score	0.96 (0.37, 1.52)
HbA1c (mmol/mol)	68 (59, 80)
Insulin dose (U/kg in 24 h)	0.71 (0.55, 0.85)
Glucagon at 90 mins (mmol/l)	2.1 (<1.5, 3.9)
C-peptide at 90 mins (pmol/l)	5 (<3, 94)
Glucose increment (mmol/l)	10 (8.7, 11.5)

Post-meal glucagon increased in the majority of participants.

Incremental glucagon from fasting to 90 minutes could be calculated in 122 participants. Median (IQR) glucagon increment was 0.3 pmol/l (0, 1.8). In the majority (65/122) glucagon secretion increased. In 18/122 participant's glucagon secretion decreased and in 39/122 participants glucagon secretion remained the same. All participants whose glucagon secretion remained the same had glucagon at the lower limit of the assay at both time points.

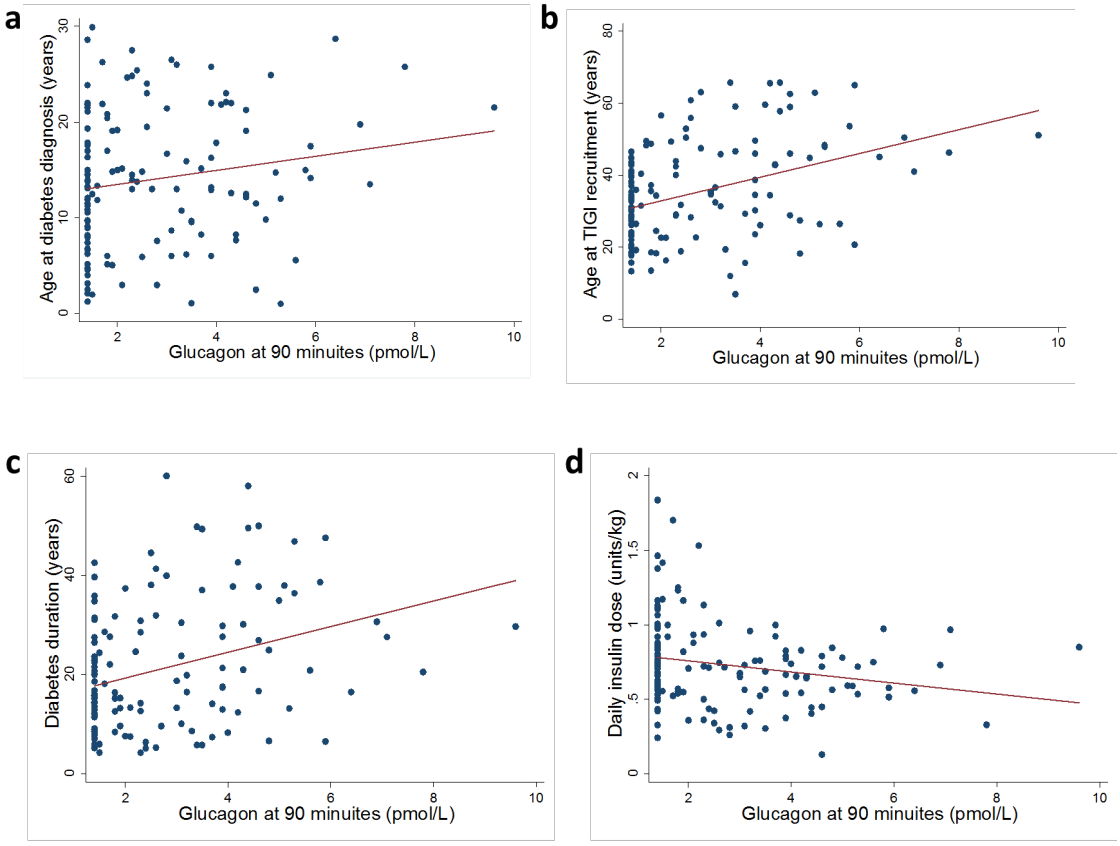
Higher 90-minute glucagon was associated with a reduced insulin dose, longer disease duration and older age at both diagnosis and recruitment, but not with C-peptide.

Analysis of glucagon at 90 minutes with key clinical characteristics was carried out in all 133 participants, with the exception of HbA1c in which one participant did not have HbA1c recorded.

Higher 90-minute glucagon was associated with older age at diabetes diagnosis, Spearman's rho = 0.18, p=0.04 (Fig. 1a); older age at recruitment to TIGI, Spearman's rho = 0.40, p<0.0001 (Fig. 1b); and longer disease duration, Spearman's rho = 0.31, p=0.0002 (Fig. 1c).

Higher glucagon at 90 minutes correlated with a lower daily insulin dose, Spearman's rho = -0.21, p=0.02 (Fig. 1d). There was no relationship between 90-minute glucagon and 90-minute C-peptide, p=0.2.

Fig. 1 – *Analysis of glucagon at 90 minutes* ***(a)*** *Scatter graph of age at diagnosis and 90-minute glucagon (n=133). Spearman's rho = 0.18, p=0.04.* ***(b)*** *Scatter graph of age at recruitment and 90-minute glucagon (n=133). Spearman's rho = 0.40, p<0.0001.* ***(c)*** *Scatter graph of disease duration and 90-minute glucagon (n=133). Spearman's rho = 0.31, p=0.0002.* ***(d)*** *Scatter graph of daily insulin dose and 90-minute glucagon (n=133). Spearman's rho = -0.21, p=0.02.*

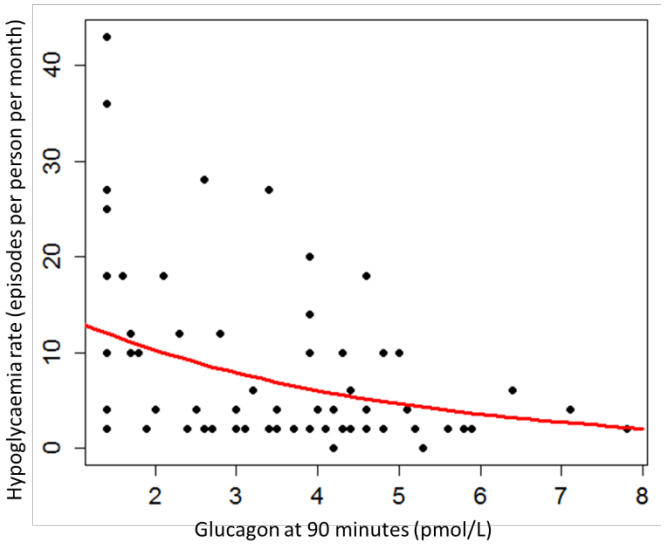


Higher 90-minute glucagon was associated with a markedly lower reported rate of hypoglycaemia.

Hypoglycaemia questionnaires were completed by 72 participants. Glucagon at 90 minutes was not associated with overall Clarke score (p=0.09) or hypoglycaemic unawareness (p=0.5).

Higher glucagon at 90 minutes was associated with a lower monthly rate of symptomatic, asymptomatic and total monthly rate of hypoglycaemia. For a 1pmol/l increase in stimulated glucagon, symptomatic hypoglycaemia rate decreased by 22% (IRR 0.78, CI 0.73-0.83, p<0.0001), asymptomatic by 33% (IRR 0.67, CI 0.56-0.80, p<0.0001) and total monthly hypoglycaemia rate by 23% (IRR 0.77, CI 0.72-0.81, p<0.0001) (Fig. 2).

Fig. 2 - *Scatter graph showing total monthly hypoglycaemia rate and glucagon at 90 minutes (n=72). Glucagon 90-minutes post-meal explains 11% of variation in total hypoglycaemia rate and a 1pmol/l increase reduces rate by 23% (IRR 0.77, CI 0.72-0.81, p<0.0001, pseudo R^2=0.11).*



Glucagon at 90 minutes in combination with C-peptide at 90 minutes and HbA1c can be used to explain 24% of variation in total hypoglycaemia rate.

Alone stimulated glucagon explains 11% of variation in total hypoglycaemia rate and a 1pmol/l increase reduces rate by 23% (IRR 0.77, CI 0.72-0.81, p<0.0001, pseudo R^2=0.11). 90-minute C-peptide when used alone explains 4% of variation in total rate of hypoglycaemia and a 1pmol/l increase reduces hypoglycaemia rate by 0.2% (IRR 0.998, CI 0.997-0.999, p<0.0001, pseudo R^2=0.04). HbA1c when used alone explains 3% of variation in hypoglycaemia rate and a 1mmol/mol increase reduces the risk of hypoglycaemia by 1% (IRR 0.99, CI 0.98-0.99, p<0.0001, pseudo R^2=0.03). When used in combination stimulated glucagon, C-peptide and HbA1c explain 24% of variation in total hypoglycaemia rate (Table 2).

Both disease duration and age at TIGI were only significant when used in the model alongside glucagon at 90 minutes and both then lost significance when used in conjunction with HbA1c. In contrast, age at diagnosis was significant when used in univariate analysis, however, lost significant when used in conjunction with glucagon and C-peptide. Similarly, glucose at 90 minutes was significant alone, however, when used in the model alongside glucagon at 90 minutes it was no longer significant.

Table 2 - *Poisson regression analysis of total monthly hypoglycaemia rate (n=71). Pseudo R^2=0.24*

Variable	IRR	Std. Error	z	p-value	95% CI	
Glucagon at 90 mins (pmol/l)	0.735	0.0335	-9.20	<0.0001	-0.3739	-0.2425
C-peptide at 90 mins (pmol/l)	0.998	0.0004	-6.41	<0.0001	-0.0032	-0.0017
HbA1c (mmol/mol)	0.979	0.0031	-6.87	<0.0001	-0.0278	-0.0154

Discussion

In people with longstanding type 1 diabetes, higher glucagon at 90 minutes was associated with a substantially lower rate of hypoglycaemia, this effect was independent of glucose at 90 minutes, disease duration, C-peptide and HbA1c. This suggests its potential as a tool for assessment of hypoglycaemia risk. In 53% (65/122) of participants glucagon inappropriately increased in response to a MMTT. Our findings support research demonstrating glucagon dysregulation in type 1 diabetes. Furthermore, they suggest that despite dysregulation, glucagon continues to play a key role in prevention of hypoglycaemia; with those that exhibit a higher glucagon 90 minutes post-meal having markedly lower rates of hypoglycaemia. Interestingly, glucagon was the biomarker that explained the most variance in rate of hypoglycaemia when used alone (11%). It is important to take into account the scales on which these biomarkers are measured when considering the impact of a single unit increase. Whilst glucagon was the biomarker with that provided the largest reduction in hypoglycaemia risk for a 1pmol/l increase (23%) (cohort range <1.5, 9.6), it was also the biomarker with the smallest range. In a multivariate Poisson regression model, C-peptide, HbA1c and glucagon were additive, with glucagon having the largest contribution to overall variation in hypoglycaemia rate. Up until now there has been very little research into the relationship between stimulated glucagon and hypoglycaemia. To our knowledge this is the first study to consider this relationship in long-duration type 1 diabetes, a cohort at high hypoglycaemic risk. Our study highlights the importance of future research into stimulated glucagon and its relationship with hypoglycaemia in type 1 diabetes.

Our study has both strengths and limitations. A strength of our study is that we assessed post-meal glucagon in a cohort with long-duration type 1 diabetes. Our cohort had a wide range of disease durations which allowed us to assess the effect of duration on glucagon dysregulation robustly. In addition, the study design meant that we recruited participants with both high and low levels of C-peptide for their duration. This enabled us to assess the effect of duration and C-peptide on glucagon separately. Furthermore, we were able to utilise a robust, well validated glucagon assay, this has previously been a barrier to glucagon studies. Another strength is that we assessed the association of post-

meal glucagon with hypoglycaemia. Most research has focused on intensive studies during hypoglycaemia, and it is not immediately intuitive to assess glucagon post meal when it would be expected to decrease. This is likely to be the reason why the association between MMTT stimulated glucagon and hypoglycaemia has not been recognised before, making this an important finding. Avoidance of hypoglycaemia is a key component of diabetes management, particularly for those with longer disease duration. As such understanding the role of glucagon in type 1 diabetes is important and has the potential to direct future management.

A limitation of the study was that monthly hypoglycaemia rate was not recorded in all participants. This was due to questionnaires not being collected at the beginning of the study period. Analysis comparing those with and without hypoglycaemia questionnaires showed the groups to be broadly similar, however, those not included in the hypoglycaemia analysis had higher HbA1c and C-peptide but lower glucagon (Table 3). A further limitation was the use of hypoglycaemia questionnaires to calculate hypoglycaemia rates. Questionnaires rely on participants to correctly recognise and recall hypoglycaemic events and the very nature of hypoglycaemia can make this difficult. It has been reported that individuals with type 1 diabetes self-report having almost half as many hypoglycaemic events as their closest cohabitant recalls. (114) Participants may not always be aware of hypoglycaemic episodes. This may be due to reduced hypoglycaemic awareness, which is a particular problem in long duration cohorts. Alternatively, this could be due to nocturnal hypoglycaemia, with almost 50% of hypoglycaemic events occurring during sleep. (25) As such, the questionnaire is likely to have vastly underestimated the true rate of hypoglycaemia. We additionally have considerable noise around the hypoglycaemia assessment, suggesting that the combined approach of glucagon, C-peptide and HbA1c may even be a stronger predictor of hypoglycaemia variation if hypoglycaemia was more precisely measured. Nonetheless, this makes our finding more remarkable as despite a noisy measure of hypoglycaemia its association with post-meal glucagon was striking.

Table 3 - *Comparison of those include in the hypoglycaemia analysis to the remaining cohort. Values reported as n (%) or median (IQR).*

	Hypoglycaemia analysis	Not in hypoglycaemia analysis	p-value
No. of participants	72	61	
No. Male (%)	31 (43%)	28 (46%)	0.7
Age at diagnosis (years)	13.4 (7.83, 21.33)	13.9 (9.75, 19.33)	0.9
Age at recruitment (years)	35.1 (23.46, 48.07)	31.3 (24.46, 41.18)	0.1
Duration of diabetes (years)	19.3 (12.59, 35.42)	15.3 (8.52, 27.52)	0.06
BMI standard deviation score	1.06 (0.51, 1.51)	0.86 (0.32, 1.54)	0.4
HbA1c (mmol/mol)	66 (56, 74)	71 (61, 82)	0.03
Insulin dose (U/kg in 24 h)	0.71 (0.55, 0.80)	0.73 (0.55, 0.98)	0.2
Glucagon at 90 mins (mmol/l)	3.0 (<1.5, 4.3)	1.5 (<1.5, 2.4)	0.0002
C-peptide at 90 mins (pmol/l)	<3 (<3, 58.5)	20 (5, 149)	<0.0001
Glucose increment (mmol/l)	10.15 (8.9, 12.2)	9.7 (8.45, 11.20)	0.3
Glucose at 90 mins (mmol/l)	21.2 (18.3, 24.5)	21.5 (18.4, 24.5)	0.9694

Glucagon is well established as an important counter-regulatory hormone protecting from hypoglycaemia in healthy people and people with diabetes. (7) Glucagon dysregulation is known, in intensive physiology studies of type 1 diabetes, to associate with less protection from hypoglycaemia. (93,94) It has been suggested that in healthy people endogenous insulin production regulates glucagon release, with falling levels stimulating glucagon secretion. In type 1 diabetes this regulatory effect is lost with declining beta cell function. Glucagon secretion becomes driven by a rise in blood glucose, resulting in inappropriate glucagon production post-meal and inadequate production in hypoglycaemia. (7) Simple measures of glucagon during stimulation tests have been less well

studied, presumably because glucagon is thought not to be relevant at this time. It has been established that glucagon inappropriately rises following a meal, with Sherr et al reporting that in individuals with type 1 diabetes glucagon levels post-MMTT were 1.5 times greater than when measured fasting. (90) In addition, Brown et al. followed 23 children for 1 year following diagnosis and carried out a MMTT every 3 months. They showed over the 12 months that as disease duration increased, stimulated C-peptide gradually declined by 45% and post-meal glucagon progressively increased by 37%. (89) However, very little is known about post-meal glucagon beyond its relationship with duration. To our knowledge this is the first study to focus on stimulated glucagon and its associations in long duration type 1 diabetes. Whilst we are unable to fully explain the relationship between higher post-meal glucagon and lower rate of hypoglycaemia it is nevertheless a key finding. Importantly, stimulated glucagon explained variation in hypoglycaemia rate better than any other variable, including duration. This highlights its potential utility as a biomarker of hypoglycaemia risk.

Interestingly, we did not see a relationship between C-peptide and 90-minute glucagon. This was also the case for Sherr et al. In contrast, Brown et al. demonstrated decreasing C-peptide was associated with an increasingly high glucagon response post-meal. (89,90) This may be due to having populations with vastly different disease durations. Alternatively it may be that a single measure was unable to detect the relationship between C-peptide and glucagon within an individual. (90) Recently Zenz et al. used hypoglycaemic clamps to compare individuals with detectable and un-detectable C-peptide. They showed that while both groups secreted glucagon in response to hypoglycaemia, levels were markedly higher in those with detectable C-peptide. Notably, median disease duration was markedly different between the C-peptide positive and negative participants (2.5 vs 23.9 years, $p<0.001$). (88) It is possible that the observed difference in glucagon response was due to differing duration rather than C-peptide level.

Glucagon at 90 minutes has the potential to be used as a biomarker of hypoglycaemia risk. Identifying individuals at risk of hypoglycaemia is a vital component of type 1 diabetes management. Intensive treatment, to minimise

development of microvascular complications, comes at the cost of increased risk of life-threatening hypoglycaemia. Currently there are no biomarkers for hypoglycaemia risk outside of HbA1c and the theoretical association of hypoglycaemia with C-peptide. Clinicians must use disease duration, hypoglycaemic awareness and history of hypoglycaemia to determine risk and decide how intensively to treat an individual. Accurately assessing and monitoring hypoglycaemia risk using a combined biomarker and clinical features approach would be beneficial in determining individualised treatment strategies. Our study shows that the combination of post-meal glucagon, C-peptide and HbA1c explains 24% of variation in hypoglycaemia rate. As a combined biomarker of hypoglycaemia risk, this would provide an invaluable tool for risk assessment. Clinicians could provide those with a lower risk with intensive treatment to prevent microvascular complications. In contrast those at higher risk might require more careful glucose monitoring and possibly a higher target HbA1c and blood glucose, to avoid hypoglycaemia. This would also highlight individuals who would benefit from hypoglycaemia education and more frequent blood glucose monitoring. Future research should further investigate the association between higher post-meal glucagon and reduced risk of hypoglycaemia, using continuous glucose monitoring to assess frequency of hypoglycaemia. In addition, other potential biomarkers, such as C-peptide, should be assessed for utility in determining risk of hypoglycaemia; with the view to provide a combined biomarker of hypoglycaemia risk to be used by clinicians when deciding individualised treatment strategies. It would also be useful to investigate the importance of glucagon being measured at 90 minutes following a standardised MMTT, when used as a potential biomarker of hypoglycaemic risk. Exploring the utility of both fasting and random glucagon measurements, alongside measurement after a non-standardised meal, would be beneficial when determining the most informative and efficient way to measure glucagon in clinical practice. In addition, our study highlighted the significance of glucagon dysregulation in type 1 diabetes and its importance in avoiding hypoglycaemia. Future work should further evaluate the inclusion of glucagon in combination with insulin in closed-loop systems. (115,116)

In conclusion, it is possible that the combined measurement of glucagon, C-peptide, HbA1c and gender may provide best prediction of hypoglycaemia risk and offer the possibility of a combined biomarker. Our study showed that higher post-meal glucagon was associated with reduced risk of hypoglycaemia. In combination with C-peptide, HbA1c and gender this explained 24% of variation in hypoglycaemia rate. The use of this combined biomarker to assess hypoglycaemia risk in clinical practice has the potential to individualise and improve type 1 diabetes management.

Chapter 5: Discussion

Chapter 5: Discussion

The work presented in this thesis demonstrates that preserved C-peptide production in long duration type 1 diabetes is associated with a reduction in hypoglycaemia. Whilst C-peptide appears to be beneficial in avoidance of hypoglycaemia we did not find any association with HbA1c or microvascular complication rates. During this analysis we also identified the potential for MMTT stimulated glucagon to be used as a biomarker of hypoglycaemia risk. Higher post-meal glucagon was associated with a lower rate of hypoglycaemia. This was independent of glucose, C-peptide and HbA1c. Furthermore, we demonstrated that when used in combination, stimulated glucagon, C-peptide and HbA1c can explain up to 24% of variance in hypoglycaemia rate.

This chapter provides a summary of the findings of this thesis and discusses the conclusions that have been drawn, implications, limitations and directions for future research.

Discussion of Chapter 3

Summary of findings

This chapter aimed to assess the impact of persistent low-level C-peptide production in long duration type 1 diabetes. Analysis was stratified by preserved (n=70) and low C-peptide group (n=151), as defined by 90-minute stimulated C-peptide adjusted for disease duration. We showed that preserved C-peptide was associated with markedly reduced rates of hypoglycaemia. Those with preserved C-peptide had 21% fewer symptomatic hypoglycaemic episodes per month (IRR 0.79, CI 0.68-0.91, p=0.001) and 65% fewer asymptomatic episodes (IRR 0.35, CI 0.25-0.48, p<0.001). However, there was no association between C-peptide group and HbA1c or prevalence of retinopathy or microalbuminuria. Interestingly, daily insulin dose was found to be lower in the preserved C-peptide group, 0.68 units/kg vs 0.81 units/kg (p=0.01).

Conclusions

Our key finding was that that higher C-peptide is associated with reduced levels of hypoglycaemia in long duration type 1 diabetes. Prior to this study little was known about the clinical impact of persistent C-peptide in long duration diabetes. Our findings are concordant with previous research assessing at individuals closer to diagnosis, the majority of which comes from the DCCT. However, we did not demonstrate the same relationship between C-peptide and reduced HbA1c and microvascular complications as seen in the DCCT. (6,54,55) There are a number of potential reasons for this. Firstly, it may be that our study was under powered, preventing us from detecting the difference in complication rates between the groups. The sample size for our complication analysis provided 80% power (alpha 0.05), to detect a difference in proportions of 30% for both retinopathy and microalbuminuria.

Secondly, it may be that the reduced HbA1c and complication rates in the DCCT were due to a duration effect. Grouped analysis completed by Steffes et al. showed that in the intensively treated individuals, persistent C-peptide reduced the incidence of retinopathy development, retinopathy progression, and development of nephropathy. However, their analysis was ultimately flawed, as duration was different between the groups. Those with undetectable C-peptide had a markedly longer disease duration when compared to the minimal,

baseline-only and sustained C-peptide groups (8.2 vs 4.5 vs 1.9 vs 2.3 years). (6) As such, shorter disease duration may explain the better HbA1c and complication rates in the higher and sustained C-peptide groups. This highlights the difficulty in disentangling the effect of duration from that of C-peptide, as ultimately the two factors are highly correlated.

A final and most likely explanation for our results comes with the additional finding that daily insulin dose was lower in those with preserved C-peptide. This could potentially explain why the benefits seen in the DCCT were not present in our cohort and suggests that those with preserved C-peptide production receiving routine clinical care may be undertreated. C-peptide is not routinely measured by clinicians and it is likely the persistent C-peptide status of participants was unknown. Currently all people with type 1 diabetes are treated to the same glycaemic targets. However, if those with persistent C-peptide were more intensively treated it is possible that we would see the HbA1c and complication benefits demonstrated by the DCCT.

Implications

Our findings raise interesting questions for diabetes management and have the potential to impact clinical practice. The apparent under-treatment of individuals with preserved C-peptide production may be preventing them from obtaining the HbA1c and complication benefits that their persistent C-peptide affords them. This demonstrates the importance of identifying individuals with preserved C-peptide production. Routine monitoring of C-peptide in clinical care would both identify such individuals and inform treatment strategy. This could be achieved easily using non-invasive UCPCR. UCPCR 120 minutes after a meal and MMTT 90-minute C-peptide are highly correlated but a single sample UCPCR is much more time and cost efficient. Furthermore, urinary C-peptide is stable for 72 hours in boric acid, allowing time for the test to be taken (in the clinical setting or at home) and the sample to be transported and analysed, making it a practical option. (53,72,73) Results could be used to individualise HbA1c and blood glucose targets. Those with preserved C-peptide production could receive more intensive treatment, with lower HbA1c and blood glucose targets, whilst maintaining their lower risk of hypoglycaemia. This may result in improvement in HbA1c and microvascular complication rates in this group.

Limitations

Our hypoglycaemia analysis was limited by use of self-reported hypoglycaemia questionnaire data. This relied upon participants accurately recognising and recalling hypoglycaemic episodes. This can be difficult due to the often-asymptomatic nature of hypoglycaemia; with impaired hypoglycaemic awareness being more common with increasing disease duration. Recording of asymptomatic episodes relies upon regular monitoring and recording of blood glucose. Furthermore, when present symptoms can be very unspecific, making hypoglycaemia difficult to recognise. Alternatively the symptoms themselves can make hypoglycaemia difficult to identify and remember, with both cognitive impairment and reduced consciousness being common. (25) Research has suggested that while severe hypoglycaemic events are typically recalled for up to a year, accurate recall of mild episodes is limited to one week. (117) A further difficultly with self-reported hypoglycaemia is that approximately 50% of hypoglycaemia occurs during sleep. (25) It is likely that the true frequency of hypoglycaemia was significantly higher than our self-reported data suggests. However, despite this noisy measure of hypoglycaemia, we found there to be a strong relationship with C-peptide. This association may be even stronger with more precise measurement of hypoglycaemia, such as continuous glucose monitoring (CGM).

Our study was cross-sectional. This prevented us from assessing the relationship between persistent C-peptide and first incidence of hypoglycaemia and complications; along with the impact of persistent C-peptide on progression of microvascular complications. A longitudinal study would allow for assessment of the effect of persistent C-peptide on these relationships. Grouped C-peptide analysis was also a limiting factor. This meant that we lost information, especially at the very low levels of persistent C-peptide within the low C-peptide group. It would be interesting to assess the impact of such low levels, which have historically been undetectable. However, such an analysis would require a remarkably large cohort.

A further limitation was the exclusion of people with renal failure. This was partly due to the recruitment process in which participants were selected from UNITED on the basis of their UCPCR. As C-peptide is renally excreted UCPCR is not a valid measure of endogenous insulin production in people with renal

impairment. (73) As such, exclusion criteria for the TIGI study included renal impairment. In addition, people with severe diabetic complications were excluded. This was because severe complications can impact T-cell function and the TIGI study was designed to investigate immunological factors impacting endogenous insulin production. Exclusion of people with severe complications means that we were not able to fully assess the impact of C-peptide on development of complications. It would be beneficial to assess the impact of persistent endogenous insulin production using alternative measures of beta cell function in people with more severe complications. However, it can often be difficult to engage individuals with poorer health status in clinical studies.

Our study suggests that under-treatment is responsible for similar HbA1c and complication rates in the two C-peptide groups. However, it is possible that there is a difference in complication rates that we were unable to detect due to a lack of power. The DCCT showed marked differences in complication rates between intensively treated responders and non-responders, such as a 50% reduced risk of retinopathy progression and 23% relative risk reduction for development of microalbuminuria. (54) However, complication rates have reduced since the DCCT highlighted the importance of intensive treatment and good glycaemic control. (48,49) As such, the difference in complications between our C-peptide groups is likely to be significantly smaller. We had 80% power (alpha 0.05), to detect a difference in proportions of 30% for both retinopathy and microalbuminuria. Our limited power was a result of having a relatively small cohort due to complication data only being available in a subset of study participants. Had we had a larger cohort, and thus better power, perhaps we would have identified a difference in complication rates.

Analysis was also restricted by assessment of retinopathy. We recorded the worst grade of retinopathy reported during the participant's most recent retinal screening appointment prior to recruitment. The diabetic retinal screening service grades retinopathy using a digital photograph of each retina following pupil dilation. (35,38) In contrast, the DCCT used seven-field stereoscopic fundus photographs taken every 6 months by certified photographers. All photographers used the Early Treatment Diabetic Retinopathy Study 25 step interim scale to grade retinopathy. Progression was defined as a three step change. (50) The method used in the DCCT was both more consistent and

reliable. In addition, it enabled precise assessment of complication progression which we were unable to do in the cross-sectional TIGI study.

Future research

The impact of persistent low-level C-peptide in long duration type 1 diabetes is an important area of clinical research. While numerous immunotherapy trials look to preserve or prolong C-peptide production, little is known about its long term effects. Our finding that preserved C-peptide production is associated with reduced risk of hypoglycaemia has important implications for clinical practice. It is important to validate our findings using a more robust measure of hypoglycaemia rate. Continuous glucose monitoring (CGM) will address the limitations of self-reported hypoglycaemia, removing participant bias and adding additional information such as glucose variability. It would be beneficial to carry out CGM across a range of C-peptide levels, looking to assess differences in incidence, severity and time spent in hypoglycaemia.

In addition, a larger prospective study assessing the impact of C-peptide on incidence and progression of microvascular complications would be highly informative. Evidence regarding the impact of preserved beta cell function mainly comes from the DCCT, however, ultimately this is limited by only focusing on individuals close to diagnosis and being carried out 30 years ago when complication rates were substantially higher. Assessment of the impact of C-peptide in long duration type 1 diabetes is needed, using the lessons learned from the DCCT regarding the importance of intensive treatment. This would require robust assessment and classification of hypoglycaemia and complications. Ideally follow-up should run routinely for a number of years, if not decades following diagnosis.

Discussion of Chapter 4

Summary of findings

During our analysis in Chapter 3 we identified that glucagon also predicted hypoglycaemia rate. This interesting finding prompted the analysis reported in chapter 3 which is discussed here. We investigated MMTT stimulated glucagon in the 133 TIGI participants that visited the CRF. Incremental glucagon was recorded in 122 participants and in response to the MMTT glucagon increased in 53% of participants (65/122), decreased in 15% (18/122) and plateaued in 32% (39/122). Higher 90-minute glucagon was also associated with older age at diagnosis ($p=0.04$), older age at recruitment ($p<0.0001$) and increasing disease duration ($p=0.0002$). While there was no association with 90 minute C-peptide ($p=0.2$) higher glucagon was associated with reduced insulin dose ($p=0.02$).

Hypoglycaemia analysis showed that higher glucagon at 90 minutes was associated with a significantly lower rate of hypoglycaemia. 11% of variation in total hypoglycaemia rate was explained by glucagon at 90 minutes alone, with a 1pmol/l increase reducing rate by 23% (IRR 0.77, CI 0.72-0.81, $p<0.0001$, pseudo $R^2=0.11$). Furthermore, when used in combination with C-peptide and HbA1c these biomarkers explained 24% of hypoglycaemic variance.

Conclusions

Our findings highlight the potential utility of MMTT stimulated glucagon as a biomarker of hypoglycaemia risk. Importantly, the relationship between glucagon and hypoglycaemia rate was independent of glucose, HbA1c and C-peptide. In addition, it was more predictive of hypoglycaemia rate than any of these measures. Whilst this is what we would expect in the hypoglycaemic setting, it was interesting that inappropriate secretion of glucagon in response to a meal explained so much variation in hypoglycaemia rate. Moreover, in combination with other biomarkers (C-peptide and HbA1c) a substantial proportion of variance in hypoglycaemia rate was explained. This suggests a role for a combined biomarker of hypoglycaemic risk which could have important implications for clinical practice.

Furthermore, unlike previous studies we did not identify an association between stimulated glucagon and C-peptide. It may be that this was due to the longer duration of our cohort. Brown et al. demonstrated that following diagnosis

stimulated C-peptide falls while glucagon rises, however, they only studied participants for their first year following diagnosis. (89) In contrast, Sherr et al. also followed individuals with >2 years duration and found no relationship between stimulated C-peptide and glucagon. (90)

Implications

Hypoglycaemia poses a significant barrier to management of type 1 diabetes. Intensive insulin treatment was demonstrated by the DCCT to significantly improve glycaemic control and reduce the development and progression of microvascular complications. (50,75) However, insulin induced hypoglycaemia remains a barrier to achieving strict glycaemic control. (29) There are currently no biomarkers used to predict hypoglycaemia risk, however, meal stimulated glucagon has the potential to do so. This would provide an invaluable tool clinicians could use to identify individuals at higher risk of hypoglycaemia, and subsequently individualise treatment strategy. Those at higher risk may require less intensive treatment, with higher blood glucose and HbA1c targets, to avoid hypoglycaemia. Furthermore, use of a combined biomarker, explaining even more variation in hypoglycaemia rate, would strengthen this tool.

Limitations

As discussed in the discussion for Chapter 3 our study is limited by its use of self-reported hypoglycaemia questionnaire data. However, despite this less precise measure, hypoglycaemia still displayed a striking association with MMTT stimulated glucagon.

A further limitation of this analysis is the stability of glucagon. Historically there have been problems with both sensitivity and specificity in glucagon assays. However, recent development of a sandwich ELISA used to identify intact glucagon through antibodies to a specific combination of N- and C- terminal epitopes has overcome this barrier. (95) While we were able to utilise the Mercodia glucagon ELISA there were concerns regarding degradation of glucagon. For this reason we did not include TIGI participants who were visited at home as we could not be certain how long samples took to arrive at the laboratory. Whilst samples collected at the CRF were rapidly spun and then stored at -80^{0}C prior to analysis, there was still a degree of uncertainty regarding the amount of degradation. While it is important to take account of

this it makes our findings more remarkable. Despite potential degradation, the association between higher MMTT stimulated glucagon and lower rate of hypoglycaemia was highly significant and explained a substantial amount of variation in hypoglycaemia rate both alone (11%) and in combination with C-peptide and HbA1C (24%).

A final limitation is the cohort size for the hypoglycaemia analysis, n=72. Unfortunately, hypoglycaemia questionnaires were not given to the participants recruited to the study in its early stages. While analysis showed that those with and without hypoglycaemia questionnaires were broadly similar, this significantly reduced the sample size.

Future research

Validating the association between glucagon and hypoglycaemia in a large cohort would add strength to our findings. We plan to analyse data from other studies where participants have both MMTT data and hypoglycaemia questionnaires, with the view to establish the utility of MMTT stimulated glucagon as a biomarker of hypoglycaemia risk.

In order to overcome the limitations of self-reported hypoglycaemia data a CGM study would be useful, as outlined in the discussion for chapter 3. This would be highly valuable in validating the use of MMTT stimulated glucagon as a biomarker of hypoglycaemia risk. Looking at C-peptide and HbA1c in the same cohort would further this research. Looking toward the development of a tool in which, these biomarkers are combined to assess hypoglycaemic risk in clinical practice and inform treatment strategy.

Further investigation is also needed into the reason for post-meal glucagon being a good marker of hypoglycaemic risk. In health glucagon is secreted in response to falling blood glucose levels and is supressed following a meal. Glucagon dysfunction has been repeatedly reported in type 1 diabetes, with inappropriate secretion following meal stimulation being demonstrated. (7,89,90) However, we are unable to fully explain why a higher MMTT stimulated glucagon is associated with reduced hypoglycaemia. This is counterintuitive as glucagon's role in protection from hypoglycaemia occurs at low blood glucose levels, not post meal. It would be logical to assume that with increasing glucagon dysregulation, such as a heightened response following a

meal, hypoglycaemia would become more common. We found this was not the case, but the opposite, higher post-meal glucagon was associated with reduced rate of hypoglycaemia. As such, hypoglycaemic clamp studies would be helpful in beginning to understand the physiology behind this association in people with a range of MMTT glucagon stimulated responses. Ideally this would be combined with CGM to assess glucose variation along with measures of hypoglycaemia.

It would also be interesting to study whether the association of glucagon with hypoglycaemia remains in those with undetectable beta cell function. The relationship between C-peptide and glucagon in type 1 diabetes is not fully understood. It has been suggested that in health glucagon secretion is mediated by endogenous insulin production, whereas, in type 1 diabetes glucagon secretion becomes reliant on blood glucose level. (7) Therefore, investigating glucagon post-meal and using hypoglycaemic clamps in people with very little or no C-peptide production could further explain this relationship. Cohorts in which this could be investigated include those selected to receive islet cell transplant and people impaired hypoglycaemic awareness recruited to HypoCOMPaSS. Our study has also highlighted the potential importance of including glucagon with insulin in closed-system pumps, an area of ongoing research. (115,116)

Overall thesis conclusions

Recent research has shown that people with long duration type 1 diabetes continue to secrete C-peptide at low levels for many years. (51–53) However, very little is known about the impact of persistent C-peptide in long duration type 1 diabetes. The findings from this thesis demonstrate that persistent C-peptide in long duration diabetes is associated with a reduced rate of hypoglycaemia. Furthermore, during this analysis we also identified that MMTT stimulated glucagon has the potential to be used as a biomarker of hypoglycaemic risk. With higher stimulated glucagon being associated with a reduced rate of hypoglycaemia.

Together these findings have the potential to change clinical practice, allowing identification of individuals at low and high hypoglycaemic risk. Currently there are no biomarkers used to assess risk of hypoglycaemia. We have shown that when used in combination, glucagon, C-peptide and HbA1c explain 24% of variance in hypoglycaemic rate. Using the combination of these biomarkers to evaluate hypoglycaemic risk would be an invaluable tool for clinical practice. Targeted intensification of treatment in people with low hypoglycaemic risk could potentially improve HbA1c and reduce microvascular complication rates within this group.

Despite the limitations associated with self-reported hypoglycaemia, both C-peptide and glucagon demonstrated strong associations with hypoglycaemia rate. Future research should look to validate these associations with a more precise measure of hypoglycaemia, such as continuous glucose monitoring. Perhaps the combination of C-peptide, glucagon and HbA1c would be an even stronger predictor of hypoglycaemia risk if hypoglycaemia was measured more precisely.

References

1. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. Lancet Lond Engl. 2014 Jan 4;383(9911):69–82.

2. Shields BM, McDonald TJ, Oram R, Hill A, Hudson M, Leete P, et al. C-Peptide Decline in Type 1 Diabetes Has Two Phases: An Initial Exponential Fall and a Subsequent Stable Phase. Diabetes Care. 2018 Jun 7;41(7):1486–92.

3. National Institute for Health and Care Excellence. Chronic kidney disease in adults: assessment and management. NICE guideline (CG182) [Internet]. [cited 2018 Jun 25]. Available from: https://www.nice.org.uk/guidance/cg182

4. Clark PM. Assays for insulin, proinsulin(s) and C-peptide. Ann Clin Biochem. 1999 Sep;36 (Pt 5):541–64.

5. Temple R, Clark PMS, Hales CN. Measurement of Insulin Secretion in Type 2 Diabetes: Problems and Pitfalls. Diabet Med. 2009 Jul 30;9(6):503–12.

6. Steffes MW, Sibley S, Jackson M, Thomas W. Beta-cell function and the development of diabetes-related complications in the diabetes control and complications trial. Diabetes Care. 2003 Mar;26(3):832–6.

7. Cryer PE. Minireview: Glucagon in the pathogenesis of hypoglycemia and hyperglycemia in diabetes. Endocrinology. 2012 Mar;153(3):1039–48.

8. Eisenbarth GS. Type I diabetes mellitus. A chronic autoimmune disease. N Engl J Med. 1986 May 22;314(21):1360–8.

9. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature. 2010 Apr 29;464(7293):1293–300.

10. Ziegler AG, Rewers M, Simell O, Simell T, Lempainen J, Steck A, et al. Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. JAMA. 2013 Jun 19;309(23):2473–9.

11. DiMeglio LA, Evans-Molina C, Oram RA. Type 1 diabetes. The Lancet. 2018 Jun 16;391(10138):2449–62.

12. VanBuecken D, Lord S, Greenbaum CJ. Changing the Course of Disease in Type 1 Diabetes. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, et al., editors. Endotext [Internet]. South

Dartmouth (MA): MDText.com, Inc.; 2000 [cited 2017 Nov 28]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK326738/

13. Bach J-F. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med. 2002 Sep 19;347(12):911–20.

14. Todd JA. Etiology of type 1 diabetes. Immunity. 2010 Apr 23;32(4):457–67.

15. Patterson CC, Dahlquist GG, Gyürüs E, Green A, Soltész G, EURODIAB Study Group. Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. Lancet Lond Engl. 2009 Jun 13;373(9680):2027–33.

16. Maahs DM, West NA, Lawrence JM, Mayer-Davis EJ. Epidemiology of type 1 diabetes. Endocrinol Metab Clin North Am. 2010 Sep;39(3):481–97.

17. Thomas NJ, Jones SE, Weedon MN, Shields BM, Oram RA, Hattersley AT. Frequency and phenotype of type 1 diabetes in the first six decades of life: a cross-sectional, genetically stratified survival analysis from UK Biobank. Lancet Diabetes Endocrinol. 2017 Nov 30;6(2):122–9.

18. Livingstone SJ, Levin D, Looker HC, Lindsay RS, Wild SH, Joss N, et al. Estimated Life Expectancy in a Scottish Cohort With Type 1 Diabetes, 2008-2010. JAMA. 2015 Jan 6;313(1):37–44.

19. Diabetes UK. Facts and Stats [Internet]. Diabetes UK; 2016. Available from: https://diabetes-resources-production.s3-eu-west-1.amazonaws.com/diabetes-storage/migration/pdf/DiabetesUK_Facts_Stats_Oct16.pdf

20. National Institute for Health and Care Excellence. Type 1 diabetes in adults: diagnosis and management NICE guideline (NG17) [Internet]. [cited 2018 Jun 25]. Available from: https://www.nice.org.uk/guidance/ng17

21. Misra S, Oliver NS. Diabetic ketoacidosis in adults. BMJ. 2015 Oct 28;351:h5660.

22. Perilli G, Saraceni C, Daniels MN, Ahmad A. Diabetic Ketoacidosis: A Review and Update. Curr Emerg Hosp Med Rep. 2013 Mar 1;1(1):10–7.

23. Farsani SF, Brodovicz K, Soleymanlou N, Marquard J, Wissinger E, Maiese BA. Incidence and prevalence of diabetic ketoacidosis (DKA) among adults with type 1 diabetes mellitus (T1D): a systematic literature review. BMJ Open. 2017 Jul 1;7(7):e016587.

24. Hepburn DA, Deary IJ, Frier BM, Patrick AW, Quinn JD, Fisher BM. Symptoms of acute insulin-induced hypoglycemia in humans with and without IDDM. Factor-analysis approach. Diabetes Care. 1991 Nov;14(11):949–57.

25. Frier BM. Hypoglycaemia in diabetes mellitus: epidemiology and clinical implications. Nat Rev Endocrinol. 2014 Dec;10(12):711–22.

26. Cryer PE. Severe Hypoglycemia Predicts Mortality in Diabetes. Diabetes Care. 2012 Sep 1;35(9):1814–6.

27. McCoy RG, Van Houten HK, Ziegenfuss JY, Shah ND, Wermers RA, Smith SA. Increased mortality of patients with diabetes reporting severe hypoglycemia. Diabetes Care. 2012 Sep;35(9):1897–901.

28. Cryer PE. The Barrier of Hypoglycemia in Diabetes. Diabetes. 2008 Dec;57(12):3169–76.

29. Cryer P. Hypoglycaemia: The limiting factor in the glycaemic management of Type I and Type II Diabetes*. Diabetologia. 2002 Jun 1;45(7):937–48.

30. Fong DS, Aiello LP, Ferris FL, Klein R. Diabetic Retinopathy. Diabetes Care. 2004 Oct 1;27(10):2540–53.

31. Mathur R, Bhaskaran K, Edwards E, Lee H, Chaturvedi N, Smeeth L, et al. Population trends in the 10-year incidence and prevalence of diabetic retinopathy in the UK: a cohort study in the Clinical Practice Research Datalink 2004–2014. BMJ Open. 2017 Feb 1;7(2):e014444.

32. Fowler MJ. Microvascular and Macrovascular Complications of Diabetes. Clin Diabetes. 2011 Jul 1;29(3):116–22.

33. Evans JR, Michelessi M, Virgili G. Laser photocoagulation for proliferative diabetic retinopathy. In: The Cochrane Library [Internet]. John Wiley & Sons, Ltd; 2014 [cited 2018 Jun 27]. Available from: http://cochranelibrary-wiley.com/doi/10.1002/14651858.CD011234.pub2/full

34. Donaldson M, Dodson PM. Medical treatment of diabetic retinopathy. Eye. 2003 Jul;17(5):550–62.

35. Public Health England. NHS Diabetic Eye Screening Programme: Information for health professionals [Internet]. 2016. Available from: https://www.hct.nhs.uk/media/1227/diabetic-eye-screening-information-sheet-for-healthcare-professionals.pdf

36. Harding S, Greenwood R, Aldington S, Gibson J, Owens D, Taylor R, et al. Grading and disease management in national screening for diabetic retinopathy in England and Wales. Diabet Med J Br Diabet Assoc. 2003 Dec;20(12):965–71.

37. Shotliff K, Duncan G. Diabetic retinopathy: summary of grading and management criteria. Pract Diabetes Int. 23(9):418–20.

38. Public Health England. NHS Diabetic Eye Screening Programme: Grading definitions for referable disease [Internet]. Public Health England; 2017. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/582710/Grading_definitions_for_referrable_disease_2017_new_110117.pdf

39. Thomas RL, Dunstan FD, Luzio SD, Chowdhury SR, North RV, Hale SL, et al. Prevalence of diabetic retinopathy within a national diabetic retinopathy screening service. Br J Ophthalmol. 2015 Jan 1;99(1):64–8.

40. Gross JL, Azevedo MJ de, Silveiro SP, Canani LH, Caramori ML, Zelmanovitz T. Diabetic Nephropathy: Diagnosis, Prevention, and Treatment. Diabetes Care. 2005 Jan 1;28(1):164–76.

41. Robertson LM, Waugh N, Robertson A. Protein restriction for diabetic renal disease. In: The Cochrane Library [Internet]. John Wiley & Sons, Ltd; 2007 [cited 2018 Jun 29]. Available from: http://cochranelibrary-wiley.com/doi/10.1002/14651858.CD002181.pub2/full

42. Caramori ML, Fioretto P, Mauer M. The need for early predictors of diabetic nephropathy risk: is albumin excretion rate sufficient? Diabetes. 2000 Sep 1;49(9):1399–408.

43. Krolewski AS. Progressive Renal Decline: The New Paradigm of Diabetic Nephropathy in Type 1 Diabetes. Diabetes Care. 2015 Jun 1;38(6):954–62.

44. Ruospo M, Saglimbene VM, Palmer SC, De Cosmo S, Pacilli A, Lamacchia O, et al. Glucose targets for preventing diabetic kidney disease and its progression. In: The Cochrane Library [Internet]. John Wiley & Sons, Ltd; 2017 [cited 2018 Jul 22]. Available from: http://cochranelibrary-wiley.com/doi/10.1002/14651858.CD010137.pub2/full

45. National Institute for Health and Care Excellence. Chronic kidney disease (partial update). Clinical Guideline 182. [Internet]. NICE; 2014. Available from: https://www.nice.org.uk/guidance/cg182/evidence/full-guideline-pdf-191905165

46. NICE-The National Institute for Health and Care Excellence. BNF: British National Formulary - Angiotensin Converting Enzyme Inhibitors [Internet]. [cited 2018 Oct 8]. Available from: https://bnf.nice.org.uk/drug-class/angiotensin-converting-enzyme-inhibitors.html#pregnancy

47. Callaghan BC, Little AA, Feldman EL, Hughes RA. Enhanced glucose control for preventing and treating diabetic neuropathy. In: The Cochrane Library [Internet]. John Wiley & Sons, Ltd; 2012 [cited 2018 Jun 28]. Available from: http://cochranelibrary-wiley.com/doi/10.1002/14651858.CD007543.pub2/full

48. Fullerton B, Jeitler K, Seitz M, Horvath K, Berghold A, Siebenhofer A. Intensive glucose control versus conventional glucose control for type 1 diabetes mellitus. In: The Cochrane Library [Internet]. John Wiley & Sons, Ltd; 2014 [cited 2018 Jul 15]. Available from: http://cochranelibrary-wiley.com/doi/10.1002/14651858.CD009122.pub2/full

49. Lachin JM, Orchard TJ, Nathan DM, for the DCCT/EDIC Research Group. Update on Cardiovascular Outcomes at 30 Years of the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study. Diabetes Care. 2014 Jan;37(1):39–43.

50. Diabetes Control and Complications Trial Research Group, Nathan DM, Genuth S, Lachin J, Cleary P, Crofford O, et al. The effect of intensive treatment

of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med. 1993 30;329(14):977–86.

51. Wang L, Lovejoy NF, Faustman DL. Persistence of Prolonged C-peptide Production in Type 1 Diabetes as Measured With an Ultrasensitive C-peptide Assay. Diabetes Care. 2012 Mar 1;35(3):465–70.

52. Oram RA, Jones AG, Besser REJ, Knight BA, Shields BM, Brown RJ, et al. The majority of patients with long-duration type 1 diabetes are insulin microsecretors and have functioning beta cells. Diabetologia. 2014 Jan;57(1):187–91.

53. Oram RA, McDonald TJ, Shields BM, Hudson MM, Shepherd MH, Hammersley S, et al. Most People With Long-Duration Type 1 Diabetes in a Large Population-Based Study Are Insulin Microsecretors. Diabetes Care. 2015 Feb;38(2):323–8.

54. DCCT. Effect of intensive therapy on residual beta-cell function in patients with type 1 diabetes in the diabetes control and complications trial. A randomized, controlled trial. The Diabetes Control and Complications Trial Research Group. Ann Intern Med. 1998 Apr 1;128(7):517–23.

55. Lachin JM, McGee P, Palmer JP, DCCT/EDIC Research Group. Impact of C-peptide preservation on metabolic and clinical outcomes in the Diabetes Control and Complications Trial. Diabetes. 2014 Feb;63(2):739–48.

56. Skyler JS. Prevention and Reversal of Type 1 Diabetes—Past Challenges and Future Opportunities. Diabetes Care. 2015 Jun 1;38(6):997–1007.

57. VanBuecken DE, Greenbaum CJ. Residual C-peptide in type 1 diabetes: what do we really know?: Residual C-peptide in T1D. Pediatr Diabetes. 2014 Mar;15(2):84–90.

58. Liu M, Wright J, Guo H, Xiong Y, Arvan P. Chapter Two - Proinsulin Entry and Transit Through the Endoplasmic Reticulum in Pancreatic Beta Cells. In: Litwack G, editor. Vitamins & Hormones [Internet]. Academic Press; 2014 [cited 2018 Jul 22]. p. 35–62. (The Pancreatic Beta Cell; vol. 95). Available from: http://www.sciencedirect.com/science/article/pii/B9780128001745000028

59. Sun J, Cui J, He Q, Chen Z, Arvan P, Liu M. Proinsulin misfolding and endoplasmic reticulum stress during the development and progression of diabetes. Mol Aspects Med. 2015 Apr 1;42:105–18.

60. Wahren J, Larsson C. C-peptide: new findings and therapeutic possibilities. Diabetes Res Clin Pract. 2015 Mar;107(3):309–19.

61. Leighton E, Sainsbury CA, Jones GC. A Practical Review of C-Peptide Testing in Diabetes. Diabetes Ther. 2017 Jun;8(3):475–87.

62. Greenbaum CJ, Mandrup-Poulsen T, McGee PF, Battelino T, Haastert B, Ludvigsson J, et al. Mixed-meal tolerance test versus glucagon stimulation test for the assessment of beta-cell function in therapeutic trials in type 1 diabetes. Diabetes Care. 2008 Oct;31(10):1966–71.

63. Field JB. Extraction of insulin by liver. Annu Rev Med. 1973;24:309–14.

64. Brundin T. Splanchnic and extrasplanchnic extraction of insulin following oral and intravenous glucose loads. Clin Sci Lond Engl 1979. 1999 Oct;97(4):429–36.

65. Van Cauter E, Mestrez F, Sturis J, Polonsky KS. Estimation of insulin secretion rates from C-peptide levels. Comparison of individual and standard kinetic parameters for C-peptide clearance. Diabetes. 1992 Mar;41(3):368–77.

66. Jones AG, Hattersley AT. The clinical utility of C-peptide measurement in the care of patients with diabetes. Diabet Med. 2013 Jul;30(7):803–17.

67. Henriksen JH, Tronier B, Bülow JB. Kinetics of circulating endogenous insulin, C-peptide, and proinsulin in fasting nondiabetic man. Metabolism. 1987 May;36(5):463–8.

68. Palmer JP, Fleming GA, Greenbaum CJ, Herold KC, Jansa LD, Kolb H, et al. C-peptide is the appropriate outcome measure for type 1 diabetes clinical trials to preserve beta-cell function: report of an ADA workshop, 21-22 October 2001. Diabetes. 2004 Jan;53(1):250–64.

69. Besser REJ, Shields BM, Casas R, Hattersley AT, Ludvigsson J. Lessons from the mixed-meal tolerance test: use of 90-minute and fasting C-peptide in pediatric diabetes. Diabetes Care. 2013 Feb;36(2):195–201.

70. Shankar SS, Vella A, Raymond RH, Staten MA, Calle RA, Bergman RN, et al. Standardized Mixed-Meal Tolerance and Arginine Stimulation Tests Provide Reproducible and Complementary Measures of β-Cell Function: Results From the Foundation for the National Institutes of Health Biomarkers Consortium Investigative Series. Diabetes Care. 2016;39(9):1602–13.

71. Zavaroni I, Deferrari G, Lugari R, Bonora E, Garibotto G, Dall'Aglio E, et al. Renal metabolism of C-peptide in man. J Clin Endocrinol Metab. 1987 Sep;65(3):494–8.

72. McDonald TJ, Knight BA, Shields BM, Bowman P, Salzmann MB, Hattersley AT. Stability and reproducibility of a single-sample urinary C-peptide/creatinine ratio and its correlation with 24-h urinary C-peptide. Clin Chem. 2009 Nov;55(11):2035–9.

73. Besser REJ, Ludvigsson J, Jones AG, McDonald TJ, Shields BM, Knight BA, et al. Urine C-peptide creatinine ratio is a noninvasive alternative to the mixed-meal tolerance test in children and adults with type 1 diabetes. Diabetes Care. 2011 Mar;34(3):607–9.

74. DCCT. Epidemiology of severe hypoglycemia in the diabetes control and complications trial. Am J Med. 1991 Apr 1;90(4):450–9.

75. Lasker RD. The Diabetes Control and Complications Trial – Implications for Policy and Practice. N Engl J Med. 1993 Sep 30;329(14):1035–6.

76. Orchard TJ, Nathan DM, Zinman B, Cleary P, Brillon D, Backlund J-YC, et al. Association between seven years of intensive treatment of type 1 diabetes and long term mortality. JAMA. 2015 Jan 6;313(1):45–53.

77. Brooks AM, Walker N, Aldibbiat A, Hughes S, Jones G, de Havilland J, et al. Attainment of Metabolic Goals in the Integrated UK Islet Transplant Program With Locally Isolated and Transported Preparations. Am J Transplant. 2013 Dec 1;13(12):3236–43.

78. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med. 2000 Jul 27;343(4):230–8.

79. Brooks AM, Oram R, Home P, Steen N, Shaw JAM. Demonstration of an Intrinsic Relationship Between Endogenous C-Peptide Concentration and Determinants of Glycemic Control in Type 1 Diabetes Following Islet Transplantation. Diabetes Care. 2015 Jan;38(1):105–12.

80. Thompson DM, Meloche M, Ao Z, Paty B, Keown P, Shapiro RJ, et al. Reduced progression of diabetic microvascular complications with islet cell transplantation compared with intensive medical therapy. Transplantation. 2011 Feb 15;91(3):373–8.

81. Vantyghem M-C, Raverdy V, Balavoine A-S, Defrance F, Caiazzo R, Arnalsteen L, et al. Continuous Glucose Monitoring after Islet Transplantation in Type 1 Diabetes: An Excellent Graft Function (β-Score Greater Than 7) Is Required to Abrogate Hyperglycemia, Whereas a Minimal Function Is Necessary to Suppress Severe Hypoglycemia (β-Score Greater Than 3). J Clin Endocrinol Metab. 2012 Nov 1;97(11):E2078–83.

82. Fioretto P, Steffes MW, Sutherland DE, Goetz FC, Mauer M. Reversal of lesions of diabetic nephropathy after pancreas transplantation. N Engl J Med. 1998 Jul 9;339(2):69–75.

83. Vantyghem M-C, Quintin D, Caiazzo R, Leroy C, Raverdy V, Cassim F, et al. Improvement of electrophysiological neuropathy after islet transplantation for type 1 diabetes: a 5-year prospective study. Diabetes Care. 2014 Jun;37(6):e141-142.

84. Keenan HA, Sun JK, Levine J, Doria A, Aiello LP, Eisenbarth G, et al. Residual insulin production and pancreatic ß-cell turnover after 50 years of diabetes: Joslin Medalist Study. Diabetes. 2010 Nov;59(11):2846–53.

85. Meier JJ, Bhushan A, Butler AE, Rizza RA, Butler PC. Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? Diabetologia. 2005 Nov;48(11):2221–8.

86. Gianani R, Campbell-Thompson M, Sarkar SA, Wasserfall C, Pugliese A, Solis JM, et al. Dimorphic histopathology of long-standing childhood-onset diabetes. Diabetologia. 2010 Apr;53(4):690–8.

87. Pipeleers D, In't Veld P, Pipeleers-Marichal M, Gorus F. The beta cell population in type 1 diabetes. Novartis Found Symp. 2008;292:19–24; discussion 24-31, 122–9, 202–3.

88. Zenz S, Mader JK, Regittnig W, Brunner M, Korsatko S, Boulgaropoulos B, et al. Impact of C-Peptide Status on the Response of Glucagon and Endogenous Glucose Production to Induced Hypoglycemia in T1DM. J Clin Endocrinol Metab. 2018 Apr 1;103(4):1408–17.

89. Brown RJ, Sinaii N, Rother KI. Too Much Glucagon, Too Little Insulin: Time course of pancreatic islet dysfunction in new-onset type 1 diabetes. Diabetes Care. 2008 Jul 1;31(7):1403–4.

90. Sherr JL, Ghazi T, Wurtz A, Rink L, Herold KC. Characterization of residual β cell function in long-standing type 1 diabetes. Diabetes Metab Res Rev. 2014 Feb;30(2):154–62.

91. Hare KJ, Vilsbøll T, Holst JJ, Knop FK. Inappropriate glucagon response after oral compared with isoglycemic intravenous glucose administration in patients with type 1 diabetes. Am J Physiol Endocrinol Metab. 2010 Apr;298(4):E832-837.

92. Kramer CK, Borgoño CA, Van Nostrand P, Retnakaran R, Zinman B. Glucagon response to oral glucose challenge in type 1 diabetes: lack of impact of euglycemia. Diabetes Care. 2014 Apr;37(4):1076–82.

93. Rickels MR, Fuller C, Dalton-Bakes C, Markmann E, Palanjian M, Cullison K, et al. Restoration of Glucose Counterregulation by Islet Transplantation in Long-standing Type 1 Diabetes. Diabetes. 2015 May;64(5):1713–8.

94. Rickels MR, Peleckis AJ, Markmann E, Dalton-Bakes C, Kong SM, Teff KL, et al. Long-Term Improvement in Glucose Control and Counterregulation by Islet Transplantation for Type 1 Diabetes. J Clin Endocrinol Metab. 2016;101(11):4421–30.

95. Wewer Albrechtsen NJ, Hartmann B, Veedfald S, Windeløv JA, Plamboeck A, Bojsen-Møller KN, et al. Hyperglucagonaemia analysed by glucagon sandwich ELISA: nonspecific interference or truly elevated levels? Diabetologia. 2014 Sep;57(9):1919–26.

96. Shepherd M, Shields B, Hammersley S, Hudson M, McDonald TJ, Colclough K, et al. Systematic Population Screening, Using Biomarkers and Genetic Testing, Identifies 2.5% of the U.K. Pediatric Diabetes Population With Monogenic Diabetes. Diabetes Care. 2016;39(11):1879–88.

97. Clarke WL, Cox DJ, Gonder-Frederick LA, Julian D, Schlundt D, Polonsky W. Reduced awareness of hypoglycemia in adults with IDDM. A prospective study of hypoglycemic frequency and associated symptoms. Diabetes Care. 1995 Apr;18(4):517–22.

98. Gold AE, MacLeod KM, Frier BM. Frequency of severe hypoglycemia in patients with type I diabetes with impaired awareness of hypoglycemia. Diabetes Care. 1994 Jul;17(7):697–703.

99. Bristow AF, Das RE. WHO international reference reagents for human proinsulin and human insulin C-peptide. J Biol Stand. 1988 Jul;16(3):179–86.

100. Graveling AJ, Frier BM. Impaired awareness of hypoglycaemia: a review. Diabetes Metab. 2010 Oct 1;36:S64–74.

101. Hope SV, Knight BA, Shields BM, Hill AV, Choudhary P, Strain WD, et al. Random non-fasting C-peptide testing can identify patients with insulin-treated type 2 diabetes at high risk of hypoglycaemia. Diabetologia. 2018 Jan 1;61(1):66–74.

102. Geddes J, Wright RJ, Zammitt NN, Deary IJ, Frier BM. An Evaluation of Methods of Assessing Impaired Awareness of Hypoglycemia in Type 1 Diabetes. Diabetes Care. 2007 Jul 1;30(7):1868–70.

103. Janssen MM, Snoek FJ, Heine RJ. Assessing impaired hypoglycemia awareness in type 1 diabetes: agreement of self-report but not of field study data with the autonomic symptom threshold during experimental hypoglycemia. Diabetes Care. 2000 Apr;23(4):529–32.

104. Davis AK, DuBose SN, Haller MJ, Miller KM, DiMeglio LA, Bethin KE, et al. Prevalence of detectable C-Peptide according to age at diagnosis and duration of type 1 diabetes. Diabetes Care. 2015 Mar;38(3):476–81.

105. Kuhtreiber WM, Washer SLL, Hsu E, Zhao M, Reinhold P, Burger D, et al. Low levels of C-peptide have clinical significance for established Type 1 diabetes. Diabet Med. 2015 Oct;32(10):1346–53.

106. Ryan EA, Paty BW, Senior PA, Lakey JRT, Bigam D, Shapiro AMJ. Beta-score: an assessment of beta-cell function after islet transplantation. Diabetes Care. 2005 Feb;28(2):343–7.

107. Skyler JS. Hope vs hype: where are we in type 1 diabetes? Diabetologia. 2017 Dec 23;1–8.

108. Fukuda M, Tanaka A, Tahara Y, Ikegami H, Yamamoto Y, Kumahara Y, et al. Correlation Between Minimal Secretory Capacity of Pancreatic β-Cells and Stability of Diabetic Control. Diabetes. 1988 Jan 1;37(1):81–8.

109. Pinckney A, Rigby MR, Keyes-Elstein L, Soppe CL, Nepom GT, Ehlers MR. Correlation Among Hypoglycemia, Glycemic Variability, and C-Peptide Preservation After Alefacept Therapy in Patients with Type 1 Diabetes Mellitus: Analysis of Data from the Immune Tolerance Network T1DAL Trial. Clin Ther. 2016;38(6):1327–39.

110. Diabetes Control and Complications Trial Research Trial. Hypoglycemia in the Diabetes Control and Complications Trial. Diabetes. 1997 Feb 1;46(2):271–86.

111. Kilpatrick ES, Rigby AS, Goode K, Atkin SL. Relating mean blood glucose and glucose variability to the risk of multiple episodes of hypoglycaemia in type 1 diabetes. Diabetologia. 2007 Dec 1;50(12):2553–61.

112. Bolli G, de Feo P, Compagnucci P, Cartechini MG, Angeletti G, Santeusanio F, et al. Abnormal glucose counterregulation in insulin-dependent diabetes mellitus. Interaction of anti-insulin antibodies and impaired glucagon and epinephrine secretion. Diabetes. 1983 Feb;32(2):134–41.

113. Madsbad S, Hilsted J, Krarup T, Sestoft L, Christensen NJ, Faber OK, et al. Hormonal, metabolic and cardiovascular responses to hypoglycaemia in Type 1 (insulin-dependent) diabetes with and without residual B cell function. Diabetologia. 1982 Dec;23(6):499–503.

114. Jørgensen HV, Pedersen-Bjergaard U, Rasmussen ÅK, Borch-Johnsen K. The Impact of Severe Hypoglycemia and Impaired Awareness of Hypoglycemia on Relatives of Patients With Type 1 Diabetes. Diabetes Care. 2003 Apr 1;26(4):1106–9.

115. Haidar A, Legault L, Dallaire M, Alkhateeb A, Coriati A, Messier V, et al. Glucose-responsive insulin and glucagon delivery (dual-hormone artificial pancreas) in adults with type 1 diabetes: a randomized crossover controlled trial. CMAJ Can Med Assoc J J Assoc Medicale Can. 2013 Mar 5;185(4):297–305.

116. Castle JR, Engle JM, Youssef JE, Massoud RG, Yuen KCJ, Kagan R, et al. Novel Use of Glucagon in a Closed-Loop System for Prevention of Hypoglycemia in Type 1 Diabetes. Diabetes Care. 2010 Jun 1;33(6):1282–7.

117. Pramming S, Thorsteinsson B, Bendtson I, Binder C. Symptomatic hypoglycaemia in 411 type 1 diabetic patients. Diabet Med J Br Diabet Assoc. 1991 Apr;8(3):217–22.

Appendix

Appendix 1 of 1: Clarke/Edinburgh Hypoglycaemia Questionnaire

Site		Patient ID	
Date Questionnaire Completed			

MODIFIED CLARKE/EDINBURGH HYPOGLYCAEMIA HISTORY

1. Tick the category that best describes you (tick one only)
   - ☐ I always have symptoms when my blood sugar is low
   - ☐ I sometimes have symptoms when my blood sugar is low
   - ☐ I no longer have symptoms when my blood sugar is low

2. Have you lost some of the symptoms that used to occur when your blood sugar was low?
   - ☐ Yes ☐ No

3. In the past year, how often have you had hypoglyaemic episodes, where you might feel confused, disorientated, or lethargic and were unable to treat yourself?
   - ☐ Never ☐ Once or twice ☐ Every other month
   - ☐ Once a month ☐ More than once a month

4. In the past 6 months, how often have you had hypoglyaemic episodes, where you were unconscious or had a seizure and needed glucagon or intravenous glucose?
   - ☐ Never ☐ 5 times ☐ 10 times
   - ☐ 1 time ☐ 6 times ☐ 11 times
   - ☐ 2 times ☐ 7 times ☐ 10 times
   - ☐ 3 times ☐ 8 times
   - ☐ 4 times ☐ 9 times

5. How often in the last month have you had readings <3.5mmol/l with symptoms?
   - ☐ Never ☐ 1-3 times ☐ 1 time/week
   - ☐ 2-3 times/week ☐ 4-5 times/week ☐ almost daily

6. How often in the last month have you had readings <3.5mmol/l **without** any symptoms
   - ☐ Never ☐ 1-3 times ☐ 1 time/week
   - ☐ 2-3 times/week ☐ 4-5 times/week ☐ almost daily

7. How low does your blood sugar need to go before you feel symptoms?

☐ 3.4-3.9 mmol/l ☐ 2.9-3.3 mmol/l ☐ 2.2-2.7 mmol/l ☐ <2.2 mmol/l

8. To what extent can you tell by your symptoms that your blood sugar is low?

☐ Never ☐ Rarely ☐ Sometimes ☐ Often ☐ Always

Continued overleaf …….

EDINBURGH HYPOGLYCAEMIA SURVEY (INCLUDING THE GOLD SCORE)

1. Please score the extent to which you experience the following symptoms during a typical daytime hypoglycaemic episode (circle a number for each symptom)

	Not present great deal						present a
Confusion	1	2	3	4	5	6	7
Sweating	1	2	3	4	5	6	7
Drowsiness	1	2	3	4	5	6	7
Weakness	1	2	3	4	5	6	7
Dizziness	1	2	3	4	5	6	7
Warmth	1	2	3	4	5	6	7
Difficulty speaking	1	2	3	4	5	6	7
Pounding heart	1	2	3	4	5	6	7
Inability to concentrate	1	2	3	4	5	6	7
Blurred vision	1	2	3	4	5	6	7
Hunger	1	2	3	4	5	6	7
Nausea	1	2	3	4	5	6	7
Anxiety	1	2	3	4	5	6	7
Tiredness	1	2	3	4	5	6	7
Tingling lips	1	2	3	4	5	6	7
Trembling	1	2	3	4	5	6	7
Headache	1	2	3	4	5	6	7

2. Do you know when your hypos are commencing? Please circle a number:

	Always aware						Never aware
Awareness	1	2	3	4	5	6	7

Further Comments: