Mechanisms of Hypoglycaemia Related Sudden Cardiac Death in Type 2 Diabetes Mellitus

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Abstract

Introduction: Hypoglycaemia has been strongly associated with cardiovascular death in trials of intensive glycaemic control in type 2 diabetes (T2DM) patients at high cardiovascular risk for up to 90 days afterwards. Hypoglycaemia may increase cardiovascular mortality via: i) proarrhythmic effects due to abnormal cardiac repolarisation and autonomic function; and ii) prothrombotic effects via platelet hyperreactivity and altered fibrin clot properties. It was hypothesised this may be mediated by both acute and mechanisms downstream of the hypoglycaemic event and these were tested experimentally.

Methods: The risk of cardiac arrhythmias was examined during spontaneous hypoglycaemia, as captured via continuous interstitial glucose and Holter monitoring in insulin-treated T2DM patients. This was complemented by hyperinsulinaemic hypoglycaemic clamp studies, where cardiovascular parameters were measured acutely and up to 7 days later in T2DM and nondiabetic subjects. Autonomic function was analysed using heart rate variability and spontaneous baroreceptor sensitivity, whilst effects on thrombosis were analysed using platelet function assays, turbidimetric and lysis assays, and scanning electron microscopy of fibrin clots.

Results: During spontaneous prolonged hypoglycaemia, an 8-fold increase in bradyarrhythmias was observed. Vagal re-activation following initial transient withdrawal was observed during spontaneous as well as experimental hypoglycaemia, that only occurred in T2DM subjects. Hypoglycaemia was found to increase platelet activation and aggregation acutely, whilst also increasing fibrin clot density and depressing fibrinolysis for up to 7 days, an effect that occurred in T2DM subjects but not nondiabetic controls.

Conclusions: Hypoglycaemia may override the benefits of intensive glucose control via proarrhythmic and prothrombotic mechanisms. These effects

may persist beyond the acute episode and mediate short term increases in cardiovascular mortality. Hypoglycaemia should be minimised in high cardiovascular risk patients through individualised glucose lowering strategies.

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Declaration

The candidate confirms that the work submitted is her own and that appropriate credit has been given except where work which has formed part of joint-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chow E, Bernjak A, Williams S, Fawdry RA, Hibbert S, Freeman J, Sheridan PJ, Heller SR Risk of cardiac arrhythmias during hypoglycaemia in patients with type 2 diabetes and cardiovascular risk. ***Diabetes*** 2014; 63(5): 1738-1747 has been included in Chapter 3

EC designed the study, collected and analysed the data, and wrote the manuscript. SW and RF collected data and reviewed the manuscript. AB and SH analysed the data and edited the manuscript. JF supported the statistical approach and analysis and reviewed the manuscript. PS and SRH designed the study, reviewed the data and edited and redrafted the manuscript.

Publications Resulting from this Work

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Chow E, Heller SR. Pathophysiology of the effects of hypoglycaemia on the cardiovascular system (2012) Diabetic Hypoglycaemia 5(1):3-8 Jun 2012

Chow E, Heller SR. 'Nocturnal Hypoglycaemia' in *Hypoglycaemia in Clinical Diabetes*, 3rd edition, Frier, BM, Heller SR, McCrimmon R (Eds.), England, Wiley, John and Sons Inc., 2014; Chapter 5

Chow E, Fisher M, Heller SR. 'Mortality, Cardiovascular Morbidity and Possible Effects of Hypoglycaemia on Diabetic Complications' in *Hypoglycaemia in Clinical Diabetes*, 3rd edition, Frier, BM, Heller SR, McCrimmon R (Eds.), England, Wiley, John and Sons Inc., 2014; Chapter 13

Manuscripts in preparation:

Chow E, Storey RF, Ajjan RA, Macdonald IA, Heller SR. Acute and subacute effects of hypoglycaemia on thrombosis in Type 2 diabetes mellitus.

Chow E, Bernjak A, Macdonald IA, Sheridan PS, Heller SR. Cardiac autonomic regulation and repolarisation during acute experimental hypoglycaemia in Type 2 diabetes mellitus

Chow E, Bernjak A, Macdonald IA, Sheridan PS, Heller SR. Hypoglycaemia impairs cardiac autonomic function in type 2 diabetes for up to seven days

Abstracts:

Chow E, Iqbal A, Bernjak A, Ajjan RA, Heller SR Effect of hypoglycaemia on thrombosis and inflammation in patients with type 2 diabetes*.* ***Lancet*** 2014; 383:S35.

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Chow EYK, Iqbal A, Walkinshaw E, Lubina-Solomon A, Daly R, Judge HM, Storey RF, Heller SR. Hypoglycaemia increases platelet reactivity in patients with Type 2 Diabetes*. Diabetes. 2013 62 (Suppl 1) A-3067*

EYK Chow, A. Bernjak, S. Williams, R.A. Fawdry, S. Hibbert, S.R Heller, and P.J. Sheridan. Effect of low glucose on risk of arrhythmia in Type 2 diabetic patients with cardiovascular risk *Europace* 2012*; 14* (suppl 4): *iv14-iv17*

Statement of contribution

The candidate, Elaine Chow, has designed the studies and was responsible recruitment, collection of data, conduct of ambulatory monitoring and hyperinsulinaemic clamp studies, platelet function analysis, analysing autonomic function tests and cardiac arrhythmia, data analysis and writing of the thesis. Prof Simon Heller advised on design of studies and analysis of data, supervised conduct of hyperinsuliaemic clamps and has reviewed the thesis. Dr Paul Sheridan advised on design of studies and cardiac electrophysiological analyses. Alan Bernjak (postdoctoral fellow) assisted with ECG recordings and analysed ambulatory and high resolution ECG recordings for heart rate variability and repolarisation analyses using customised software. Prof Rob Storey has advised on design of the clamp studies and platelet function assays. Part of platelet function assays were assisted by research technicians Sam Outteridge, Rebecca Daly, Jemma Walker. Dr Ramzi Ajjan has advised on all aspects on analysis of fibrin clot properties. The turbidimetry & lysis assay, fibrinogen, C3, PAI-1 and electron microscopy of fibrin clots were performed by Fladia Phoenix, research technician (Leeds). Cytometric bead array for cytokines were performed at the flow cytometry facility, University of Sheffield.

Acknowledgements

The idea for this project came on a grey November afternoon when browsing through diabetes journals in the library and reading about the then just-published ACCORD study. This has been a tremendous journey, through the conception of the project, being funded by the NIHR, recruiting patients, performing clamps with a baby in tow, and seeing the fascinating results and the impact they have within the diabetes community. I would like to thank Emma Walkinshaw, Alexandra Lubina-Solomon, and Ahmed Iqbal, who as fellow clinical researchers have helped me, particularly in the clamp studies. I would also like to thank our research nurses, Sue Hudson and Chloe Nisbet, and all the clinical research facility staff who have been instrumental to the smooth running of the experimental work. I also thank the laboratory support staff, Sam, Rebecca, Jemma and Fladia, without whom this work would not have been possible.

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Finally, I am indebted to all the diabetic and nondiabetic volunteers who have selflessly contributed their time towards this research. You have made this journey so enjoyable and I sincerely hope that we will be better able to care for individuals with diabetes.

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List of Abbreviations

ACCORD	Action to Control Cardiovascular Risk in Diabetes
ACS	Acute Coronary Syndromes
ADP	Adenosine Diphosphate
ADVANCE	Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation
AGEs	Advanced Glycation End Products
AMI	Acute Myocardial Infarction
APTT	Activated Partial Thromboplastin Time
AUC	Area Under The Curve
BRS	Baroreceptor Sensitivity
cAMP	Cyclic Adenosine Monophosphate
CAN	Cardiac Autonomic Neuropathy
CBA	Cytometric Bead Array
CGM	Continuous Glucose Monitoring
CGMS	Continuous Glucose Monitoring System
CRP	C Reactive Protein
CVD	Cardiovascular Disease
DAD	Delayed Afterdepolarisations
DBP	Diastolic Blood Pressure
E:I	Expiration:Inspiration
EAD	Early Afterdepolarisations
ECAM-1	Endothelial Cell Adhesion Molecule-1
FFA	Free Fatty Acid
FITC	Fluorescein Isothiocyanate
GLUT-4	Glucose Transporter Type 4
GPCR	G Protein Coupled Receptors
HbA1c	Glycated Haemoglobin
HDL	High Density Lipoprotein
HF	High Frequency
HOMA	Homeostatic Model Assessment
HPLC	High Performance Liquid Chromatography
HR	Hazard Ratio
HRV	Heart Rate Variability
hsCRP	High Sensitivity C-Reactive Protein
ICAM	Intracellular Cell Adhesion Molecules
IFN-γ	Interferon-Gamma
IG	Interstitial Glucose
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin 8

IRR	Incident Rate Ratios
IRS-1	Insulin Receptor Substrate 1
LDL	Low Density Lipoprotein
LF	Low Frequency
LQTS	Long QT Syndromes
LT	Lysis Time
LVEF	Left Ventricular Ejection Fraction
MA	Maximum Absorbance
MAP	Mean Arterial Pressure
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein-1
MFI	Median Fluorescence Intensity
NF-κB	Nuclear Factor Kappa B
NO	Nitric Oxide
PAI-1	Plasminogen-Activator-Inhibitor-1
PE	Phycoerythrin
PEG	Polyethylene Glycol
PI3K	Phosphatidylinositol-3-Kinase
PI3K	Phosphoinositide 3-Kinase
PKC	Protein Kinase C
QTd	QT Dispersion
REM	Rapid-Eye-Movement
RMSSD	Root-Mean Square Of The Difference Of Successive R-R Intervals
ROS	Reactive Oxygen Species
SBP	Systolic Blood Pressure
SCD	Sudden Cardiac Death
SDNN	Standard Deviation Of Normal R-R Intervals
SMBG	Self-Monitored Blood Glucose
T1DM	Type I Diabetes mellitus
T2DM	Type 2 Diabetes mellitus
TNF-α	Tumour Necrosis Factor Alpha
tPA	Tissue Plasminogen Activator
UKPDS	United Kingdom Prospective Diabetes Study
VADT	Veteran Administration Diabetes Trial
VCAM	Vascular Cell Adhesion Molecules
VF	Ventricular Fibrillation
VLDL	Very-Low-Density Lipoprotein
VPB	Ventricular Premature Beats
VT	Ventricular Tachycardia
vWF	Von Willebrand Factor
WBSPC	Whole Blood Single Platelet Counting

Chapter 1 - Introduction

Diabetes mellitus is defined as a metabolic disorder characterised by raised blood glucose, and currently affects 4% of the UK population with numbers expected to reach half a billion worldwide by 2030. The consequences of chronic hyperglycaemia include microvascular complications (nephropathy, retinopathy, neuropathy) and macrovascular complications (cardiovascular, peripheral arterial and cerebrovascular disease). The leading cause of death is cardiovascular disease (CVD) which accounts for 50% of all deaths of diabetic individuals (Morrish et al., 2001), and of these, sudden cardiac death (SCD) is the first presentation of CVD in half of all cases. In 400 BC, Hippocrates noted that 'sudden death is more common in those who are naturally fat than in the lean', and epidemiological studies have shown that the risk of SCD is increased by two- to three-fold among subjects with type 2 diabetes (T2DM) compared with nondiabetic individuals (Curb et al., 1995).

Current prevention of cardiovascular complications is dependent on a multifactorial approach, addressing risk factors such as hypertension, hyperlipidaemia and lifestyle factors, and glycaemic control. Intensive glycaemic control reduces microvascular complications and was demonstrated in the landmark United Kingdom Prospective Diabetes Study (UKPDS), where levels were reduced by a quarter (UK Prospective Diabetes Study Group, 1998a, b). The potential of intensive glycaemic control to reduce the incidence of CVD is less established and has been tested

recently by three international randomised controlled trials - Action to Control Cardiovascular Risk in Diabetes (ACCORD), Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) and Veteran Administration Diabetes Trial (VADT). These trials were designed to examine the effect of intensive glycaemic treatment versus conventional treatment on cardiovascular endpoints, including major adverse cardiovascular events and death (Duckworth et al., 2009; Gerstein et al., 2008; Patel et al., 2008). A total of nearly 25,000 T2DM patients worldwide with existing CVD or two or more cardiovascular risk factors were randomised into an intensive glycaemic control group (target glycated haemoglobin A1c, HbA1c <6.0% in ACCORD and VADT, <6.5% in ADVANCE) versus a group receiving standard glycaemic therapy. My research question was prompted by the premature termination of the ACCORD trial in 2008 due to excess mortality in the intensive glucose lowering arm.

In this introductory chapter, the pathophysiological mechanisms of T2DM and SCD will be outlined, and the relationship between T2DM, glycaemia and an increased risk of CVD will be explored, together with the paradoxical effectiveness of intensive glucose lowering strategies in CVD prevention. Evidence from large scale trials of intensive glycaemic control in T2DM will be presented that implicate hypoglycaemia as a causal factor in increased risk of cardiovascular death, a key observation which has prompted this research. The existing literature on cardiovascular effects of

hypoglycaemia and potential mechanistic links between hypoglycaemia and SCD will be reviewed. Finally, I will present the research question based on current gaps in the knowledge and the overall aims of my thesis.

1.1 Pathogenesis of Type 2 Diabetes

Diabetes mellitus describes a group of metabolic disorders characterised by hyperglycaemia, either as a result of deficient insulin secretion, resistance to the action of insulin, or a combination of both. The World Health Organization (WHO) has defined the glucose thresholds for diagnosis: fasting plasma glucose >7 mmol/L; random plasma glucose >11.1 mmol/L, or a 2 hour plasma glucose >11.1 mmol during 75 g oral glucose tolerance test (2009). While these definitions are widely accepted, the aetiological classification of diabetes is not straightforward, particularly as the understanding of pathogenesis of diabetes has increased. T2DM, which accounts for 90-95% of diabetes, is a heterogeneous disorder, where individuals may present with a range of defects, from predominantly insulin resistance with relative insulin deficiency, to predominantly a secretory defect with insulin resistance (2009).

It has previously been assumed that insulin resistance is the primary defect in T2DM, leading to eventual beta cell failure as a result of compensatory hyperinsulinaemia. However, current evidence challenges this view. T2DM develops as a consequence of a progressive, largely genetically determined, decline in beta cell function in addition to acquired insulin resistance secondary to factors such as physical activity and obesity (Alsahli

M, 2010). In the pre-diabetic state, individuals are at least as insulin resistant as patients with T2DM, and normally there is a hyperbolic relationship between insulin secretion and insulin sensitivity. Individuals with T2DM fall off this curve and have inadequate insulin secretion in the face of insulin resistance (Figure 1-1). In the United Kingdom Prospective Diabetes Study (UKPDS), the use of a mathematical model relating insulin and glucose measurements through homeostatic model assessment (HOMA), indicated that there is already a 50% loss of beta cell function at the point of diagnosis (1995). Both glucotoxicity secondary to hyperglycaemia and lipotoxicity from free fatty acids (FFA), impair the capacity of beta-cell for glucose sensing, regeneration and the propensity for apoptosis (Alsahli M, 2010). There is growing evidence that endoplasmic reticulum stress induced apoptosis and cytokines, such as interleukin-1 (IL1), may be implicated in beta-cell decline.

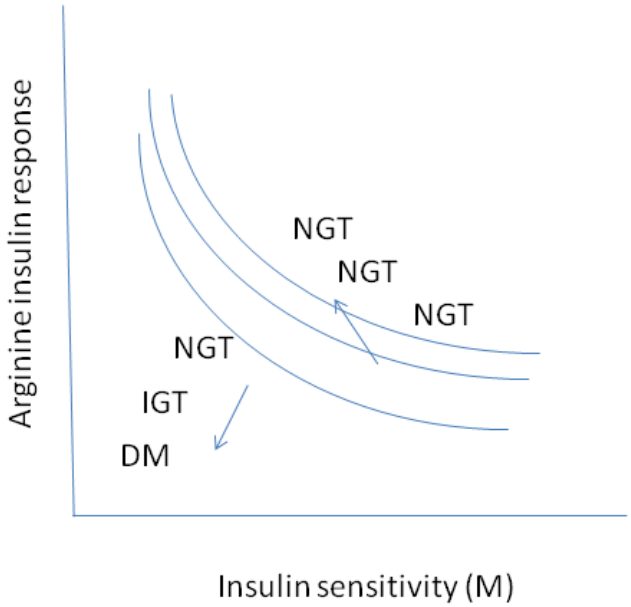

Figure 1-1: Relationship between insulin secretion and insulin sensitivity

The curvilinear relationship between beta cell function (as represented by the acute insulin response) and insulin sensitivity as measured during a hyperinsulinaemic clamp. Individuals with normal glucose tolerance (NGT) remain on the curvilinear line, whereas progressors went on to develop impaired glucose tolerance (IGT) and eventually diabetes (DM), failing to demonstrate adequate beta cell compensation for insulin resistance. Modified from (Alsahli M, 2010).

Insulin resistance is defined as the inability of insulin to exert its normal biological actions. The molecular mechanisms of insulin resistance are incompletely understood. There are multiple intramyocellular defects concerning insulin action, including impaired glucose transport and phosphorylation, reduced glycogen synthesis, and decreased glucose oxidation (Defronzo, 2009). However, a notable abnormality is the impaired ability of insulin to phosphorylate the insulin receptor substrate 1 (IRS-1), which leads to the reduced activation of phosphatidylinositol-3-kinase (PI3K). PI3K normally mediates insulin-stimulated muscle glycogen synthase activity and insulin stimulated glucose disposal (DeFronzo, 2004). The key sites of insulin resistance, with respect to glucose homeostasis, are the liver and muscle. Hepatic insulin resistance leads to the inability of insulin to suppress basal hepatic glucose production and fasting hyperglycaemia. Skeletal muscle is responsible of 80% of the total body glucose uptake, whereas adipose tissue is responsible for only 5% of glucose disposal. Muscle insulin resistance has been demonstrated in lean and obese T2DM individuals, and impaired glucose uptake by leg muscles has been shown to occur in response to physiological increases in plasma insulin (DeFronzo, 2004).

Insulin resistance is clearly associated with obesity, and in epidemiological studies, there is a clear association between total body fat and insulin resistance across a broad range of body mass indices and ethnic groups (Buse et al., 2011). Central adiposity is more closely linked to insulin resistance and there is considerable evidence that fat distribution and adipocyte metabolism are involved in the pathogenesis of T2DM. Insulin is normally an antilipolytic hormone which restrains the release of FFA release from adipocytes via hormone-sensitive lipase. The impaired antilipolytic effect of insulin leads to chronic FFA elevation, and FFA has been shown to induce hepatic and muscle insulin resistance. Furthermore, it is now thought that fat cells are not merely sites of lipid storage but in fact active endocrine organs. Dysfunctional adipocytes have been shown to produce excessive amount of proinflammatory, insulin-resistance inducing adipocytokines, such as tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), and reduced amounts of protective adipocytokines, such as adiponectin. The pathogenesis of T2DM is clearly multifactorial and complex. Defronzo (2009) described the 'ominous octet', where in addition to beta cell dysfunction and liver and muscle insulin resistance, other factors, including accelerated lipolysis of fat cells, incretin deficiency/resistance, hyperglucagonaemia, increased renal glucose reabsorption and brain insulin resistance leading to altered appetite regulation, all contribute to disease pathogenesis.

1.2 Pathophysiology of Sudden Cardiac Death

SCD is defined as death attributed to cardiac causes occurring suddenly or unexpectedly within one hour of the onset of symptoms (Myerburg and Wellens, 2006). The incidence of SCD is 0.1-0.2% per year in the adult population (Myerburg and Wellens, 2006), which amounts to 50,000-70,000 people annually in the UK (NICE, 2007). The terminal event in SCD is usually due to fatal ventricular tachycardia (VT) or ventricular fibrillation (VF). In Western countries, the underlying cause in 80% of cases is coronary artery disease, with cardiomyopathies and ion channel abnormalities accounting for the remaining 20% of cases. SCD is often the first expression of ischaemic heart disease and accounts for 50% of all deaths due to ischaemic heart disease.

Diabetes has been associated with an increased risk of SCD (Balkau et al., 1999) but whether the risk increases beyond that attributed to CVD is debated (Kucharska-Newton et al., 2010; Suarez et al., 2005). In the Rochester diabetic neuropathy study, 21 SCDs were reported during a 15 year follow up, all of whom had coronary or myocardial disease (Suarez et al., 2005). In the Honolulu heart programme, a 23 year follow up of over 800 patients found that diabetes conferred an increased relative risk of SCD of 2.76 (Curb et al., 1995). Similarly, a population based case control study found that high glucose levels were associated with an increased risk of SCD irrespective of microvascular complications (Jouven et al., 2005). The Framingham study also revealed a strong association between

diabetes and SCD in women (Kannel and McGee, 1985). In the Paris Prospective Study 1, diabetes was an independent risk factor for SCD even after adjusting for other risk factors (Balkau et al., 1999).

The pathophysiology of SCD is notoriously difficult to study, as these are sudden, unheralded events, and it is increasingly recognised that SCD involves an underlying substrate and a transient trigger. The pathophysiology of SCD is a multifactorial process involving: 1) conventional risk factors promoting atherogenesis; 2) rupture of the vulnerable plaque; 3) activation of the thrombotic cascade; and 4) a final transient trigger, such as autonomic balance in patients with an electrophysiological predisposition for arrhythmia (Myerburg and Wellens, 2006)

Figure 1-2). The pathogenesis of SCD in diabetic patients is likely to be multifactorial due to the combination of atherosclerosis as well as specific thrombosis and plaque-related and electrophysiological factors that enhance the risk. In the following section an overview of the pathophysiological processes underpinning the evolution of SCD is discussed.

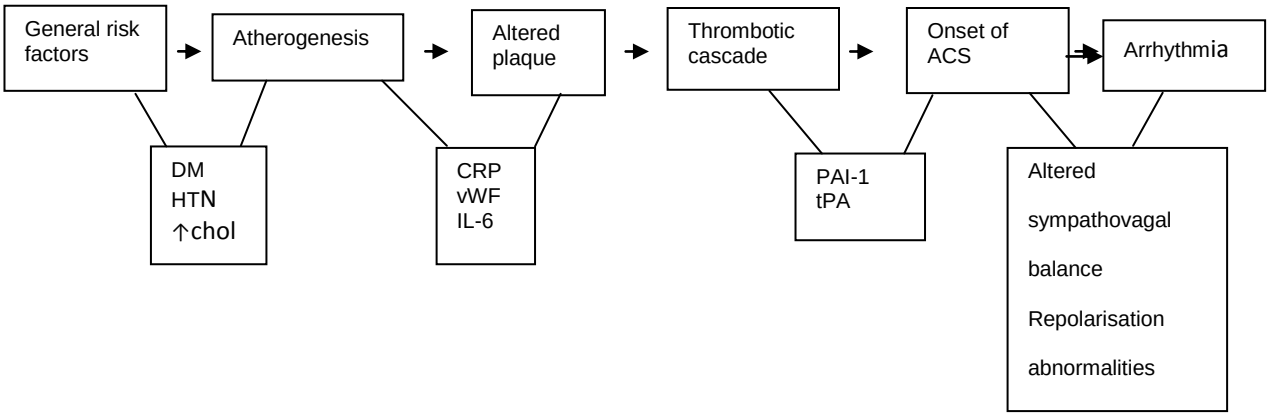

Figure 1-2: Multifactorial cascade leading to SCD

General risk factors, such as diabetes and hypertension promote atherogenesis. The transition from a stable to a vulnerable plaque is driven by diffuse subclinical inflammation. Clotting factors and the fibrinolytic balance determine the fate of a ruptured plaque and the development of coronary thrombosis. Transient factors, such as an altered sympathovagal balance and cardiac repolarisation abnormalities, can trigger the final fatal event of a ventricular tachyarrythmia. DM - diabetes mellitus, HTN - hypertension, CRP - C reactive protein, vWF - von Willebrand factor, IL-6 - interleukin 6, PAI-1 - plasminogen activator 1, tPA - tissue plasminogen activator.

1.2.1 Atherogenesis

Atherogenesis is a chronic inflammatory condition characterised by endothelial injury, modified lipoproteins and immune cells (Boyle, 2007). Low density lipoprotein (LDL) cholesterol is deposited across the vascular endothelium, mediated by angiotensin II, and oxidation of LDL cholesterol leads to a chain of metabolic responses, which attract monocytes to the subendothelial compartment. These monocytes differentiate into macrophages under the influence of IL1 and turn into foam cells secreting cytokines, such as interleukin 2 (IL-2) and TNFα, which drive the expression of vascular cell adhesion molecules (VCAM) on the endothelial surface. In turn, these adhesion molecules attract T lymphocytes which secrete interferon-gamma (IFN-γ) and stimulate smooth muscle proliferation

(Boyle, 2007). There is eventual elaboration of the extracellular matrix which turns into a fibrous cap overlying the atheroma.

1.2.2 Vulnerable Plaques

Acute myocardial infarction (AMI) and unstable angina are the result of acute plaque disruption and subsequent thrombosis overlying the plaque, leading to coronary vascular occlusion. An understanding of the concept of the 'vulnerable plaque' is fundamental to the current concept of acute coronary syndromes (ACS). One of the factors thought to promote transitioning from a stable to a vulnerable plaque is subclinical inflammation (Libby, 2008). Markers of subclinical inflammation, such as high C reactive protein (CRP) levels, predicted a 2-fold increase in SCD risk over a 17 year follow up period in men (Albert and Cobbe, 2006), whilst elevated levels of von Willebrand factor (vWF) and IL-6 predicted an increased risk of a fatal over that of a nonfatal coronary event (Empana et al., 2010; Kucharska-Newton et al., 2009).

1.2.3 Thrombosis and Coronary Occlusion

Plaque rupture precipitates a series of events leading to the formation of a thrombus over the eroded or ruptured plaque causing ACS. In a large post mortem study of SCD victims, 74% had evidence of intramural thrombosis (Davies and Thomas, 1984). Activation of the thrombotic cascade is a critical event in the precipitation of SCD and involves both cellular and fluid phases of the coagulation system. Platelets are involved in the initial response by adhering to the ruptured plaque, which is followed by the

activation of the thrombotic cascade and the generation of a platelet rich fibrin mesh (Grant, 2007). The fate of a ruptured plaque and whether it becomes a sustained occlusive thrombus is dictated by the fibrinolytic balance. Increased levels of hepatic derived coagulation factors promote clot formation, while impaired fibrinolysis can result from a decrease in fibrinolytic enzymes or an increase in endogenous inhibitors, such as plasminogen-activator-inhibitor-1 (PAI-1).

1.2.4 Cardiac Electrical Instability and Arrhythmogenesis

The final event in SCD in 80% of cases is a fatal ventricular tachyarrhthmia. Based on observational studies, SCD stratifiers with prognostic value in patients with established cardiac disease fall into 3 categories (Spooner, 2008):

1) Structural - left ventricular ejection fraction (LVEF) <30-35%, left ventricular hypertrophy

2) Markers of enhanced sympathetic activity - heart rate variability (HRV)

3) Markers of electrical instability - prolonged QTc, arrhythmia inducibility during invasive electrophysiological studies

Left ventricular hypertrophy of any cause is one of the strongest predictors of SCD, and the risk of SCD post myocardial infarction is increased by left ventricular dysfunction (Solomon et al., 2005). Autonomic function has a strong contributory influence on SCD. Impaired baroreceptor sensitivity (BRS) and HRV are strongly associated with an increased SCD risk in cohort studies (La Rovere et al., 1998). The risk of SCD is highest during the first 3

hours after waking, coinciding with maximal sympathetic activation (Albert and Cobbe, 2006). In prospective studies among T2DM patients, those with a depressed cardiac vagal function were 1.5 to 2 times more likely to suffer a SCD (Kataoka et al., 2004; Wheeler et al., 2002).

Arrhythmias can result from abnormal automaticity, triggered activity, or both. Triggered activity may be caused by early or delayed afterdepolarisations (EAD and DAD), which are secondary repolarisations during or after the repolarisation phase, respectively. Long QT syndromes (LQTS), both congenital and acquired have provided a paradigm for the understanding of the relationship between abnormal cardiac repolarisation and ventricular arrhythmias. In LQTS, changes in cardiac ion channels lead to altered repolarisation currents and a prolonged cardiac repolarisation phase (Goldenberg et al., 2008) (Figure 1-3). It has been hypothesised that increased dispersion creates pockets of inexcitable tissue predisposed to re- entrant arrhythmias and a polymorphic form of VT (Taggart et al., 1990). Transmural and apical basal heterogeneity are responsible for the morphology of the T wave on surface electrocardiogram (ECG). QT parameters have been shown to be accurate predictors of cardiac mortality in newly diagnosed T2DM patients (Naas et al., 1998). Another study has shown that QT interval abnormalities present at diagnosis and were better predictors of cardiac death than the ankle brachial pressure index and autonomic function tests in type 2 diabetes patients (Rana et al., 2011).

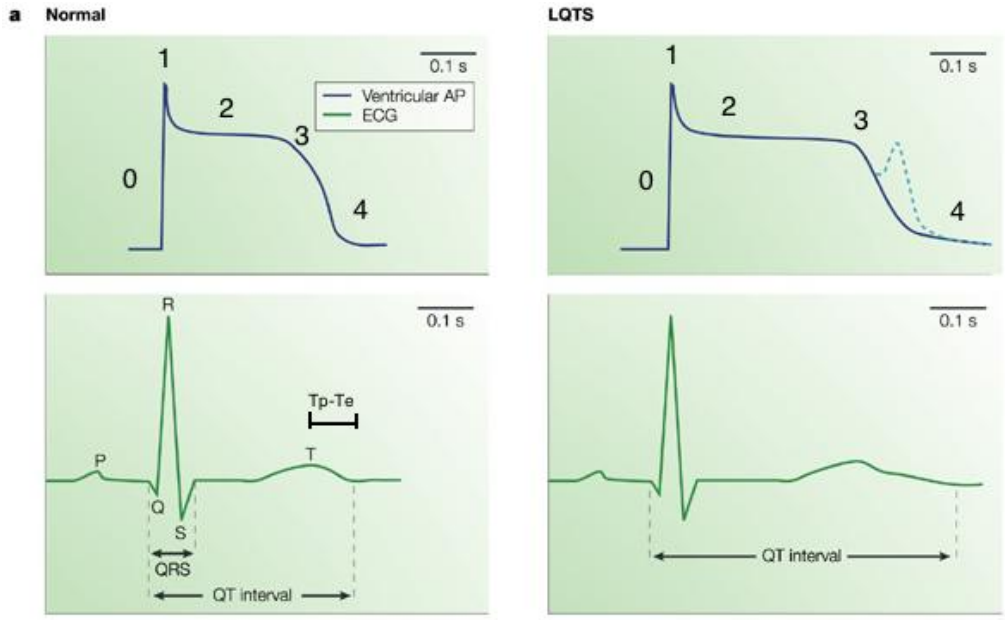

Figure 1-3: Ionic basis of abnormal cardiac repolarisation in LQTS and relation to ECG morphology

(A) The top panel shows the phases of a normal cardiac action potential (AP). In phase 0, rapid depolarisation occurs caused by an influx of Na^+ via the i_{na} channel. In phase 1, inactivation of i_{na} and the movement of K^+ via i_{to} result in a transient net outward current and the notch appearance of the AP. The plateau phase (2) is mainly sustained by the opening of type l calcium channels which results in a net inward current. During phase 3, rapid repolarisation occurs via rapid delayed inward rectifying K^+ current, $i_{kr.}$ The i_{kr} channels close when the membrane is repolarised to its resting potential (phase 4). The QT interval corresponds to the total duration from depolarisation to repolarisation of a cardiac AP (bottom). T wave morphology is affected by gradients of repolarisation across the myocardium. (B) The AP in LQTSs is abnormally prolonged in the plateau phase due to a decrease in repolarisation currents (e.g. Mutated i_{kr}) or an increase in depolarising currents (e.g. Mutation in i_{na}). This leads to a prolonged AP as well as QT interval. The prolonged plateau phase increases the risk of early after depolarisations (EAD) (dotted line) which predisposes to re-entrant arrhythmias and VT. Adapted from (2004).

1.3 Type 2 Diabetes and Cardiovascular Disease: Role of Glycaemia and Other Risk Factors

The relationship between type 2 diabetes and CVD is well established, however, the relative contribution of individual risk factors is debated. Unlike type 1 diabetes where macrovascular risk is primarily mediated by

hyperglycaemia, in T2DM other factors including insulin resistance, adiposity, dyslipidaemia, hypertension and subclinical inflammation are also implicated. The present section briefly outlines the role of these risk factors followed by a discussion of endothelial, thrombotic and myocardial mechanisms of diabetic cardiovascular disease. This section prefaces the subsequent section on the rationale for intensive glucose lowering therapies in reduction of CVD.

1.3.1 Risk Factors for Cardiovascular Disease in Type 2 Diabetes

T2DM is associated with a 2- to 4-fold increase in cardiovascular mortality compared to nondiabetic subjects (Laakso, 2010). These findings have been confirmed in several cohorts, including the European Prospective Investigation of Cancer and Nutrition (EPIC Norfolk) (Khaw et al., 2001) and the Atherosclerosis Risk in Communities (ARIC) studies (Selvin et al., 2010). Laakso and colleagues compared the seven year incidence of fatal and nonfatal myocardial infarction in 1373 nondiabetic and 1059 T2DM subjects (Haffner et al., 1998). In nondiabetic subjects the 10 year risk of myocardial infarction was 3.5%. However, in those with a history of previous myocardial infarction, the risk increased to 19%, whilst in diabetic patients the 10 year risk of myocardial infarction was 20% even those with no with prior history of CVD. This gave rise to the concept of diabetes as a 'coronary heart disease equivalent'. Similar findings have been replicated in other cohorts (Juutilainen et al., 2005).

Traditional risk factors (low levels of high density lipoprotein (HDL), high levels of LDL cholesterol, obesity and hypertension), together with non-traditional or diabetes specific risk factors (hyperglycaemia, insulin resistance, inflammation, and nephropathy), are involved in thed development of CVD in T2DM. It is important to note that these factors tend to cluster and do not occur in isolation. Stern (1995) originally proposed the 'common soil hypothesis' that both diabetes and CVD arise from common antecedents, and Reaven (1988) described the components of the insulin resistance syndrome, or the metabolic syndrome, which associates insulin resistance with obesity, hyperglycaemia, high plasma triglycerides, low HDL cholesterol and hypertension. Subsequent to its original description, there is strong epidemiological data to support the association of the metabolic syndrome with CVD (Fonseca et al., 2004). However, it is not always easy to dissect the individual contribution of each of these factors since they tend to aggregate. Figure 1-4 depicts the interaction between these factors and their role in pathogenesis of CVD.

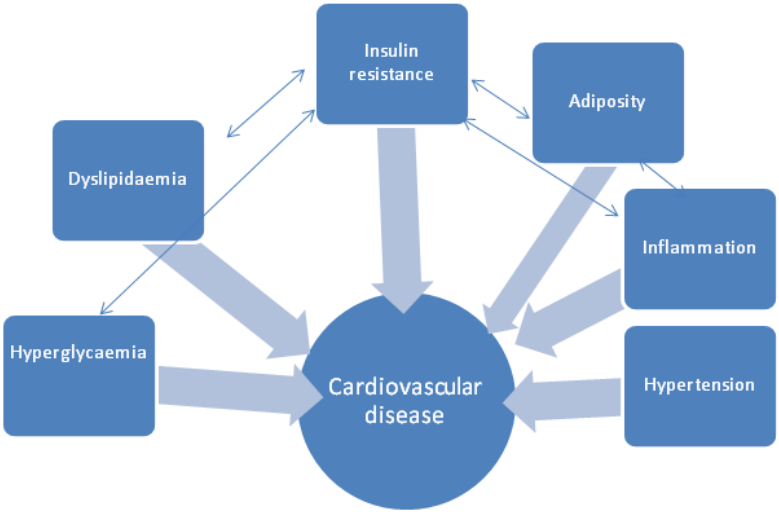

Figure 1-4: Interaction of risk factors in T2DMand development of CVD

With respect to hyperglycaemia, epidemiological studies indicate a clear association between glucose levels and cardiovascular risk, and a systematic review of 26 cohort studies showed that 1% increase in HbA1c was associated with a relative risk of 1.15 95% confidence intervals (CI, 1.11-1.20) for all causes of mortality and 1.15 95% CI (1.05-1.17) for CVD. There is evidence of a dose response relationship appearing to extend to within the normoglycaemic range (Zhang et al., 2010). The Honolulu Heart Programme demonstrated a predictive correlation between fasting plasma glucose levels (nondiabetic, impaired glucose tolerance, and diabetic ranges) and cardiovascular events and mortality (Donahue et al., 1987), whilst Levitan et al. (2004) found that hyperglycaemia in the nondiabetic range was associated with an increase in CVD in a linear fashion for two hour post-prandial glucose and fasting glucoses above 5.6 mmol/L.

Brownlee (2005) proposed that hyperglycaemia may mediate tissue damage through a unifying pathway via overproduction of reactive oxygen species (ROS) by the mitochondrial electron transport chain. These then activate four damaging pathways: the polyol, hexoamine, and protein kinase C (PKC) pathways and the formation of advanced glycation end products (AGEs). Production of ROS leads to the up-regulation of nuclear factor kappa B (NF-κB) and transcription of proinflammatory cell adhesion molecules and monocyte chemoattractant protein-1 (MCP-1), which attract

immune cells to the endothelium. Activation of the PKC pathways also decreases endothelial nitric oxide (NO) availability and induces endothelial dysfunction. Further hyperglycaemia has been associated with the abnormal post-translational modification of plasma coagulation proteins and altered clot properties (Grant, 2007).

The increased cardiovascular risk appears to begin prior to the development of frank hyperglycaemia (Laakso, 2010).In the UKPDS the risk of coronary heart disease was increased even at normal or low HbA1c with CVD risk being substantially higher compared with microvascular complications such as retinopathy at the equivalent HbA1c level (UK Prospective Diabetes Study Group, 1998b). The risk of coronary heart disease and stroke is already substantially elevated at the time of diagnosis of T2DM. Haffner et al. (1990) developed the 'ticking clock' hypothesis of complications, asserting that the clock for microvascular risk starts ticking at the onset of hyperglycaemia, while the clock for macrovascular risk starts ticking at some antecedent point . It is thought that insulin resistance may explain the accumulation of cardiovascular risk factors in the years before diagnosis (FizeI'ova et al., 2014) (Figure 1-5).

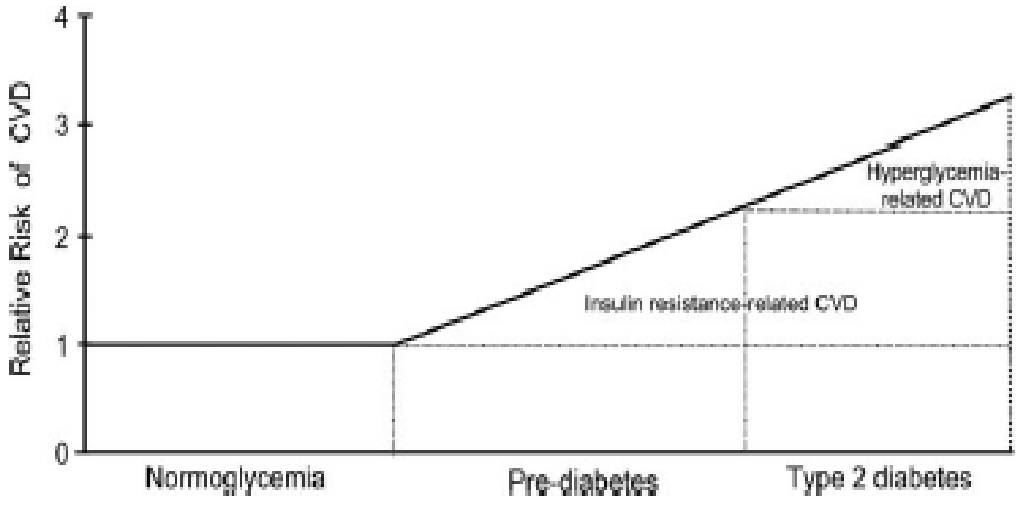

Figure 1-5: Relative risk of CVD in normoglycaemia, pre-diabetes and T2DM and relative contribution of insulin resistance and hyperglycaemia
Modified from (Laakso, 2010)

In the following section, the mechanisms involved in the development of diabetic CVD will be described, together with the contributory roles of the above risk factors.

1.3.2 Mechanisms of Diabetic Cardiovascular Disease

T2DM is associated with multiple pathophysiological abnormalities that predispose to an increased risk of CVD. Figure 1-6 summarises the constellation of cardiovascular abnormalities which culminate in a vulnerable individual with T2DM, who is at a significantly increased risk of cardiovascular complications as further detailed below.

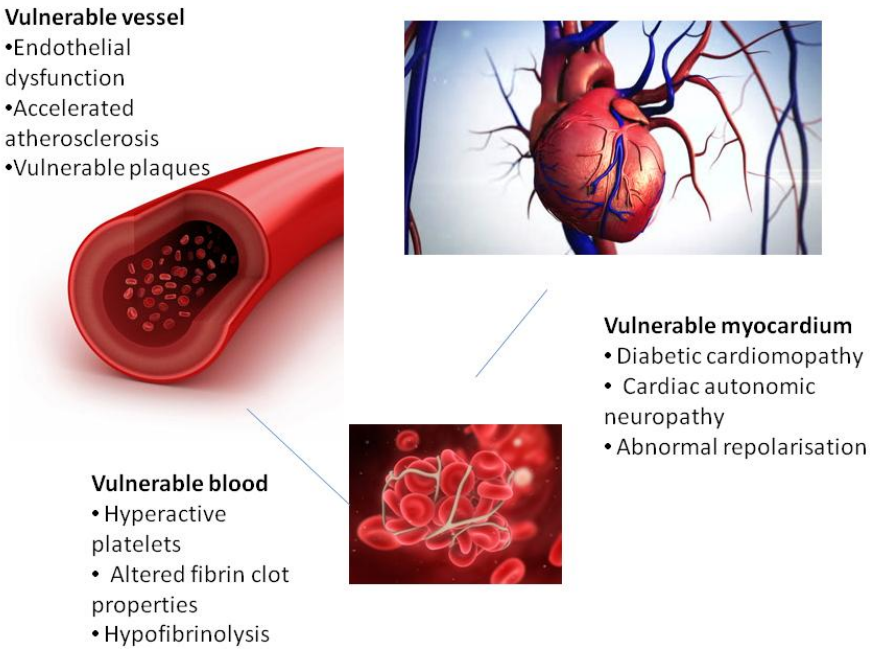

Figure 1-6: The vulnerable T2DM patient - mechanisms of increased cardiovascular risk
Modified from (Hess et al., 2012b)

1.3.2.1 Atherogenesis

Patients with T2DM exhibit accelerated atherosclerosis compared to nondiabetic individuals. One of the earliest events in the pathogenesis of atherosclerotic lesions is impaired endothelial function. It is well known that individuals with T2DM exhibit impaired nitric-oxide mediated vasodilatation. In the Hoorn study, markers of endothelial dysfunction, such as vWF and the endothelial cell adhesion molecule 1 (ECAM-1) explained 43% of the increase in cardiovascular mortality in T2DM (De Jager et al., 2005). Hyperglycaemia also increases vascular smooth muscle cell proliferation and macrophage migration into the media, promoting fatty streak formation within atherosclerotic lesions (Moreno and Fuster, 2004).

T2DM patients tend to have more diffuse and distal coronary artery lesion, and advanced techniques, such as intravenous ultrasound-virtual histology, studies have found that diabetes patients have more thin-cap fibroatheroma, which have a higher propensity to rupture and cause coronary thrombosis (Hess et al., 2012b). Both subclinical inflammation from adipose tissue and hyperglycaemia-related oxidative stress, have been linked to the development of thin cap fibroatheroma. Higher levels of inflammatory mediators originating from adipose tissue and systemically, and as well as increased numbers of monocytes, can contribute to the weakening of the fibrous cap in T2DM (Viswanathan and Zaman, 2012). Diabetes-induced oxidative stress has been linked to adventitial inflammation in porcine coronary arteries, with cytokines such as IL-6 and TNF-α, MCP-1 and adhesive molecules playing a role (Zhang et al., 2003a).

1.3.2.2 Platetet Hyperactivity

Hyperactive platelets contribute to CVD through triggering thrombus formation, microcapillary embolisation, and the release of cytokines that can accelerate the progression of vascular lesions (Kakouros et al., 2011). Platelets in T2DM are hyperactive and demonstrate greater aggregation responses compared to controls for given concentration of agonists. A fundamental characteristic of the diabetic platelet is higher baseline intracellular calcium levels and reduced levels of cyclic adenosine monophosphate (cAMP); thus the platelet is capable of activation at lower level of agonist stimulation. Many of these changes are associated with

hyperglycaemia, which is capable of inducing markers of platelet activation, including P-selectin and the CD40 ligand even in nondiabetic individuals (Kakouros et al., 2011). Platelets respond to sub-threshold stimuli under hyperglycaemic conditions and this results in increased platelet turnover (Hess et al., 2012b). T2DM platelets are larger and possess more integrin receptors (GP1b/GPIIb/IIIa) responsible for formation of the platelet plug which correlates with a level of glycaemic control (Kakouros et al., 2011). Hyperglycaemia can also induce thromboxane synthesis, mimicking conditions of shear stress and consequent platelet activation (Hess et al., 2012b).

1.3.2.3 Altered Fibrin Clot Characteristics and Hypofibrinolysis

Haemostatic abnormalities are also observed in type 2 diabetes which may contribute to increased coronary thrombosis. There are quantitative changes to level of coagulation proteins such as tissue factor and Factor VII which are key initiators of clot formation (Alzahrani and Ajjan, 2010). Thrombin levels are increased which produces denser, less permeable clots. IL6 production and elevated fibrinogen levels predict CVD (Alzahrani and Ajjan, 2010). Fibrinolysis is also impaired as high levels of PAI-1, a key inhibitor in the fibrinolysis pathway is feature of T2DM (Alzahrani and Ajjan, 2010). Type 2 diabetes individuals display both quantitative and qualitatitive changes to clot structure. An analysis of plasma-purified fibrinogen clots from type 2 diabetes patients showed increased fibre thickness, a more compact structure, smaller pore size and increased

number of fibrinogen branching points compared with healthy controls (Dunn et al., 2005).Qualitatively, hyperglycaemia has been shown to increase post-translational glycation of fibrinogen leading to more compact clots (Alzahrani and Ajjan, 2010). At the same time clots in type 2 diabetes patients are slower to lyse, due to decrease in plasminogen binding to fibrin networks and decreased plasmin generation (Alzahrani and Ajjan, 2010).

1.3.2.4 Cardiac Metabolism

The effect of an occlusion of the coronary artery leads to disparate clinical courses in different patients, ranging from silent ischaemia to SCD (Hess et al., 2012b), and these outcomes are probably influenced by a number of myocardial characteristics. Factors associated with atherosclerosis-dependent ischemia, such as perfusion, myocardial viability and wall motion abnormalities, as well as those independent of ischaemia, e.g. sympathetic hyperactivity, left ventricular hypertrophy and cardiac repolarisation abnormalities, may play critical roles (Naghavi et al., 2003).

In the presence of insulin resistance there is a shift in substrate utilisation from glucose to FFA within cardiac myocytes. This energetic inflexibility leads to increases in oxygen demand, which is reflected in a larger infarct size in response to a comparable ischemic insult in diabetic patients compared to nondiabetic individuals (Hess et al., 2012b). Although the increased risk of heart failure in diabetes is in part linked to ischaemic cardiomyopathy, some individuals develop a distinct diabetic

cardiomyopathy (Boudina and Abel, 2010) as a result of remodelling, apoptosis of cardiac myocytes and interstitial fibrosis.

1.3.2.5 Cardiac Autonomic Neuropathy

Diabetes can 'attack' the cardiovascular autonomic system due to either functional or structural nerve damage (Vinik and Ziegler, 2007). Whilst the pathogenesis of diabetic autonomic neuropathy is incompletely understood, it is thought to be part of a polyneuropathy that parallels the development of peripheral neuropathy. Metabolic insults and oxidative stress, neurovascular insufficiency and a deficiency in neurohormonal growth factors, have all been thought to contribute (Vinik and Ziegler, 2007). CAN may range from asymptomatic depression of autonomic reflexes that are only present on testing to devastating syndromes such as postural hypotension and nocturnal diarrhoea. Cardiac autonomic neuropathy (CAN) can increase the risk of SCD via a number of mechanisms. These include sympathetic over-activity and the loss of vagal inhibition leading to an increased propensity for tachyarrhythmias, regional dysinnervation leading to an intracardiac sympathetic imbalance, silent ischaemia, abnormal repolarisation, adverse cardiac remodelling leading to systolic and diastolic dysfunction.

1.3.2.6 Prolonged QT Interval

There is a higher prevalence of QT abnormalities, an index of cardiac repolarisation in T2DM patients compared with normal subjects (Veglio et al., 2002), and this appears to be present at the time of diagnosis of T2DM

(Rana et al., 2011). One of the characteristics of abnormal repolarisation is an intracellular calcium overload within cardiac myocytes. Impaired ventricular relaxation can lead to consecutive diastolic calcium overload (Hess et al., 2012b), and there is evidence that impaired calcium handling is linked to insulin resistance. Fasting insulin concentrations have been associated with QT independent of obesity-related parameters (Kazumi et al., 1999). In animal studies, the L-type calcium current displays defective inactivation in obese insulin-resistant rats (Lin et al., 2012). There is decreased expression of calcium transporters, such as the sarco/endoplastic reticulum calcium ATPase (SERCA2), and other alterations of the persistent sodium current are linked to down-regulation of PI3K (Lu et al., 2013). The overall effect is an abnormal repolarisation substrate that is more vulnerable to stressors, such as ischemia, electrolyte disturbances, autonomic imbalance, triggering afterdepolarisations and cardiac arrhythmias.

1.3.3 The Contribution of Glycaemia to CVD in Type 2 Diabetes

From the above discussion, several points are worthy of consideration before approaching the subject of the effect of intensive glycaemic control on cardiovascular mortality in T2DM in clinical trials. Firstly, CVD in T2DM is multifactorial with a clustering of risk factors and hyperglycaemia partly contributing, but the majority of cardiovascular risk may be accumulated secondary to insulin-resistance- related factors while blood glucose is in the pre-diabetic range. Thus patients are likely to have accumulated a

significant atherogenic burden at the time of presentation, and this may account for the lack of cardiovascular benefit from intensive glucose lowering during advanced disease. Secondly, hyperglycaemia can impact on different stages in the evolution of CVD. Hyperglycaemia can have an early influence in the atherogenic process through oxidative stress and endothelial dysfunction, but can also impact on more proximate mechanisms, such as increased thrombogenicity and myocardial metabolism. Thirdly, not only the absolute glucose concentration, but degree of fluctuation has been associated with cardiovascular risk (Esposito et al., 2004) (Monnier et al., 2012) (Hanefeld et al., 2004), and this may be influenced by patient factors and the type of glucose lowering therapy employed. Finally, pharmacological and non-pharmacological interventions, even if its primary aim is glucose lowering, can have pleiotropic effects where it may simultaneously modify other risk factors, such as weight and inflammation, in a protective or adverse manner.

1.4 Intensive Glycaemic Control and Cardiovascular Mortality in Type 2 diabetes

The association between hyperglycaemia and CVD reported both in observational studies and epidemiological analyses of trials has formed the rationale for interventional trials of intensive glucose lowering to reduce macrovascular complications in T2DM (Stratton et al., 2000). The landmark UKPDS randomised 5102 newly diagnosed T2DM patients to receive either conventional therapy (dietary restriction) or intensive therapy (either

sulphonylurea or insulin, or in overweight patients metformin) for glucose control (UK Prospective Diabetes Study Group, 1998a, b). Over 10 years HbA1c averaged 7.0% (6.2-8.2) in the intensive group compared to 7.9% (6.9-8.8) in the conventional group, and overall, there was a reduction in 12% for any diabetes-related endpoint, together with a significant reduction of 25% for microvascular complications. In addition, there was a trend towards a non-significant reduction in myocardial infarction by 16% (p=0.052). The study was limited by the duration of the follow up (median 10.7 years when published in 1998) and the sample size was not adequate to test the effects of intensive glycaemic control on CVD. However, at 10 year followup, risk reduction in myocardial infarction emerged with time (15% p=0.01) despite loss of glycaemic differences between arms after the first year(Holman et al., 2008).

Three multicentre international randomised controlled trials were designed to test the hypothesis that intensive glucose therapy reduces mortality as the primary outcome in T2DM. The ACCORD, ADVANCE and VADT randomised 24,000 patients with a high cardiovascular risk to intensive glycaemic control or standard control groups (Duckworth et al., 2009; Gerstein et al., 2008; Patel et al., 2008). The characteristics of these three trials are summarised in Table 1-1.

Table 1-1: Summary of ACCORD, ADVANCE and VADT characteristics

	ACCORD	ADVANCE	VADT
n	10 251	11 140	1791
Mean age (years)	62	66	60
Sex (%male/female)	39/61	42/58	97/3
Duration of diabetes (years)	10	8	11.5
History of CVD (%)	35	32	40
Median baseline HbA1c (%)	8.1	7.2	9.4
Target HbA1c (%)*	<6.0 vs 7.0-7.9	<6.5 vs based on local guidelines	<6.0 vs planned separation of 1.5
Achieved HbA1c (%)*	6.4 vs 7.5	6.3 vs 7.0	6.9 vs 8.5
Median follow up (years)	3.5	5	5.6
On Insulin at end of study* (%)	75 vs 55	40 vs 24	89 vs 74
On TZD at end of study* (%)	91 vs 58	17 vs 11	53 vs 42
Weight change* (kg)	+3.5 vs +0.4	-0.1 vs -1.0	+7.8 vs + 3.4
Severe hypoglycaemia * (%)	16.2 vs 5.1	2.7 vs 1.5	21.2 vs 9.9
Definition of primary outcome	Nonfatal MI, nonfatal stroke, CVD death	Microvascular and macrovascular outcomes	Nonfatal MI, nonfatal stroke, CVD death, hospitalisation for heart failure, revascularisation
HR for primary outcome (95% CI)	0.90 (0.78-1.04)	0.9 (0.82-0.98); macrovascular 0.94 (0.84-1.06)	0.88 (0.74-1.05)
HR for mortality findings (95% CI)	1.22 (1.01-1.46)	0.93 (0.83-1.06)	1.07 (0.81-1.42)

In all three trials no significant benefit in overall mortality or cardiovascular events was observed in the intensively treated groups. Furthermore, in

2008 the diabetes community was alarmed by the premature termination of the ACCORD trial due to the excess number of deaths that had occurred within the intensive treatment arm of the trial (Hazard ratio, HR 1.22 95% CI 1.01-1.46, p=0.04, Figure 1-7).

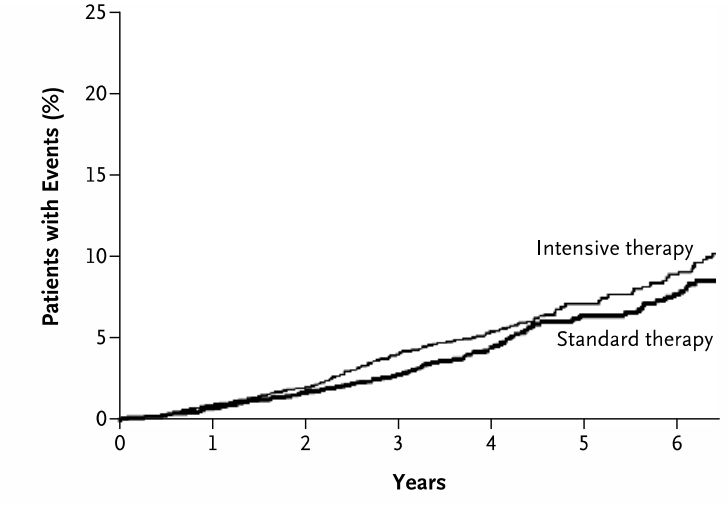

Figure 1-7: Differences in survival following intensive and standard glycaemic therapy in the ACCORD trial
Adapted from (Gerstein et al., 2008)

Cardiovascular deaths accounted for a third of all deaths in the ACCORD and VADT studies (Duckworth et al., 2009; Gerstein et al., 2008) and half of all deaths in the ADVANCE trial (Patel et al., 2008). In the ACCORD study, there was an excess of fatal cardiovascular events in the intensive treatment group, and in particular, there was an excess of SCDs which accounted for 46% of all the excess cardiovascular deaths associated with the intensive control group. In the VADT, SCDs were 3-fold higher in the intensive treatment group as compared to the standard arm. However, although the overall rates of fatal cardiovascular deaths were greater in the intensive arm, this did not apply to nonfatal events, as the rates of nonfatal

myocardial infarction were lower in the intensive treatment group (HR 0.76, 95% CI 0.67-0.92, p = 0.004). Similar findings have been reported in a meta-analysis of randomised controlled trials of intensive glycaemic control in T2DM (Boussageon et al., 2011). Thus, there may be some evidence that intensive glycaemic control appears to selectively promote fatal cardiovascular events, in particular SCD, over nonfatal events.

A further paradoxical observation is that although intensive glycaemic control had no apparent benefit or increased the risk of death overall, it may have had protective effects in subgroups with a low atherogenic burden based on *post hoc* analyses. In the ACCORD study, a significant reduction in the primary cardiovascular endpoint was observed in patients with no prior cardiovascular events (Gerstein et al., 2008). This view is supported by an ancillary analysis of the VADT that showed intensive glycaemic therapy reduced future CVD events in those patients with low baseline coronary artery calcium scores (Duckworth et al., 2009). In the 10 year follow up of the UKPDS trial, patients randomised to intensive glycaemic control group who at the time of diagnosis had a low atherogenic burden were protected from cardiovascular events beyond the period of the intervention (Holman et al., 2008). The benefits of intensive glycaemic control may be due to a reduction in hyperglycaemia, which is linked to increased oxidative stress as outlined earlier (Laakso, 1999). This supports the notion of a 'hyperglycaemic memory', where even transient exposures to high glucose can cause persistent damage to the vasculature.

Thus early intervention in the course of T2DM may be cardioprotective but the benefits may not extend to those with established CVD.

These two paradoxical observations raise the possibility that although intensive glycaemic therapy may reduce adverse effects of hyperglycaemia through mechanisms such as oxidative stress (Laakso, 1999), it may also increase the risk of fatal cardiovascular events, including SCD, by another mechanism in those with established CVD. A number of explanations have been proposed, including greater weight gain or medication effects, and a high proportion of patients were reported to be taking thiazeliediones, which have been associated with heart failure and excess cardiovascular mortality (Gerstein et al., 2008). However, a strong candidate was the effect of hypoglycaemia, since this was 3-fold higher in frequency in the intensive arm of the study (Gerstein et al., 2008) (Figure 1-8). The increased likelihood of hypoglycaemia during strict glycaemic control is not unexpected (Turnbull et al., 2009), particularly during a strategy in which the overall aim was to lower HbA1c levels to below 6.5% as rapidly as possible.

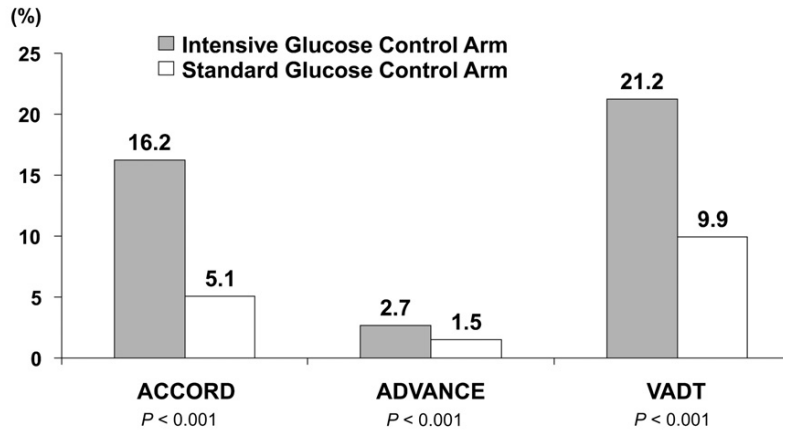

Figure 1-8: Percentage of patients with at least one episode of severe hypoglycaemia in trials of intensive glycaemic control in T2DM patients
Adapted from (Frier et al., 2011)

In the following section it will be argued that hypoglycaemia is a strong causal explanation for an increase in cardiovascular mortality.

1.5 Can Hypoglycaemia be a Potential Mechanism for Increased Cardiovascular Death Observed with Intensive Glycaemic Control in Type 2 Diabetes Patients

There has been considerable controversy concerning the question of whether hypoglycaemia is causal in the increase in cardiovascular mortality in trials of intensive glycaemic control in T2DM (Yakubovich and Gerstein, 2011). One issue is that many of the subsequent analyses are based on *post hoc* analyses, which were not designed *a priori* to test the effect of hypoglycaemia on cardiovascular outcomes. Additional limitations include the lack of uniform definitions of hypoglycaemia and variable reporting of hypoglycaemic events. In the ACCORD, ADVANCE and VADT studies, a history of hypoglycaemia was consistently associated with an increase in

subsequent cardiovascular mortality (Bonds et al., 2010; Duckworth et al., 2009; Zoungas et al., 2010). Even though this relationship was attenuated after adjusting for multiple covariates, it nevertheless remained significant, and the crucial question is whether a true causal relationship exists between hypoglycaemia and mortality. The possibility cannot be excluded that the association between hypoglycaemia and death could, at least in part, be an artefact of residual confounding factors and is simply a marker for vulnerability. Goto et al. (2013) conducted a bias analysis to evaluate the effect of severe comorbid illness as a confounding factor on the association between severe hypoglycaemia and the risk of CVD. Their analysis indicated that comorbid illness alone is unlikely to explain the association, as it requires a severe morbid illness to be ten times more prevalent in individuals with a history of hypoglycaemia compared to those without, which is unlikely.

The Bradford Hill criteria defines a list of factors to be considered when trying to determine whether an association is truly causal (Hill, 1965). These include the strength of an association, the temporal relationship, dose-response relationship, consistency with other studies, specificity, biological plausibility, reversibility, coherence, and analogy. Evidence for and against hypoglycaemia as a causal factor for increased cardiovascular mortality will now be discussed in the context of these criteria.

1.5.1 Strength of Association

In the ACCORD trial patients who had experienced one or more severe hypoglycaemic episodes had higher mortality rates than those with no hypoglycaemia across both study arms (Bonds et al., 2010). One third of all deaths were attributed to CVD and hypoglycaemia was associated with a higher cardiovascular mortality. A similar pattern was also reported in an analysis of the ADVANCE trial (Zoungas et al., 2010), where patients with a history of hypoglycaemia had higher rates of cardiovascular death with an adjusted HR of 3.79 (2.36-6.08). In the VADT, a recent severe hypoglycaemic event was the strongest independent predictor of death at 90 days (Duckworth et al., 2009). In both intensive and standard treatment arms, patients who experienced severe hypoglycaemia, sufficient to cause an impairment in consciousness, had an 88% increase in primary cardiovascular events and a 3-fold increase in cardiovascular death (Duckworth, 2009). Recent severe hypoglycaemia was associated with a HR of 3.7 for cardiovascular death (p=0.01) and Figure 1-9 demonstrates the association between severe hypoglycaemia and mortality.

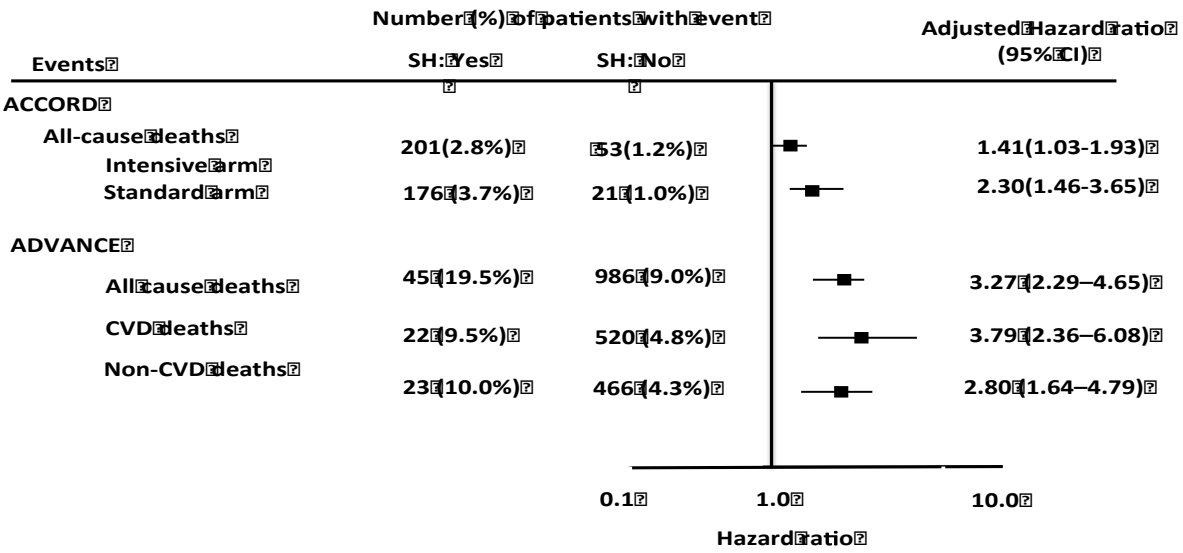

Figure 1-9: Association between severe hypoglycaemia and mortality in the ACCORD and ADVANCE studies

HR for death by any cause in patients with at least one severe episode of hypoglycaemia versus no hypoglycaemia.

1.5.2 Consistency with Other Studies

The association between hypoglycaemia and cardiovascular mortality has also been reported in trials of intensive insulin therapy in critical care patients. A study by van den Berghe et al. (2001) demonstrated that intensive insulin therapy resulted in a reduction in mortality in surgical critical care patients. However, subsequent studies have reported no change or an increase in mortality when intensive insulin therapy has been applied in other medical or mixed intensive care settings (Brunkhorst et al., 2008; Finfer et al., 2009; Van den Berghe et al., 2006), with the risk of hypoglycaemia being increased by up to 6-fold in some reports in the intensive treatment arm. Hypoglycaemia was also observed to be an independent risk factor for death following adjustment for disease severity

(Brunkhorst et al., 2008; Griesdale et al., 2009; Van den Berghe et al., 2006).

1.5.3 Dose Response

There is some evidence for a dose-response relationship between the severity of a hypoglycaemic event and the subsequent risk of cardiovascular death. In a *post hoc* analysis of the ORGIN trial which compared the addition of glargine versus standard therapy, the HR between cardiovascular death and hypoglycaemia was stronger for severe than for nonsevere hypoglycaemia in both treatment groups (HR 1.38 vs 1.08 for severe vs nonsevere hypoglycaemia in the glargine group, HR 2.89 versus 0.95 in standard group) (Mellbin et al., 2013). In the NICE-SUGAR study, the adjusted HRs for death among patients was stronger for those with moderate or severe hypoglycaemia, compared to those with no hypoglycaemia (HR 1.41 (1.21 to 1.62; P<0.001) and 2.10 (95% CI, 1.59 to 2.77; P<0.001), respectively). Furthermore, the association with death was increased among patients who had moderate hypoglycaemia on more than one day (Finfer et al., 2012).

In the ACCORD trial, although hypoglycaemia was associated with an increased risk of death in both study arms, the relationship was stronger in the standard therapy group than the intensive arm (Gerstein et al., 2008), and similar observations have been reported in other studies (Mellbin et al., 2013). This counterintuitive observation has been used as an argument against hypoglycaemia as a causal explanation for increased cardiovascular

mortality (Yakubovich and Gerstein, 2011). However, the ACCORD study group have further analysed the frequency of self-monitored blood glucose (SMBG) <3.9 mmol in the seven days prior to the four month study visit, and found that there was a lower risk of death (HR 0.68 95% CI 0.36-1.24) in subjects randomly assigned to the intensive treatment group who experienced frequent episodes of low blood glucose determine through self-monitoring during the previous week (Seaquist et al., 2012). This relationship was more pronounced in subjects with a history of severe hypoglycaemia requiring medical assistance in the intensive treatment group. Similarly, in a *post hoc* analysis of the ADVANCE trial, the risks of major macrovascular outcomes and death were reduced among patients reporting minor hypoglycaemia compared to those who did not report such episodes (Zoungas et al., 2010). The fact that the association between hypoglycaemia and cardiovascular death was weaker in the intensive treatment arm may be explained by more frequent asymptomatic hypoglycaemia. Repeated episodes of hypoglycaemia result in blunting the sympathodrenal response (Heller and Cryer, 1991). However, this weaker association in the intensive treatment arm does not itself negate a potential causal association between hypoglycaemia and cardiovascular death.

1.5.4 Specificity

Some analyses have examined the specific cause of death associated with hypoglycaemia. For example, in the NICE-SUGAR study of intensive insulin

therapy in critically ill patients, the strongest association found for hypoglycaemia was cardiovascular death, particularly due to distributive shock (HR 4.35, p<0.001) (Finfer et al., 2012). In the ORIGIN study, an increase in arrhythmic death was associated with severe hypoglycaemia (adjusted HR 1.77, 1.17-2.67, p<0.007) (Mellbin et al., 2013). In *post hoc* analyses of the ADVANCE trial, severe hypoglycaemia was associated with an increase in major macrovascular events (HR 2.88, 2.01-4.12), cardiovascular death (HR 2.69, 1.72-4.19), and total mortality (HR 2.69, 1.97-3.67) all p<0.001 (Zoungas et al., 2010). However, hypoglycaemia was also related to death due to seemingly unrelated nonvascular causes in the ADVANCE trial, such as skin disorders (adjusted HR 4.73, 1.96-11.40) and respiratory disorders (adjusted HR 2.4, 1.43-2.43), casting doubt on the specificity of the association and suggesting the possibility of residual confounding factors.

1.5.5 Temporal Relationship

In the ACCORD study, of the 74 participants who reported any hypoglycaemic event, six died with 30 days of the event, whilst only one death in the intensive treatment group was identified as definitely related to hypoglycaemia. Therefore hypoglycaemia was identified as having a probable role in only 0.7% of deaths. A *post hoc* analysis of the NICE-SUGAR study found excess mortality in the intensive treatment group, with the median time to death from the occurrence of a hypoglycaemic event being seven days for moderate hypoglycaemia and eight days for severe

hypoglycaemia (Finfer et al., 2012). However, pathogenic mechanisms in critically ill patients may be different compared to those in individuals with T2DM and 'stable' CVD. It has been difficult to identify a direct immediate link between hypoglycaemia and cardiovascular death. However, given the high prevalence of asymptomatic hypoglycaemia and the variability in self-monitoring of blood glucose, it is possible that hypoglycaemia could have been unrecognised, even if it was contributory. Furthermore, if a death is unexpected, it would be difficult to determine if hypoglycaemia had occurred immediately before death.

Most studies have reported that the risk of death associated with severe hypoglycaemia was not immediate; rather it increased in the months following the event. In the VADT, severe hypoglycaemia predicted death at 90 days (Duckworth, 2009). In the ADVANCE study, Zoungas et al. (2010) examined the incident of vascular outcomes in patients with severe hypoglycaemia, and found that the HR for cardiovascular death at three months was 5.35 ($p<0.01$); this was attenuated at 6 months (HR 3.57) but remained significant ($p=0.01$).

The risk of a cardiovascular event is also elevated following other conditions, such as after a systemic respiratory infection (Smeeth et al., 2004). In a case control study, acute respiratory-tract infections were associated with an increased risk of a first ever AMI in low cardiovascular risk patients for a period of about two weeks (Meier et al., 1998).There has been a burgeoning of the literature in the past 15 years linking systemic

sepsis and inflammation to increased arterial thrombotic events within 30 days (Donze et al., 2014). Thus the fact that hypoglycaemia at the time of the event may not have been an immediate precipitant of cardiovascular death, does not negate a potential causal role further downstream. Hypoglycaemia has been associated with increases in proinflammatory markers and impairment in autonomic function for at least 24 hours after the event (Adler et al., 2009; Wright et al., 2010). However, whether there are other mechanisms that can promote a sustained elevation for the risk of CVD in the weeks to months after the event is a question which remains to be answered.

1.5.6 Reversibility, Analogy, Coherence, and Biological Plausibility

The question of reversibility is difficult to address since the risk of hypoglycaemia is inherent in glycaemic management and is not an exposure that can simply be removed. Although there is currently little evidence linking hypoglycaemia and SCD in T2DM, there is circumstantial evidence linking hypoglycaemia to sudden nocturnal deaths in T1DM. Tattersall and Gill (1991) published a series of investigations into 50 cases of sudden deaths among young adults with T1DM, and in 22 cases there was a similar mode of death, whereby the patient was found in an undisturbed bed having been well the day before, and the term 'dead-in-bed' syndrome was coined to describe the clinical picture (Campbell, 1991). Subsequent cohorts in Sweden and Australia have reported similar findings (Sartor and Dahlquist, 1995; Tu et al., 2008).

There is evidence linking hypoglycaemia and the dead-in-bed syndrome, as in Tattersall and Gill's original study, fourteen out of the 50 patients had known problems with recurrent nocturnal hypoglycaemia. Another cohort study of T1DM patients found that sudden overnight death was more common in the last three years of the study (1988-1980), coinciding with the greater use of intensive insulin therapy (Thordarson and Søvik, 1995). Recently, Tanenberg et al. (2009) described a 23 year old with T1DM who was experiencing recurrent nocturnal hypoglycaemia and was fitted with an insulin pump and a continuous glucose monitoring system (CGMS). He was then found dead in an disturbed bed 20 hours after the fitting of the continuous glucose monitor, and his recorded glucose was <30 mg/dL or 1.8 mmol/L around the time of his death (Fig1-10) . Thus, there is circumstantial evidence linking sudden deaths in T1DM to nocturnal hypoglycaemia, although it is unknown whether this could also happen in T2DM.

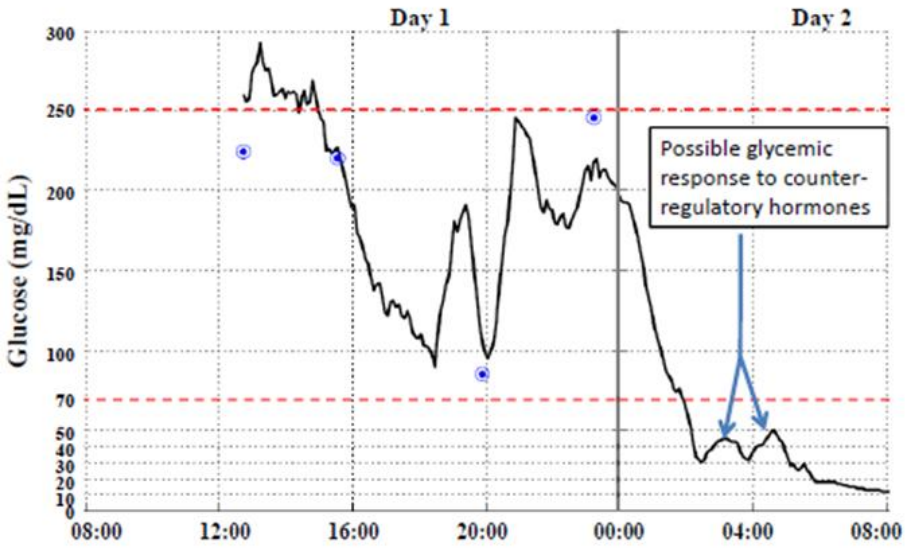

Figure 1-10: Low interstitial glucose at the time of death as captured by continuous glucose monitoring in a T1DM subject

A 23 year old patient with T1DM had a continuous glucose monitor fitted and subsequently died suddenly overnight. The time of death is estimated between 5-8 am, at which point he was severely hypoglycaemic. Source: (Tanenberg et al., 2009)

An association between hypoglycaemia and SCD is biologically plausible, as hypoglycaemia provokes sympathoadrenal activation that can result in cardiac ischaemia, changes in the cardiac autonomic balance, abnormal cardiac repolarisation, and an increased risk of arrhythmic death (Chow and Heller, 2012). Hypoglycaemia can also be prothrombotic via increased platelet activation and aggregation, an increase in coagulant factors and pro-inflammatory molecules. In addition, hypoglycaemia has also been shown to impair autonomic responses to hypotensive stress for at least 24 hours after an event (Adler et al., 2009). Much of the evidence for this has been obtained from nondiabetic and T1DM individuals, but remains plausible in patients with T2DM. In the following sections, the physiology of hypoglycaemia will be reviewed, followed by the postulation of a number of mechanisms by which hypoglycaemia can increase SCD in T2DM.

1.6 The Physiology of Hypoglycaemia

1.6.1 Epidemiology

The problem of hypoglycaemia has been under-recognised in patients with T2DM, as it has traditionally been thought that the risk of hypoglycaemia is low in comparison to T1DM. The risks associated with metformin and incretin based therapies is low, and in the UKPDS, only 0.3% metformin treated newly diagnosed T2DM patients reported one or more episodes of hypoglycaemia per year (Amiel et al., 2008). T2DM patients on insulin for

>5 years are at a significant risk of hypoglycaemia occurring in 25% of subjects and at rates similar to patients with recently diagnosed T1DM (Group, 2007). A number of factors predispose those with T2DM to hypoglycaemia, including advancing age, as counter-regulatory responses to hypoglycaemia decrease, even in nondiabetic individuals (Zammitt and Frier, 2005). Counter-regulatory responses also diminish with the duration of disease, as glucagon release during hypoglycaemia is reduced as patients become progressively insulin deficient (Segel et al., 2002). Renal failure reduces the clearance of many glucose lowering agents, including insulin, and predicts hypoglycaemic risk (Bruderer et al., 2014). Impaired hypoglycaemia awareness has not been intensively studied in the T2DM population, but it is well known that the elderly may experience a different symptom profile that can often be confused with strokes. There is a high prevalence of comorbid conditions, such as cognitive dysfunction, which impairs the ability to recognise and self-treat. Interestingly, epidemiology studies have shown no association between rates of hypoglycaemia and HbA1c (Akram et al., 2006), although some reports indicate a relationship with glycaemic variability (Zammitt and Frier, 2005). It is estimated that 28% of severe hypoglycaemia results in admission to hospital, with the associated healthcare costs amounting to around £1000 per admission (Leese et al., 2003). T2DM patients experience longer hospitals stays due to a greater burden of morbidity compared to T1DM patients (Zammitt and Frier, 2005).

1.6.2 Counter-regulatory Responses in Hypoglycaemia

Homeostatic mechanisms exist to maintain the glucose concentration within narrow limits because of the dependence of the central nervous system on glucose. A complex network of biochemical, functional and haemodynamic changes can restore glucose concentrations and produce symptoms which protect the body from the effects of hypoglycaemia. The body responds to hypoglycaemia in two ways: 1) increased endogenous glucose production through glycogenolysis and gluconeogenesis; and 2) behavioural responses to prompt an individual to eat. At the same time, energy is diverted to vital organs, such as the brain and liver, and away from less essential organs, such as the kidneys, during acute hypoglycaemia.

Counter-regulatory hormones are released in a hierarchical response at reproducible thresholds in normal humans (Figure 1-11). When plasma glucose levels fall to around 4.6 mmol/L, the first physiological defence is a decrease in insulin secretion by pancreatic beta cells (Cryer et al., 2003). Experiments have been performed to determine the relative importance of these hormones by producing isolated deficiencies of these hormones in response to insulin-induced hypoglycaemia (Rizza et al., 1979). When glucose falls to 3.8 mmol/L, the secondary defence of secreting glucagon by pancreatic alpha cells is initiated in an attempt to restore glucose level (Cryer, 2008). A third line of defence is adrenomedullary secretion of adrenaline secondary to the activation of the autonomic nervous system.

Adrenaline has a variety of metabolic effects, including increasing glycogenolysis and gluconeogenesis, reducing glucose utilisation peripherally, and inhibiting insulin secretion (Macdonald and King, 2007). Adrenomedullary adrenaline secretion becomes more relevant when glucagon is deficient, as in the case of T1DM and advanced T2DM. Growth hormone and cortisol secretion occur during more prolonged periods of hypoglycaemia lasting longer than one hour. The depth and duration of hypoglycaemia determines the magnitude of counter-regulatory response (Davis et al., 1997), but the overall resultant effect is an increase in hepatic glucose production and the limitation of glucose utilisation by non-neural tissues.

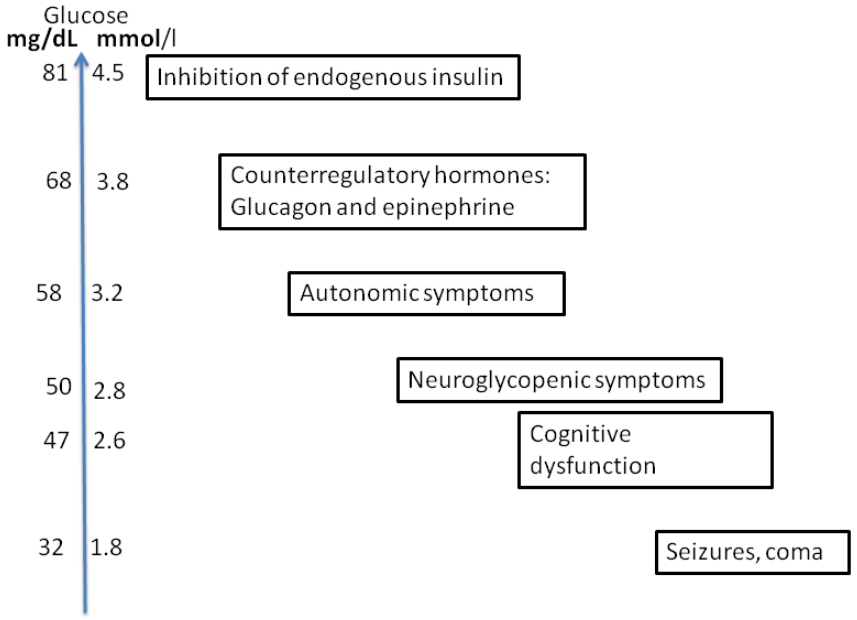

Figure 1-11: Thresholds for counter-regulatory responses and symptoms during hypoglycaemia in a nondiabetic adult

Counter-regulatory responses to hypoglycaemia have been extensively studied in normal subjects and in T1DM, but less systematically investigated in T2DM. Adrenaline secretion is preserved in individuals with

T2DM, although the glucagon response has been reported in different studies as either modestly diminished or preserved (Zammitt and Frier, 2005). In a study comparing nondiabetic, non-insulin treated, and insulin treated diabetic subjects, the glucagon response was almost absent in the insulin-treated group but intact in the others (Segel et al., 2002). Counter-regulatory hormonal release in some T2DM patients occur at higher glucose levels than in nondiabetic controls or T1DM patients (Korzon-Burakowska et al., 1998; Levy et al., 1998; Spyer et al., 2000).

The autonomic nervous system is responsible for neurogenic symptoms, such as sweating or tremor, as well as haemodynamic changes observed during hypoglycaemia. Hypoglycaemia is accompanied by a number of haemodynamic changes that include an increase in heart rate, systolic blood pressure, myocardial contractility, and cardiac output mediated via β-1 adrenoreceptors and a decline in peripheral resistance (Fisher et al., 1990). Changes in regional blood flow also occur, such as an increase in cerebral and splanchnic blood flow, thereby enhancing the delivery of substrates to vital organs. Experiments by Fagius et al. (1986) showed that during hypoglycaemia, muscle sympathetic activity increases after insulin is injected, peaking after 25-30 minutes, which coincides with a glucose nadir and persists for 90 minutes after euglycaemia is restored.

Recent antecedent hypoglycaemia causes attenuation in sympathoadrenal activation against subsequent hypoglycaemic episodes, leading to reduced neurogenic symptoms and hypoglycaemic unawareness (Dagogo-Jack et

al., 1993; Heller and Cryer, 1991), However, it can be reversed by scrupulous avoidance of hypoglycaemia for as little as two to three weeks (Dagogo-Jack et al., 1994). There is evidence that this phenomenon is not limited to T1DM, as Segel et al. (2002) demonstrated antecedent hypoglycaemia resulted in lower glucose thresholds required to activate glucagon, catecholamine and symptomatic responses in advanced T2DM and that these responses were attenuated in subsequent hypoglycaemia. The mechanisms underlying this impairment of counterregulation are uncertain, however it is likely to be centrally mediated. Based on brain imaging studies, the paraventricular nuclus of the thalamus has been implicated in the habituation of sympathoadrenal responses following hypoglycaemia. Other groups have reported alterations in glucose sensing by glucose excitatory and gluose inhibitory neurones in ventomedial hypothalamus (Cryer, 2013) . A number of studies have found augmentation of sympathoadrenal responses to repeated hypoglycaemia with selective serotonin reuptake inhibitors, alpha and beta blockade, opiate antagonists and selective K_{ATP} agonists suggesting multiple mediators and complex neural circuitry are involved (Cryer, 2013).

1.7 Potential Mechanisms of Hypoglycaemia-Related Sudden Cardiac Death

In the above section, I have argued that hypoglycaemia may be causal factor in the increase in cardiovascular mortality during intensive glycaemic control in T2DM patients. Mechanisms by which hypoglycaemia can exert

adverse CV events are incompletely understood. Early studies have investigated the effects of transient hypoglycaemia induced by an insulin bolus. Subsequen studies have examined the effects of more sustained hypoglycaemia using hyperinsulianemic clamps. However, most studies have been in T1DM and nondiabetic individuals with few studies in T2DM patients. The purpose of this section is to review the existing literature on CV effects of hypoglycaemia and propose putative mechanisms by which hypoglycaemia may predispose to CV events in T2DM individuals.

1.7.1 Endothelial Function and Atherogenesis

Human and animal studies have indicated the mechanisms by which hypoglycaemia might contribute to early atherogenesis. Human endothelial cells, when exposed to acute hypoglycaemia, show a rapid reduction in NO availability (Wang et al., 2011). There is some data to suggest that acute hypoglycaemia can impair flow-mediated dilatation by both NO-dependent and -independent mechanisms in people with T2DM (Gogitidze Joy et al., 2011). Acute hypoglycaemia can also induce the release of potent vasoconstrictors, such as endothelin (Wright et al., 2007).

Atherogenic cell adhesion molecules appear to be up-regulated following hypoglycaemia. VCAMs and intracellular cell adhesion molecules (ICAMs) are higher during hyperinsulinaemic hypoglycaemia in both healthy individuals and people with T1DM (Gogitidze Joy et al., 2010). Rats that were exposed to repetitive episodes of hypoglycaemia had an increased number of adherent monocytes in the aorta (Jin et al., 2011). The effect of

hypoglycaemia on leukocyte trafficking has also been studied, with Wright et al. (2010) reporting an increase in CD40 ligand and platelet monocyte adhesion in both healthy participants and those with T1DM diabetes. Some of these mechanisms may be mediated by adrenaline, as adrenergic blockade prevents the up-regulation of ICAM and VCAM induced by hypoglycaemia in a rat model of diabetes (Jin et al., 2011). Clinically, there is evidence that people with T1DM diabetes and a history of recurrent hypoglycaemia have increased carotid and femoral intima-media thickness suggesting early atherosclerosis, compared with individuals with a similar duration of disease and matched risk factors (Gimenez et al., 2011).

1.7.2 Inflammation

Diffuse inflammation is important in promoting transition to a vulnerable plaque that may rupture. Inflammatory markers, such as CRP and IL-6, are strong predictors of the risk of ACS in cohort studies (Packard and Libby, 2008). In one study, CRP increased for 24 hours after transient insulin induced hypoglycaemia in T1DM subjects and healthy volunteers (Galloway et al., 2000). During insulin tolerance tests performed on normal individuals, a rise in TNF-α and interleukin 8 (IL-8) has been observed that is likely to be adrenaline mediated, along with a later rise in IL-1 that is likely to be cortisol mediated. Gogitidze Joy et al. (2010) reported an increase in IL-6 during acute hypoglycaemia in T1DM and healthy individuals. However, the effect of acute hypoglycaemia on proinflammatory cytokines has not always been consistent, as Wright et al.

(2010) reported significant increases in hsCRP in healthy but not T1DM subjects following insulin-induced hypoglycaemia. Furthermore, the authors reported rises in IL-6 that occurred during both hyperinsulinaemic euglycaemia and hypoglycaemia. These discrepancies may be due to differences in the method of inducing experimental hypoglycaemia and the biological variability of these markers.

1.7.3 Platelet Activation and Aggregation

Platelets make important contributions to the atherothrombotic process, as they adhere to the damaged endothelium and contribute to the development of atherosclerotic lesions. Activated platelets also contribute to the onset of arterial thrombosis following plaque rupture. In human studies, platelet activation occurs in response to acute hypoglycaemia, resulting in the release of beta-thromboglobulin from platelet granules and the expression of cell surface receptors, such as P-selectin (Gogitidze Joy et al., 2010; Trovati et al., 1986). These changes are inhibited by the alpha-2-adrenoreceptor blockade (Kishikawa et al., 1987). Hypoglycaemia has also been shown to potentiate platelet aggregation induced by other agonists, such as adenosine diphosphate (ADP) and collagen (Trovati et al., 1986) via an alpha-2 adrenergic mechanism (Kishikawa et al., 1987).

1.7.4 Coagulation and Fibrinolysis

Hypoglycaemia is associated with an increase in procoagulant factors, such as factor VIII activity (Grant et al., 1987). Receptor blockade with propranolol prevents a rise in factor VIII associated with hypoglycaemia,

which suggests that this is mediated by adrenergic control (Grant, 1990). vWF, an endothelial-derived factor that promotes thrombosis, is increased in response to hypoglycaemia and is raised in patients with diabetes (Fisher et al., 1991), whilst a reduction in activated partial thromboplastin time (APTT) was observed following insulin-induced hypoglycaemia (Dalsgaard-Nielsen et al., 1982).

In contrast, some studies suggest that the fibrinolytic system is activated in response to hypoglycaemia induced by an insulin bolus. An increase in tissue plasminogen activator (tPA), plasminogen activator activity, and a decrease in inhibitory factors, such as PAI-1 (Fisher et al., 1991; Wieczorek et al., 1993), have been reported during hypoglycaemia. Most of these earlier studies did not control for the effects of insulin and it is unclear whether these observations were due to the effects of insulin per se. Hypoglycaemia stimulated by an insulin bolus was highly unphysiological does not represent hypoglycaemia in a diabetic individual during therapy. Preliminary data published by my collaborators have showed increases in clot lysis times during sustained hyperinsulinaemic hypoglycaemia in T1DM subjects (Ajjan et al., 2009; Gogitidze Joy et al., 2010). However, more studies are needed to delineate the role of hypoglycaemia in coagulation and fibrinolysis.

1.7.5 Cardiac Ischemia

Hypoglycaemia is accompanied by sympathoadrenal activation and catecholamine release, which can have widespread effects on the

cardiovascular system, including a transient increase in heart rate mediated by beta-adrenoreceptors (Fisher et al., 1990; Russell et al., 2001). Cardiac output increases during hypoglycaemia, mainly via increased myocardial contractility stimulated by β1-adrenergic receptors (Fisher et al., 1987). The demand for myocardial oxygen would be expected to rise with this increase in workload, and under normal circumstances there should be a matched increase in myocardial blood flow; however, there is evidence that myocardial blood flow may be reduced during insulin-induced hypoglycaemia. This has been observed in healthy volunteers and people with T1DM without known coronary artery disease (Rana et al., 2011). Release of potent vasoconstrictors into the coronary circulation can mediate this effect, and in patients with diabetes and existing flow-limiting disease, it is possible that such a reduction in flow leads to myocardial ischemia or even infarction. In an experimental canine model of coronary artery occlusion, the area of early myocardial necrosis increased by nearly 50% following exposure to hypoglycaemia (Libby et al., 1975).

There have been isolated reports of myocardial ischemia observed during diabetic hypoglycaemic comas (Ben-Ami et al., 1999; Markel et al., 1994), but given the intense haemodynamic changes associated with hypoglycaemia, one would expect ischaemia to be more frequently observed. De Desouza et al. (2003) monitored 19 individuals with T2DM with a history of frequent hypoglycaemia and coronary artery disease. Patients underwent 72 hour continuous glucose monitoring (CGM) with

simultaneous cardiac Holter monitoring for ischemia. Among the 54 recorded episodes of hypoglycaemia, 10 were associated with symptoms of chest pain and 4 with ischaemic ECG changes. Silent ischaemia is more common in patients with diabetes, and there have been several case reports of silent myocardial infarctions associated with hypoglycaemia where low glucose was the presenting feature (Chang et al., 2007; Pladziewicz and Nesto, 1989). Therefore, cardiac ischemia secondary to hypoglycaemia may occur more commonly than is recognised.

1.7.6 Cardiac Arrhythmias

There are anecdotal reports of a variety of arrhythmias precipitated by hypoglycaemia. Atrial fibrillation has been commonly reported, and is reversible through the administration of intravenous dextrose (Baxter et al., 1990; Collier et al., 1987; Odeh et al., 1990). There have also been other reports of premature ventricular contractions (Shimada et al., 1984) and atrioventricular junctional tachycardia (Pezzarossa et al., 1993), although VT has rarely been reported, perhaps because such events are usually fatal (Chelliah, 2000). Hypoglycaemia has also been associated with bradyarrhythmias in several reports (Gill et al., 2009; Navarro-Gutiérrez et al., 2003; Pollock et al., 1996). This appears counterintuitive given that sympathoadrenal activation is expected to increase the heart rate (see discussion below). In a study by Gill et al. (2009), 25 T1DM patients underwent ambulatory ECG monitoring with CGM. Thirteen episodes of nocturnal hypoglycaemia occurred and eight were associated with cardiac

arrhythmias, including bradycardia, ventricular ectopics, atrial ectopics and P wave abnormalities.

1.7.7 Arrhythmia Mechanisms: Autonomic

It is well established that hypoglycaemia is accompanied by neural-mediated activation of the sympathetic nervous system and adrenaline release. As alluded to above, there is strong evidence to support a sympathovagal imbalance as a risk factor for SCD. Several studies have examined the effect of hypoglycaemia on the sympathovagal balance using spectral analysis of HRV. Koivikko and colleagues studied six T1DM diabetics and eight healthy subjects over a two day single blinded cross over design of euglycaemic clamps and hypoglycaemic clamps one week apart (Koivikko et al., 2005). Cardiac vagal activity, as reflected by the high frequency (HF) component of the spectral analysis, decreased progressively during hypoglycaemia in both controls and diabetic subjects. The increase in heart rate and decrease in high-frequency power were not related to an increase in adrenaline or noradrenaline. This suggests that adrenomedullary sympathoexcitation was not the primary cause of the altered HRV, but were most likely a result of reduced cardiac vagal outflow caused by the hypoglycaemia itself. Laitinen et al. (2003) performed serial euglycaemic clamps (target glucose 5 mmol/L for 2 hours) followed by hypoglycaemic clamps (target glucose 3 mmol/L for 2 hours) on 18 healthy subjects, and reported a trend towards a decrease in the HF component while the low frequency (LF) band remained the same during

hypoglycaemia. However, conflicting results were reported by Schachinger et al. (2004), who found an increase in HRV and vagal activity during hypoglycaemia in healthy volunteers. This discrepancy may be attributed to shorter HRV recordings in the Schachinger study which are less accurate and differences in the hypoglycaemic clamp protocols. Consequently, further studies are required to investigate if similar autonomic changes occur in T2DM patients during hypoglycaemia.

1.7.8 Arrhythmia Mechanisms: Repolarisation

Hypoglycaemia can affect repolarisation characteristics and the action potential duration of cardiac cells. Low glucose may reduce the repolarisation reserve by a direct effect on the main repolarising potassium channels in cardiac cells (Zhang et al., 2003b). In normal subjects, hypoglycaemia produced by an insulin infusion resulted in lengthening of the QT interval (Eckert and Agardh, 1998). This also occurs in T1DM and T2DM patients (Marques et al., 1997). Observational studies suggest a lengthening of the QT interval occurs during nocturnal hypoglycaemia in T1DM subjects (Gill et al., 2009; Murphy et al., 2004; Robinson et al., 2004).

There have been relatively few studies on the effect of hypoglycaemia on changes in QT interval in T2DM subjects. Marques et al. (1997) performed controlled hypoglycaemia on seven noninsulin dependent individuals with T2DM and eight insulin dependent T1DM subjects with no CVD, and the subjects participated in hyperinsulinaemic hypoglycaemic clamps with a target glucose of 3 mmol/L for 120 minutes. In the non-insulin dependent

diabetics, the QT interval increased after 60 minutes of hypoglycaemia and continued to increase for 120 minutes. This was paralleled by an increase in adrenaline. T1DM subjects had a similar degree of QT prolongation, but QT plateaued by 120 minutes of hypoglycaemia. Another study by Landstedt-Hallin et al. (1999) examined 13 patients with T2DM on insulin and glibenclamide with a mean HbA1c 7.6% and mean duration of diabetes of 9 years. An increase in the QT interval and QT dispersion (QTd) were found following 60 minutes of hypoglycaemic clamp with at blood glucose of 2.5-3.0 mmol/L.

Increases in catecholamine levels appear to be the main driver of QT prolongation. Adrenaline causes QT lengthening when infused into humans in a dose dependent manner, while a beta-blockade with atenolol prevented hypoglycaemia induced QTc and QTd lengthening in healthy humans (Robinson et al., 2003). Pre-treatment of a group of T1DM subjects with atenolol similarly attenuated the QTc lengthening induced by hypoglycaemia in physiological studies (Lee et al., 2005). QT lengthening seems to occur independently of a fall in extracellular potassium as QT increases were observed in hyperinsulinaemic hypoglycaemia despite potassium clamping (Robinson et al., 2003). Thus adrenaline may have a direct effect on the myocardium independent of potassium.

1.7.9 Effects of Hypoglycaemia beyond the Acute Episode

The cardiovascular effects of hypoglycaemia in the hours and weeks that follow an acute episode remain largely uninvestigated. Hypoglycaemia-

associated autonomic failure is a term that has been coined to describe the attenuation of counter-regulatory responses mediated by the autonomic nervous system, following an episode of hypoglycaemia (Cryer, 2005). Our group has shown that impaired counter-regulatory responses can persist for at least several days following an episode of hypoglycaemia (George et al., 1995). However, autonomic impairment caused by hypoglycaemia may be more generalised and not limited to hypoglycaemic responses alone. One study examined the effect of antecedent hypoglycaemia on cardiac autonomic function in healthy participants, and found that two episodes of insulin-induced hypoglycaemia lasting 60 minutes resulted in decreased baroreceptor sensitivity, cardiovagal function, and autonomic responses to hypotensive stress for at least one day (Adler et al., 2009). In rats, antecedent hypoglycaemia also reduced the subsequent adrenaline responses to non-hypoglycaemic stimuli, such as hypotension, by 2- to 3-fold (Herlein et al., 2006). Cardiac autonomic impairment can increase the risk of fatal arrhythmias and reduce the response to physiological stressors. In people with existing diabetic autonomic neuropathy, this risk might be further increased. This highlights a plausible pathological pathway which might increase cardiovascular risk in the long-term.

The cardiovascular effects of recurrent hypoglycaemia are not known, and recurrent episodes may exert a cumulative effect on atherothrombotic risk (Jin et al., 2011). Alternatively, sympathoadrenal responses are attenuated by repeated episodes of hypoglycaemia and this might actually protect

against the cardiovascular effects mediated by adrenergic mechanisms. In a recent analysis of the ACCORD study, although mortality was higher in the intensively treated group, those with the highest number of recurrent hypoglycaemic episodes exhibited a slightly lower risk of death, which would be consistent with this hypothesis (Seaquist et al., 2012). A summary of the potential mechanisms by which hypoglycaemia may predispose to SCD are shown in Figure 1-12.

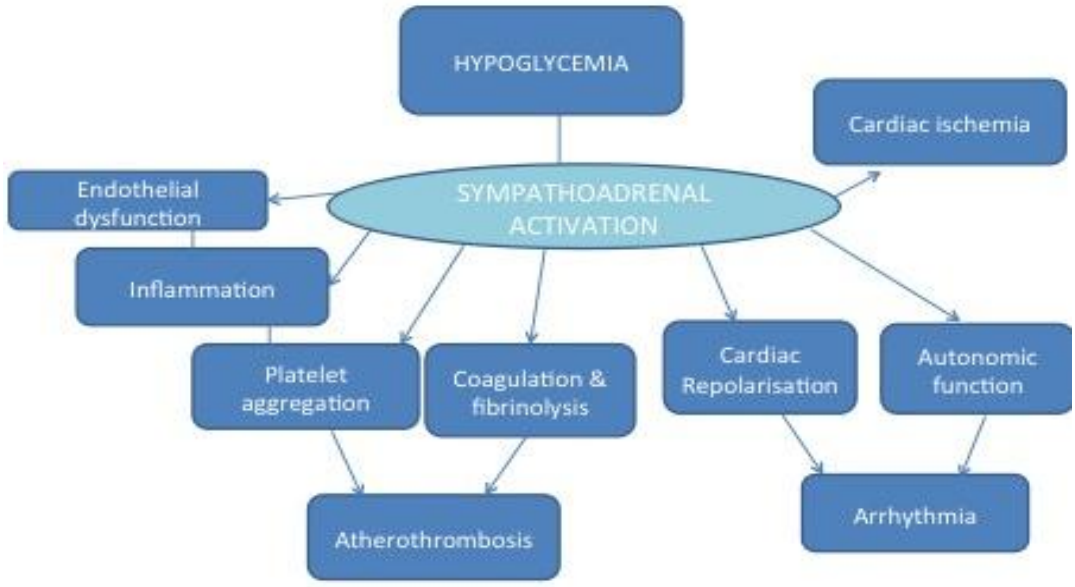

Figure 1-12: Potential mechanisms of hypoglycaemia-related SCD
Hypoglycaemia is accompanied by sympathoadrenal activation which can promote inflammation and platelet aggregation and hence the development of a vulnerable plaque. These effects are likely to be cumulative over months to years. The effect of hypoglycaemia on coagulation and fibrinolysis can contribute to acute coronary thrombosis and can affect autonomic function during an acute episode but also cause more sustained impairment. This in conjunction with abnormal repolarisation that is associated with acute hypoglycaemia, which may act as a final pathophysiological trigger for a fatal tachyarrhythmia.

1.8 Summary

To summarise, T2DM is associated with an increased risk of CVD through multiple pathways involving premature atherosclerosis, increased

thrombogenicity and altered myocardial metabolism and function. Hyperglycaemia may be responsible for some but not all of the excess risk, and clearly insulin resistance, dyslipidaemia, hypertension, and adipocity-related inflammation can contribute even in the pre-diabetic state. Intensive glycaemic control reduces microvascular complications, but has not consistently reduced cardiovascular mortality in subjects at known high cardiovascular risk (Duckworth et al., 2009; Patel et al., 2008); in one trial there was increased mortality (Gerstein et al., 2008) including an excess of SCDs. Hypoglycaemia, which was increased in the intensive treatment arm, was one of the strongest predictors of mortality in *post hoc* analyses. Although the association could be due to shared confounding factors, the relationship is strong and has been consistently observed in other settings. Experimental studies in T1DM and nondiabetic individuals have shown that hypoglycaemia is capable of inducing a wide range of adverse cardiovascular effects, many of which can predispose to SCD, thereby making the association biologically plausible.

Given the global epidemic of T2DM which is set to rise in coming decades, it is a pressing question as to the optimal glycaemic strategy for CVD reduction. Since the publication of the ACCORD, ADVANCE and VADT studies, there has been considerable controversy as to 'how low to go'. Whether hypoglycaemia, which is unfortunately a common iatrogenic complication of some glucose lowering therapies, is causal in increasing the risk of cardiovascular death, is an important question which cannot easily

be answered from observational studies and trial data. Therefore exploring mechanisms by which hypoglycaemia can increase the risk of SCD using experimental studies may help answer these questions.

1.9 Aims of the Thesis

There is a dearth of experimental studies on the cardiovascular effects of hypoglycaemia in T2DM patients. Nearly all the studies mentioned earlier have been performed in nondiabetic and T1DM individuals and consequently the results must be extrapolated with some caution to T2DM populations, as there are physiological differences. In general, T2DM patients have preserved adrenaline responses to hypoglycaemia compared to T1DM individuals (Segel et al., 2002; Zammitt and Frier, 2005). Furthermore, counter-regulation may occur at a higher threshold compared to T1DM or nondiabetic individuals (Spyer et al., 2000). The magnitude of the adrenaline released is expected to be greater for the same level of hypoglycaemia, thus catecholamine-mediated adverse cardiovascular effects may be even more pronounced in T2DM individuals. Insulin resistance that characterises T2DM is associated with prothrombotic and proinflammatory effects (Grant, 2007), for example endogenous levels of PAI-1 are known to be higher in T2DM patients (Grant, 2007). The prevalence of ischaemia and cardiac structural abnormalities are higher than in T1DM or nondiabetic populations and provide further substrates for SCD in T2DM individuals.

Secondly, existing experimental studies have concentrated on the cardiovascular effects of acute hypoglycaemia up to 24 hours, yet there is ample evidence to suggest that acute hypoglycaemia may have downstream effects. In the ACCORD trial, few deaths were judged to have occurred at the time of acute hypoglycaemia (Bonds et al., 2010). Instead, hypoglycaemia appears to predict the risk of death in the weeks and months following an episode. In the VADT study, hypoglycaemia was the strongest independent predictor of mortality at 90 days (Duckworth et al., 2009). Therefore an important and novel aspect of this work is to explore whether hypoglycaemia exerts more sustained effects on the cardiovascular system beyond the immediate episode. I will investigate whether autonomic, thrombotic and inflammatory changes may persist up to seven days after an episode of hypoglycaemia.

The choice of mechanisms to investigate is based on theoretical and practical considerations. It is known that the increase in mortality associated with intensive glucose lowering occurs in patients with established CVD but not in those with a low atherogenic burden; furthermore, there was an excess of fatal myocardial infarcts over nonfatal myocardial infarcts in those receiving intensive glycaemic therapy. Thus the more proximate mechanisms of acute coronary thrombosis, transient autonomic imbalance and abnormal repolarisation, as triggers for fatal arrhythmias, may be more relevant than earlier processes of endothelial dysfunction and atherogenesis. The acute effects of hypoglycaemia on

cardiac ischaemia are clearly important; however, there are ethical considerations of experimentally inducing hypoglycaemia in T2DM patients with established ischaemic heart disease. Thus the main mechanisms chosen to be investigated are the effects of hypoglycaemia on thrombosis (platelet function, fibrin clot formation and lysis), cardiac autonomic function, and cardiac repolarisation.

1.9.1 Aims and Hypothesis

Aim: To explore potential mechanisms of hypoglycaemia-related SCD in T2DM

Hypothesis: Hypoglycaemia predisposes to SCD in patients with T2DM through the following mechanisms:

i) Adverse effects on thrombosis - increases in platelet reactivity, fibrin network characteristics and hypofibrinolysis

ii) Adverse effects on cardiac autonomic function

iii) Abnormal cardiac repolarisation

The effects of hypoglycaemia are aimed to be investigated over the course of an acute hypoglycaemic episode and up to seven days after an episode of hypoglycaemia.

Chapter 2 - Research Design and Methodology

The purpose of this chapter is to justify the choice of the experimental method and the reasoning behind using complementary approaches for studying both spontaneous and experimentally induced hypoglycaemia. The choice of study population will be discussed together with the advantages and limitations of each approach. The design of the hyperinsulinaemic clamp protocol will be presented and in addition the choice of electrophysiological and atherothrombotic outcome measures, including the theoretical underpinnings for each will be discussed.

2.1 Overview of the Study Design

The aims of the thesis were to investigate the acute and downstream effects of hypoglycaemia on: 1) fibrin characteristics and fibrinolysis; 2) cardiac autonomic function; and 3) cardiac repolarisation. Hypoglycaemia is difficult to study as it occurs relatively rarely, even in tightly controlled individuals with diabetes, and is mostly self-treated before presentation to a health professional. The introduction of CGMS has allowed the capture of spontaneous hypoglycaemia in ambulatory patients which can be combined with ambulatory ECG monitoring. Synchronous monitoring allows the determination of arrhythmias, non-invasive indices of autonomic function and cardiac repolarisation at the time of the episode. Furthermore, patients at high cardiovascular risk or with a history of CVD may be studied who otherwise may be unsuitable for more invasive

investigations employing experimentally induced hypoglycaemia. However, this is dependent on capturing sporadic, unpredictable episodes of spontaneous hypoglycaemia and it is not be possible to take biochemical measurements of thrombosis and counter-regulatory hormones at the time of an event. It is also impossible to control for the time, depth and duration of spontaneous hypoglycaemia, as well as the environmental conditions surrounding a hypoglycaemic episode.

The advantage of experimentally induced hypoglycaemia is that the intensity and duration of the hypoglycaemic stimulus can be controlled, together with the environmental conditions that may influence cardiovascular parameters. In addition, physiological measurements can be taken under stable glucose conditions and the exact timing of biochemical measurements for thrombosis and counter-regulatory hormones can be controlled and recorded at the time of hypoglycaemia, as well as at intervals after the episode. The hyperinsulinaemic hypoglycaemic clamp is a well-established method of simulating an episode of experimental hypoglycaemia under controlled conditions (DeFronzo et al., 1979). However, supraphysiological doses of insulin are used and this induces larger electrolyte fluxes and counter-regulatory responses than clinical episodes. The nature of experimental hypoglycaemia may be different as subjects are resting and supine as opposed to spontaneous hypoglycaemia when subjects are mostly ambulant, at least during the day. Further, the hyperinsulinaemic clamp technique is invasive and can only be performed

in a relatively small number of subjects, and it would be unethical to subject T2DM patients with high cardiovascular risk to experimental hypoglycaemia, who are the group of interest in this study.

It was therefore decided to use complementary approaches of ambulatory monitoring to investigate the acute electrophysiological response to hypoglycaemia in T2DM patients at high cardiovascular risk (study 1) and using experimentally induced hypoglycaemia (study 2) to examine the electrophysiological and thrombotic effects effects in patients with no CVD both during and after an episode. An overview of the two studies is presented below.

2.2 Study 1: Cardiac Electrophysiological Response to Spontaneous Hypoglycaemia in Individuals with Type 2 Diabetes and with a Cardiovascular Risk

2.2.1 Overview of the Study

The primary outcome of this study is the frequency of arrhythmia during spontaneous hypoglycaemia compared to euglycaemia in T2DM patients with known cardiovascular risk. Changes in cardiac autonomic function, as shown by heart rate variability (HRV), and changes in cardiac repolarisation, as shown by QTc intervals, were investigated as potential mechanisms that can predispose to arrhythmias.

Patients with T2DM taking insulin for > 4 years and with a history of CVD or at high CV risk were recruited to the study. Following a baseline

assessment which included autonomic function testing, all subjects underwent simultaneous continuous interstitial glucose (IG) and ambulatory ECG monitoring for five days. The frequency of arrhythmias, HRV and corrected QT were compared between hypoglycaemia and euglycaemia matched for the time of day. A hypoglycaemic episode was defined as interstitial glucose (IG) <3.5 mmol/L for at least 20 minutes and euglycaemia as 5-10 mmol/L. To account for diurnal variation in cardiac electrophysiology and counter-regulatory responses, episodes were further divided into daytime and nocturnal (2300 to 0700 hours) episodes for the analyses.

2.2.2 Study Participants

T2DM patients were recruited from Sheffield Teaching Hospitals diabetes outpatient clinics. Subjects who had been treated with insulin for at least four years were recruited, as these individuals were at highest risk of hypoglycaemia due to disease duration. In a recent clinical observational study, 25% of T2DM patients on insulin >5 years experienced one or more episodes of low IG <2.2 mmol/L during the study week, as recorded by CGM (Group, 2007). In this present study, the inclusion criteria were chosen such that patients had either established CVD or two or more CVD risk factors and were designed to mirror the characteristics of the ACCORD study cohort (Gerstein et al., 2008).

Inclusion criteria:

- T2DM patients on insulin treatment for 4 or more years

- History of CVD (ischemic heart disease, peripheral vascular or cerebrovascular disease) and/or two additional cardiovascular risk factors (hypertension and dyslipidaemia, both defined as requiring medication, a current smoker, obesity)

Exclusion criteria:

- Pregnancy
- Bundle branch block on resting ECG
- Permanent atrial fibrillation
- Prescription of QT prolonging drugs
- Severe diabetic complications (e.g. blindness) affecting the ability to participate in the study
- Myocardial infarction or coronary artery bypass graft <12 months ago
- Inability to communicate in English
- Inability to provide informed consent

2.2.3 Research Governance

Informed consent was obtained from all the participants according to the principles of good clinical practice. The study received local ethics approval (Appendix 3).

2.2.4 Baseline Assessment

Baseline data were obtained from patients, including age, sex, BMI, duration of diabetes, and duration of insulin treatment. Past medical

history and information on diabetes treatment and other medications were also obtained and a baseline 12 lead ECG was recorded. Blood pressure was measured using an automatic oscillometric sphygmomanometer (DINAMAP® GE Medical Systems Information technologies, Inc.) after lying supine for five minutes. For HbA1c, venous blood was collected into tubes containing ethylenedimiaminetetraacetic acid (EDTA) (BD Vacutainer™ K3E) and analysed via ion exchange high performance liquid chromatography (HPLC) techniques at Sheffield Teaching Hospital central laboratories. Blood (2 mL) was collected into tubes with a clot accelerator and separation gel (BD Vacutainer® SST™ Advance) and subjected to centrifugation at 300 ×*g* at room temperature for 10 minutes. The resulting serum was analysed for urea and electrolytes using an automated system (Cobas® analyser, Roche Diagnostics, UK) at Sheffield Teaching Hospital laboratories.

2.2.5 Cardiac Autonomic Reflex Tests

Cardiac autonomic reflex testing was performed to determine the presence of cardiac autonomic neuropathy (CAN) at baseline. The cardiovascular reflex tests were performed to assess the integrity cardiovagal and sympathoeffector responses via reflex pathways triggered by physiological manoeuvres. They consisted of three tests which predominantly assess cardiovagal function (heart rate to deep breathing, the Valsava manoeuvre, and heart rate response to standing) and two tests for sympathetic function (blood pressure response to standing and change in diastolic

blood pressure (DBP) in response to a sustained handgrip). Participants rested for three minutes before each test and the results were compared against age-adjusted normal ranges (O'Brien et al., 1986), with results below the 95% confidence limits regarded as abnormal. These simple bedside tests have been validated for diagnosis and staging, and as trial endpoints in CAN (Tesfaye et al., 2010; Vinik and Ziegler, 2007). Based on the most recent consensus (Tesfaye et al., 2010), the presence of two or more abnormal cardiovagal tests were defined as definite CAN and these participants excluded from the trial. A number of factors are known to influence autonomic function and these were controlled for. Venous glucose was concurrently measured to ensure participants were euglycaemic during the tests, and all participants were asked to avoid caffeine or smoking on the day of the tests and vigorous exertion 24 hours prior to the tests. The tests were performed according to the standardised protocols outlined below.

ECG signals were obtained using a three lead ECG monitor (Ivy Cardiac Trigger Monitor 3000, Ivy Biomedical Systems Inc., Branford CT, USA), which generates a synchronised pulse at the peak of each R wave. This was connected to the WR TestWorks™ Analogue Interface (WR Medical Electronics Company, Maplewood, USA) and ECG signals was digitised using a sampling frequency of 1000 Hz via a data acquisition device (NI-DAQCard-6062E, National Instruments™, UK) connected to a laptop computer (Toshiba Satellite 1800-S274). Data were recorded and analysed

using the WR-TestWorks™ software (version 2.4.0, WR Medical Electronics Co., Maplewood, USA). This software contained dedicated modules for the heart rate response to deep breathing, Valsava, and standing. A marker was placed on the ECG recording denoting the beginning of the manoeuvre and R-R intervals (measured in ms) were generated within the programme, with the tests results calculated according to definitions below.

2.2.5.1 Heart Rate Response to Deep Breathing

The subjects remained supine and breathed deeply at the rate of one breath per 10 seconds (i.e. six breaths per minute) for one minute while being monitored by ECG. Reproducibility was improved by visual cues and the participants breathed in and out according to a timed light display. The heart rate increases on inspiration and falls with expiration (sinus arrhythmia), and the expiration:inspiration (E:I) ratio is the ratio of the mean of the longest R-R intervals during deep expirations to the mean of the shortest R-R intervals during deep inspirations.

2.2.5.2 Heart Rate Response to the Valsalva Manoeuvre

The supine patient was connected to an ECG monitor and asked to forcibly exhale into a modified sphygmomanometer to a pressure of 40 mmHg for 15 seconds. Once the Valsalva was released, the subjects were asked to lie still without talking and recording continued for a further 45 seconds. This was repeated three times with one minute of rest in between. Normally, the reflex response to the Valsalva manoeuvre includes tachycardia and peripheral vasoconstriction during strain, followed by an overshoot in

blood pressure and bradycardia after release of the strain. The response to the performance of the Valsalva manoeuvre has four phases:

Phase I: Transient rise in blood pressure and a fall in heart rate due to compression of the aorta and propulsion of blood into the peripheral circulation. Haemodynamic changes are mostly secondary to mechanical factors.

Phase II: Early fall in blood pressure with a subsequent recovery of blood pressure later in the phase. Blood pressure changes are accompanied by an increase in heart rate, and there is a fall in cardiac output due to impaired venous return causing compensatory cardiac acceleration, increased muscle sympathetic activity, and peripheral resistance.

Phase III: Blood pressure falls and heart rate increases with the cessation of expiration.

Phase IV: Blood pressure increases above the baseline value (overshoot) because of residual vasoconstriction and restored normal venous return and cardiac output.

The Valsalva ratio is the longest R-R divided by the shortest R-R occurring within 45 seconds of the peak heart rate. The mean ratios from three attempts were recorded.

2.2.5.3 Heart Rate Response to Standing

Participants remained supine for at least five minutes and then stood to a full upright position. The normal response is a characteristic and rapid

increase in heart rate in response to standing that is maximal at approximately the 15th beat after standing, followed by a relative bradycardia that is maximal at approximately the 30th beat after standing. Because the maximum and minimum R-R intervals may not always occur at exactly the 15th or 30th beats after standing, the 30:15 ratio has been redefined as the longest R-R interval during beats 20–40 divided by the shortest R-R interval during beats 5–25 (Ziegler et al., 1992).

2.2.5.4 Systolic Blood Pressure Response to Standing

Blood pressure was measured using an automatic sphygmomanometer with the patient having remained supine for >5 minutes and then repeated one minute after standing. A drop of systolic blood pressure (SBP) <10 mmHg was classified as normal, 10-30 mmHg as borderline, and >30 mmHg as abnormal. A drop in DBP >10 mmHg was also considered abnormal.

2.2.6 Continuous Glucose and Ambulatory ECG Monitoring

All patients underwent five days of simultaneous 12 lead Holter and interstitial CGM. Patients carried on with their usual daily activities and diabetes treatments. Twelve lead ambulatory ECGs (Lifecard 12, Spacelabs Healthcare, Hertford, UK) were recorded at a sampling rate of 128 Hz with electrodes in a Mason-Likar configuration. IG was monitored using a CGMS which employed a wired enzyme electrochemical method of glucose detection (Freestyle Navigator Continuous Glucose Monitoring System, Abbott Diabetes Care, Maidenhead, UK) (Heller and Feldman, 2010).

Calibrations against capillary blood glucose were performed at least four times during the study week according to the manufacturer's instructions. This calibration was performed to convert sensor-detected currents into glucose readings and to deduce blood glucose levels from glucose values measured in interstitial fluid (Vaddiraju et al., 2010). One of the limitations of CGM is its reduced accuracy in the hypoglycaemic range due to a low sensor signal. A further limitation is that changes in IG lag behind that of blood glucose by 12-20 minutes. This has implications for the accuracy of calibration; hence the device has built-in functions which only permit calibration when IG is stable within a pre-specified range. The accuracy of CGM devices has been tested in hyperinsulinaemic clamps against arterialised blood glucose (Clarke et al., 2005). Mindful of the limitations of CGM, a system was selected that has been reported to follow the descent in blood glucose to the hypoglycaemic nadir (Clarke et al., 2005), with a lowest detection limit of 1.1 mmol/L (20 mg/dL).

IG was measured every minute by the CGM and 10 minute averages were reported (CoPilot Healthcare, Abbott Diabetes Care, Maidenhead, UK), with hypoglycaemia defined as IG ≤ 3.5 mmol/L in accordance with previously published studies (Gill et al., 2009), euglycaemia as 5-10 mmol/L, and hyperglycaemia defined as IG ≥ 15 mmol/L. These glycaemic thresholds were designed to allow for a margin of error in the CGM. Predictive alarms were switched off and participants were instructed not to view the glucose values except during calibrations. Patients were also

asked to keep a record of any symptomatic hypoglycaemia. An episode of low IG <3.5 mmol/L on CGM without the simultaneous self-reporting of symptoms was regarded as asymptomatic.

2.2.7 Arrhythmia Analysis

The 12 lead ambulatory ECG data was analysed with the Pathfinder Ambulatory ECG analysis system (v. 8.701, Delmar Reynolds Medical Ltd, Edinburgh & Hertford, UK). Leads I, II and V5 were used for analysis as they represented the orthogonal leads. Normal and aberrant beats were labelled by the Pathfinder system using a commercial algorithm with pre-set sensitivity to optimise the trade-off between preserving useful information versus eliminating artefacts. The ECG was manually screened for gross arrhythmias, whilst the software automatically detected arrhythmic events according to predetermined event definitions, which included atrial ectopic beats, bradycardia (defined as four or more consecutive beats at less than 45 beats per minute), ventricular premature beats (VPB) and complex VPB (bigeminy, trigeminy, couplet, Salvos and VT). Definitions of these terms are described in Appendix 1. All identified arrhythmic events were manually verified for accuracy, with investigators blinded to glucose values during arrhythmia analysis. Hourly counts for each type of arrhythmia were paired against hourly mean IG, which was categorised into hypoglycaemia (IG ≤ 3.5 mmol/L), hyperglycaemia (IG ≥ 15 mmol/L) and euglycaemia (5 mmol/L < IG < 10 mmol/L). Analyses were

separated into day and night (2300-0700 hours) in order to take into account diurnal variation.

2.2.8 Heart Rate Variability

Direct recordings of cardiac sympathetic and vagal neural activity are difficult to measure in the conscious human, thus non-invasive assessment of cardiac autonomic balance relies on measuring the efferent activity of end organs, such as the heart. At rest, short term heart rate changes are predominantly influenced by neural inputs from sympathetic and parasympathetic traffic, and the right atrial stretch. The R-R interval and fluctuations are linearly related to vagal cardiac activity, and this has been validated in anaesthetised dogs where the R-R interval has been shown to be prolonged in direct proportion to right vagal stimulation (Eckberg and Sleight, 1992). There are limited data for humans, but Carlsten et al. (1957) performed vagal stimulation in six patients undergoing radical neck surgery for cancer and found that the R-R interval increased linearly with stimulation frequency and plateaus at a higher range. Vagal activity leads to rapid changes in heart periods as the acetylcholine is rapidly hydrolysed. There is general accepted that rapid changes in the R-R interval are almost entirely mediated by changes in vagal cardiac activity (Eckberg and Sleight, 1992; Fouad et al., 1984).

Different computational methods may be applied to represent the variation in R-R intervals over time. The time domain measures include the standard deviation of 5-minute averages of normal R-R intervals (SDNN),

and the root-mean square of the difference of successive R-R intervals (RMSSD), which represent long and short term variations in parasympathetic activity. HRV can also be studied using power spectral density analysis, which describes how variance is distributed as a function of frequency (Figure 2-1). As mentioned previously, the vagus nerve acts rapidly on the sinus node. Therefore fluctuations of the heart rate at a HF (0.15-0.4 Hz) are thought to represent parasympathetic activity. In dogs and humans, parasympathetic blockade by atropine eliminates most heart rate fluctuations above 0.15 Hz (Parati et al., 1995)

The interpretation of the low frequency component (0.04-0.15 Hz) of HRV is a matter of great controversy, and the concept of normalised LF as a measure of relative sympathetic contribution was introduced by the Malliani group (Pagani et al., 1986). In healthy subjects who underwent a graded tilt, there was an observed correlation between the degree of tilt and the LF and HF power expressed as a proportion of the total power (LFnorm and HF norm) in the spectra, and the LF-to-HF ratio. However, there was no correlation between the tilt angle and the absolute values of LF power (Montano et al., 1994). Rather than increasing or decreasing fluctuations in heart rate, sympathetic activity appears to act as a low-pass filter

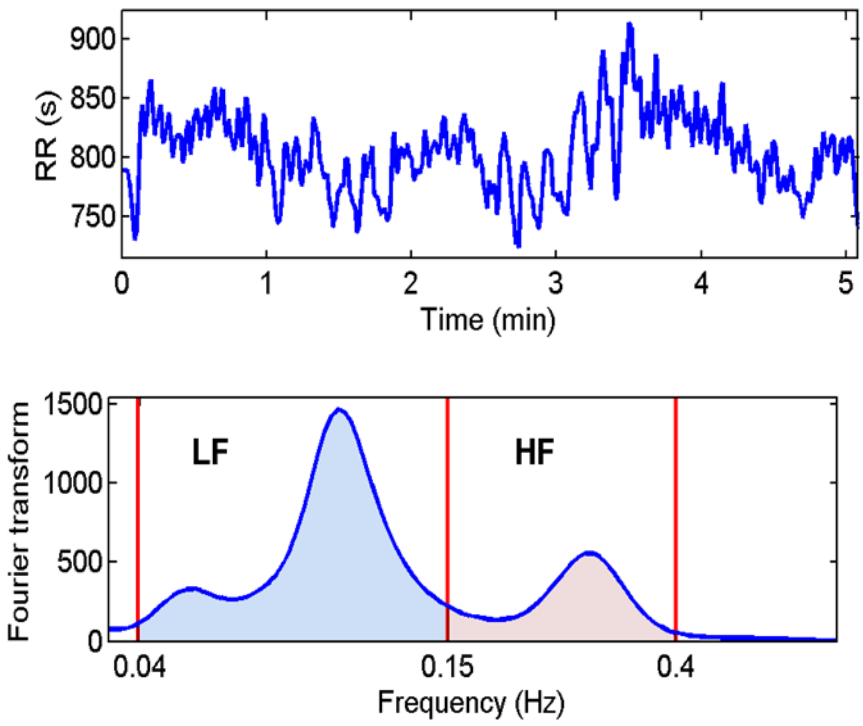

Figure 2-1: Power spectra of heart rate variability
The variation in R-R interval is mathematically transformed using the Fourier transformation into a power spectrum. Power spectra are further divided according to frequency domains: Low frequency LF (0.04-0.15 Hz) and high frequency HF (0.15-0.4 Hz).

(Bernardi et al., 2011). Thus during sympathetic activation (e.g. upright posture, exercise or heart failure), the increasing sympathetic contribution reduces the respiratory-mediated HF component and there is a global reduction in total power in the heart rate spectra. However, the relative contribution of LF power to the total power is increased, thus LF expressed only in normalised units is meaningful for expressing the sympathovagal balance (Bernardi et al., 2011). Another important concept to bear in mind is that HRV measures fluctuations in autonomic inputs to the heart, rather than the mean level of autonomic inputs.

There are a number of limitations to the use of LF as a measure of cardiac sympathetic activity. In animals, heart rate fluctuations in the LF domain are affected by electrical stimulation of both the vagal and sympathetic cardiac nerves. In humans, heart rate variations in the LF range are reduced by either parasympathetic or sympathetic pharmacological blockade (Parati et al., 1995), and there is a weak correlation between cardiac norepinephrine spill-over and LF, and the LF:HF ratio (Goldstein et al., 2011). Furthermore, heart rate variation in the LF range is also influenced by respiration, as slow-breath-induced low frequencies can occur during spontaneous breathing (Bernardi et al., 2011), although this effect can be controlled by paced respiration during HRV recordings. There is some evidence that LF is more closely correlated with BRS (Goldstein et al., 2011). However, this has also been challenged (Martelli et al., 2014). Despite these limitations, practically there are few non-invasive methods of measuring cardiac sympathetic activity, and the use of normalised LF or LF:HF as an index of relative sympathetic contribution has been supported by the Toronto Consensus panel on Diabetic Neuropathy (Bernardi et al., 2011).

2.2.8.1 Heart Rate Variability Analysis

The reliability of HRV analysis can be influenced by a number of factors, such as the length of recording, and the occurrence of ectopic beats and artefacts. HRV analysis in this study was performed in accordance with the recommendations of the Taskforce on Heart Rate Variability (1996), and R-

R intervals were extracted only from annotated normal beats (NN intervals) using the Pathfinder Ambulatory ECG analysis system, as ectopic beats will distort the spectra. For reliable analysis, the ECG recording must be at least 10 times longer than the wavelength of interest; therefore a 5 minute segment of successive NN intervals was selected around each reported IG value. HRV analysis was performed on each 5 minute ECG segment, and the time domain measures of SDNN and RMSSD were calculated. Spectral analysis of HRV was performed using the Fourier transformation. The LF band was defined as 0.04-0.15 Hz and the HF band as 0.15-0.4 Hz, and the ratio between the LF power and total power was calculated (LFnorm).

2.2.9 Cardiac Repolarisation

The QT interval is defined from the onset of the QRS complex, which marks the depolarisation of the first myocardial ventricular cell, to the end of the T wave, which marks repolarisation of the last ventricular myocyte. QT prolongation results from an increased action potential in the ventricular myocardium, and it remains the most widely used surface ECG marker for abnormal repolarisation given its ability to predict potentially fatal ventricular tachyarrhythmias.

Although automatic QT detection algorithms are available, these are not reliable, especially when T wave morphologies are abnormal, which our group has shown to occur during hypoglycaemia (Marques et al., 1997). Thus a semi-automatic algorithm was employed which allows the manual verification of fudicial points on the ECG calculated by the computer

algorithm. First, annotated normal ECG beats were identified using the Pathfinder system (v.8.701, Delmar Reynolds Medical Ltd, Edinburgh and Hertford, UK) from the orthogonal leads I, II and V5 to represent global repolarisation. On each lead, a 40 Hz high pass filter was implemented to reduce the noise (Badilini et al., 1999), and a cubic spline interpolation was then applied to remove LF baseline wander without affecting the higher frequency ECG components. A composite wave was then generated from the orthogonal leads I, II and V5 to represent global repolarisation. Analysis of the composite wave was performed on a five minute window centred on each IG value.

QT intervals from ambulatory recordings were analysed based on a selective beat averaging approach, as described by Badilini et al. (1999).The principle of selective beat averaging is to obtain higher signal-to-noise ratio templates for QT analysis (Badilini et al., 1999). In this method, the beats with a stable preceding heart rate were selected in order to respect the restitution properties of ventricular repolarisation. Beats that occur during heart rate changes may exhibit a different repolarisation shape compared to those occurring at a stable heart rate because the QT interval has not fully adapted. In this study, beats with a preceding R-R interval within ± 15 ms and a second preceding R-R interval within ± 50 ms of the prevailing mean R-R across the five-minute segment were averaged.

The composite wave was then calculated from the averaged beats derived from leads I, II and V5. In the composite wave, the onset of the Q wave was

marked as the first positive deflection from the isoelectric line above 10 mV (Lee et al., 2003), whilst the end of the T wave was determined using the tangent method, whereby the tangent to the steepest downslope of the T wave crosses the isoelectric line (Figure 2-2). The tangent method has previously been employed in determining QT changes during experimental hypoglycaemia (Robinson et al., 2003), and has advantages over other methods, such as the threshold method, which require a stable isoelectric baseline. All median beats were manually reviewed and the fiducial points adjusted if necessary by two independent observers blinded to the glucose values. Furthermore, all the T waves were manually classified as normal, notched or fusion, according to predetermined criteria described in Appendix 2.

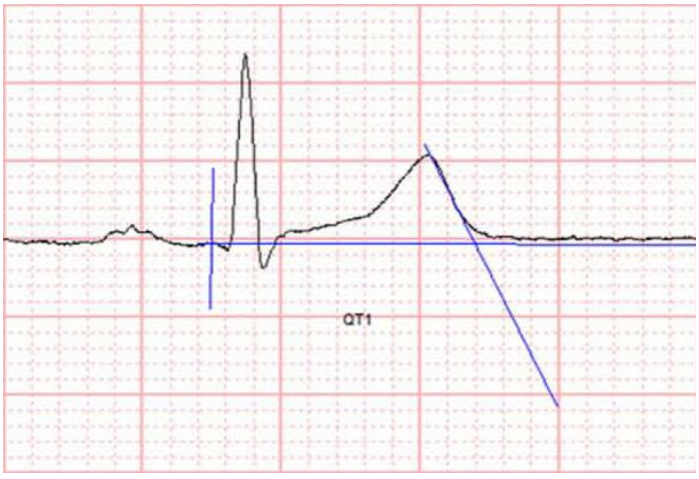

Figure 2-2: Tangent method of T end determination
The peak of the T wave is identified by finding the return to the baseline of the first derivative, with the subsequent maximal negative dv/dt identifying the point of peak downslope of a positive monophasic T wave. Where the tangent intersects the baseline is defined as the T end. The baseline is defined by the PR segment and/or the TP segment if repolarisation does not interfere with the subsequent P wave (Lepeschkin and Surawicz, 1952).

The QT interval is affected by the heart rate, so a heart rate correction is necessary to compare QT intervals at different heart rates. The QT interval is most commonly corrected using Bazzett's formula:

$$\text{Corrected QT} = \frac{\text{QT Interval}}{\sqrt{\text{R}} - \text{R interval}}$$

However, Bazett's formula tends to overcorrect at high heart rates and under correct at low heart rates, and in addition, fixed correction formulae assume that QT/R-R relationships are uniform between individuals. It has been shown that there are significant inter-individual differences in QT/R-R relationships due to effects such as age and gender. Therefore, QT intervals were corrected for heart rate (QTcS) using subject-specific regression formulae, as has been previously employed in studies of hypoglycaemia (Christensen et al., 2010). Individualised linear regression formulae were derived that were based on all the QT/R-R values obtained for each individual during euglycaemia.

2.2.10 Statistical Analysis

2.2.10.1 Power Calculation

This was an observational study. There was no prior information available as to any of these potential relationships and so a power calculation would have been based upon guesswork. Consequently, no power calculations were performed, but the numbers chosen were based upon an assessment of the number of patients it was possible to examine given the constraints

on recruitment and the projected hypoglycaemia rates of approximately 1 in 4 patients during the study week (2007).

2.2.10.2 Statistical analysis

Data were inspected for normality and data that was found to follow an approximate normal distribution were summarised using the mean (SD), whilst skewed data were summarised using the median (IQR). Demographic data between patients who experienced at least one hypoglycaemic episode versus those that experienced none were compared using an independent t-test, Mann-Whitney U test, or Fisher's exact test for the effect of the insulin regimen and insulin type on hypoglycaemia.

The generalised estimated equations approach was employed to investigate the effect of the glycaemic status on arrhythmia counts while taking into account correlated measurements from individuals who experienced more than one episode of hypoglycaemia or hyperglycaemia. The Poisson model, which is usually used to analyse count data, was not optimal in this study as there were many individuals who did not experience arrhythmic events. For this reason, the data were fitted with a negative binomial model which takes into account the exposure time and the individuals experiencing no arrhythmic events. A first order autoregressive correlation structure was applied to adjust for the within-individual correlation. Exponentiated regression coefficients represent the

incident rate ratios (IRR), and the IRR of arrhythmias during hypoglycaemia and hyperglycaemia compared with euglycaemia were calculated.

HRV parameters and QTc were compared at the glucose nadir of the hypoglycaemic or glucose maxima of a hyperglycaemic episode against an equivalent euglycaemic time point on a different day. Where there was more than one matching hypoglycaemic-euglycaemic episode in an individual participant over the course of the recording period, the mean from all daytime and nocturnal episodes from that individual were taken respectively. Data were analysed using a paired t-test and statistical analysis was performed using SPSS (version 20.0, IBM, Chicago, Illinois), with a p-value of ≤ 0.05 deemed to be statistically significant.

2.3 Study 2: Acute and Downstream Cardiovascular Effects of Experimental Hypoglycaemia in Type 2 Diabetes

2.3.1 Overview of the Study

The aim of this study was to investigate cardiovascular responses during acute hypoglycaemia and downstream effects at days 1 and 7 using an experimental model of hyperinsulinaemic hypoglycaemia. The study was performed in T2DM individuals with no known CVD and an age and BMI matched nondiabetic group. Following the screening visit, each individual underwent paired euglycaemic and hypoglycaemic studies on two separate occasions where the rates and duration of insulin infusion were identical. Thus we were able to control the effects of hyperinsulinaemia. The advantage of this design is that each subject serves as his/her own control.

There is considerable intersubject variability in measures such as platelet reactivity and inflammation. It would be harder to match these parameters using a two group design where different sets of subjects undergo euglycaemia and hypoglycaemic studies. Euglycaemic studies and hypoglycaemic clamps were separated by at least four weeks to reduce the risk of carry over effects, but were separated by no more than twelve weeks. Euglycaemic studies preceded hypoglycaemic clamp studies for each individual. The reason for this is that interventional trials suggest hypoglycaemia has potential cardiovascular effects up to 90 days after the event and hence the clamps were not performed in a randomised fashion. The study was single blinded. Subjects did not know the glucose levels or the order with which euglycaemic and hypoglycaemic studies were performed.

In the euglycaemic arm, glucose was maintained at 6 mmol/L for two 60 minute periods in the morning and the afternoon, whilst in the hypoglycaemic arm, glucose was maintained at 2.5 mmol/L for two 60 minute periods (Figure 2-3). The rationale for inducing two periods of hypoglycaemia was based on previously published antecedent hypoglycaemia studies, which performed two 90-120 minute hypoglycaemic clamps on the same day in order to produce a sufficient stimulus (Adler et al., 2009; Davis et al., 2009).

To examine the downstream effects of hypoglycaemia, cardiovascular parameters were measured at baseline, and days 1 and 7 following

hypoglycaemia or euglycaemia. The study visits were conducted in the morning to control for effects of circadian variation. Spontaneous hypoglycaemia in the intervening period may alter the responses under study, therefore the selection criteria included diabetic patients at low risk of hypoglycaemia (on oral agents +/- GLP-1 analogues or on insulin <2 years), and the diabetic subjects were instructed to keep a diary of symptomatic hypoglycaemia during the study period. High resolution ECG and cardiac autonomic reflex testing was performed at baseline and days 1 and 7. Catecholamines, cortisol, thrombotic, and inflammatory biomarkers were also measured at these time points in both arms of the study (Figure 2-3).

The response to acute hypoglycaemia was compared between morning euglycaemic and morning hypoglycaemic clamps. No measurements were taken from afternoon clamps as the responses would have been altered by the hypoglycaemia induced in the morning. Blood pressure and heart rates were monitored every 30 minutes, and high resolution ECGs were performed at 0, 30, 60, 90, and 120 minutes, and following recovery (150 minutes) (Figure 2-4). Venous blood for the analysis of urea and electrolytes, free insulin, and inflammatory biomarkers and counter-regulatory hormones was sampled at the baseline and end of the clamp in both the euglycaemic and hypoglycaemic arms (120 minutes). Platelet function assays, fibrin network properties, and coagulation proteins were

measured at the baseline, end of the clamp and following recovery (150 minutes).

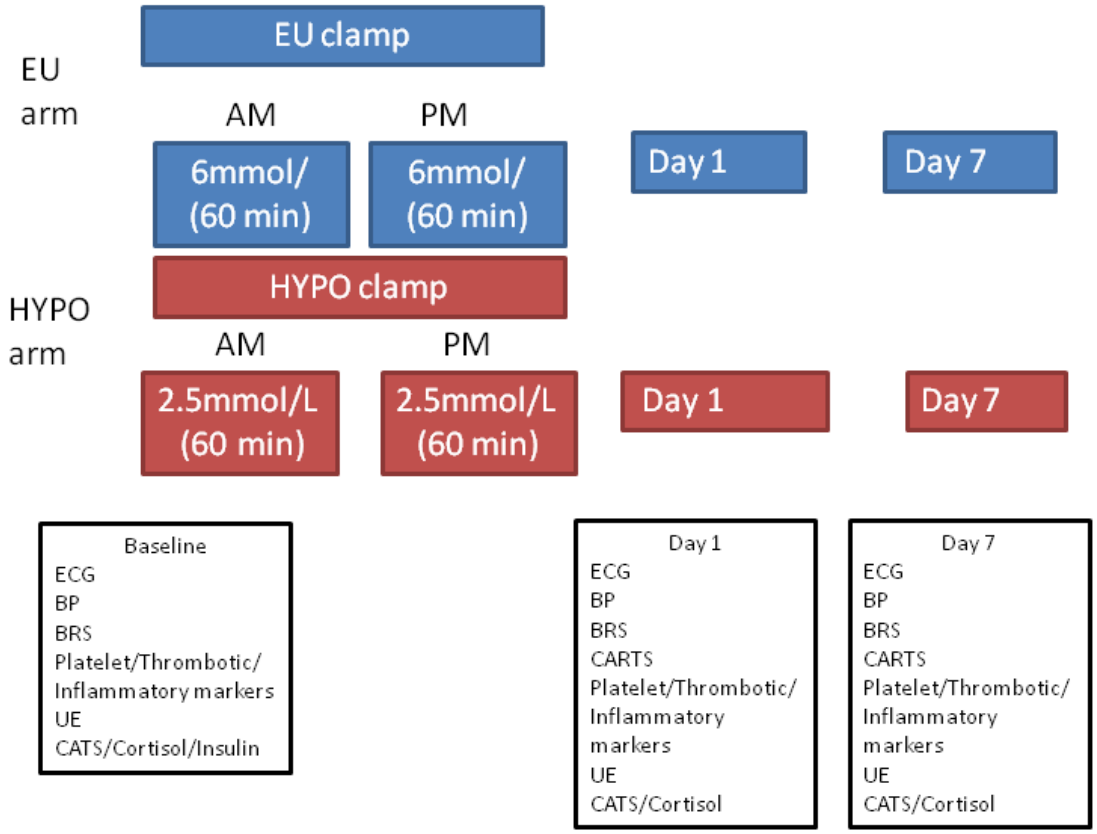

Figure 2-3: Flowchart for the hyperinsulinaemic clamp study
In the euglycaemic (EU) arm, subjects were maintained at a glucose level of 6 mmol/L for 60 minutes in the morning and 60 minutes in the afternoon, whilst in the hypoglycaemic arm, glucose was maintained at 2.5 mmol/L for 60 minutes in the morning and 60 minutes in the afternoon. Follow up visits were arranged in the morning on days 1 and 7. Abbreviations: BP-blood pressure, BRS -baroreceptor sensitivity, UE-urea and electrolytes, CARTs-cardiovascular autonomic reflex tests, CATAS-catecholamines.

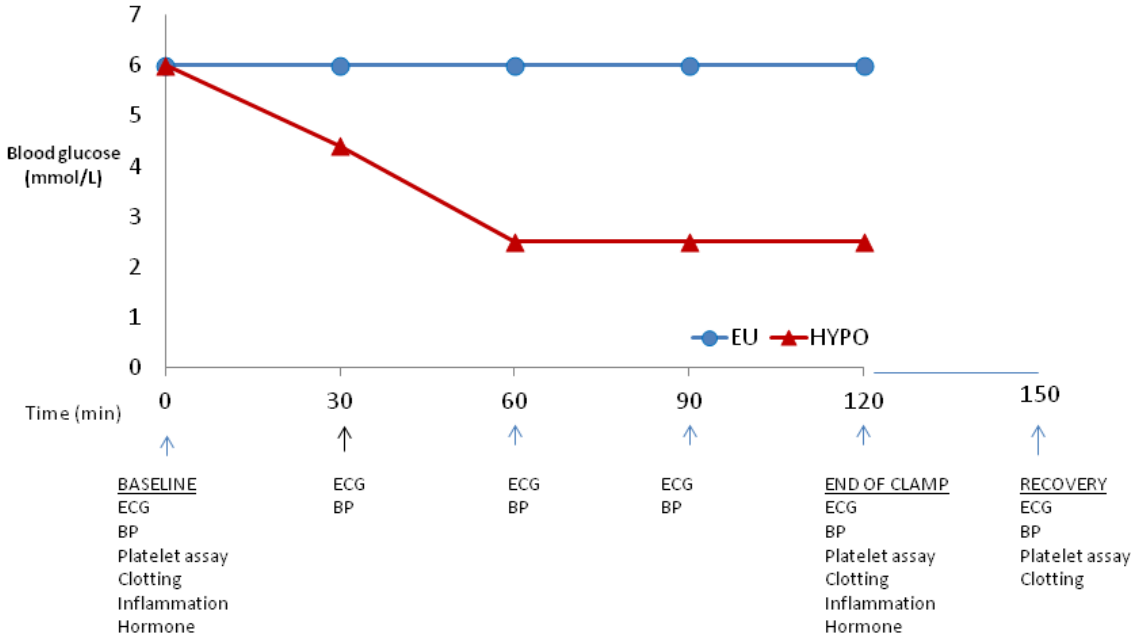

Figure 2-4: Measurement of cardiovascular parameters during acute hypoglycaemia

Cardiovascular parameters were measured during the morning hypoglycaemic (HYPO) clamp and compared against the euglycaemic (EU) clamp. Physical and biochemical measurements were made every 30 minutes during the clamp and after recovery.

This experimental approach was piloted in four diabetic and four nondiabetic individuals, with the purpose of testing the feasibility of the hyperinsulinaemic clamp protocol, subject recruitment, and measurement time points. During the pilot experiments, target blood glucoses were achieved and hence no changes were made to the hyperinsulinaemic clamp protocol. However, difficulty was experienced in recruiting healthy male volunteers and there was high screen failure of female diabetic participants. Consequently, recruitment of healthy volunteers was widened to both university and hospital staff to increase the pool of potential participants. Data from the pilot phase were included in the main analysis.

2.3.2 Study Participants and Recruitment

Individuals with T2DM aged between 18 and 65 were recruited to the study and the control group consisted of an age and BMI matched group of nondiabetic individuals, as these factors and gender can affect cardiac autonomic and thrombotic parameters and counter-regulatory responses. The reason for matching for age and BMI factors only were due to practical difficulties in recruiting matched nondiabetic male volunteers which emerged from the pilot phase. For reasons of safety, only participants with uncomplicated diabetes and no history, signs or symptoms suggestive of CVD were recruited. Inclusion and exclusion criteria are outlined below.

Inclusion criteria for diabetic patients:

- T2DM on oral medications and/or insulin for less than 2 years and/or GLP1 analogues
- Age 18-65
- Attending Sheffield Teaching Hospital/GP Practices in Sheffield
- Most recent HbA1c 6.5-10.5% (48-91 mmol/mol)
- Informed Consent

Inclusion criteria for nondiabetic individuals:

- Age 18-65
- Informed Consent

Exclusion criteria for diabetic and nondiabetic individuals:

- Documented myocardial infarction, ischaemic heart disease, cardiac arrhythmia, heart failure, peripheral vascular disease, stroke, visual

impairment due to retinopathy, or nephropathy as demonstrated by elevated serum creatinine accompanied by proteinuria.

- Abnormal resting 12 lead ECG
- Significant symptoms suggestive of CVD
- Pregnancy
- Known untreated hyperthyroidism
- Epilepsy or previous seizures
- Patients on beta-blockers or QT prolonging drugs
- Patients on anticoagulants or antiplatelet therapy (except aspirin)
- Cardiac autonomic neuropathy
- Serious intercurrent illness
- Malignant disease
- Previous history of deep venous thrombosis or pulmonary embolus.
- Inability to communicate in English
- Unwilling to self-monitor blood glucose levels (diabetic patients)
- Family history of sudden death

Potential diabetic participants were identified from diabetes outpatient clinics where an initial approach was made by their usual clinicians and interested individuals were provided with information about the study; this was the main route of recruitment. In addition, diabetes consultants, specialist nurses and community diabetes team were contacted and asked to refer any potential participants. Nondiabetic participants were recruited from staff, students and the general public attending Sheffield Teaching

Hospitals and the University of Sheffield. Advertising posters were placed around the diabetes centre, hospitals and the university, and the study was also advertised via the hospital email distribution list through which the majority of the nondiabetic volunteers were identified.

2.3.3 Research Governance

Informed consent was obtained from all participants according to the principles of good clinical practice. The study has received local ethics approval (Appendix 4).

2.3.4 Screening

Screening included the collection of demographic information, a full history and an examination, including an enquiry into cardiovascular symptoms and medications, a full blood count, urea and electrolytes, determination of fasting glucose levels, lipids and HbA1c, and the urine albumin creatinine ratio. Nondiabetic participants were screened for diabetes, which was defined as a fasting glucose >7 mmol/L or HbA1c >48 mmol/L (6.5%). A baseline 12 lead electrocardiogram was performed, and participants were screened for CAN as described for study 1. Participants with definite CAN, as defined by two or more abnormal tests based on published reference ranges, were excluded (O'Brien et al., 1986). Participants who fulfilled the edibility criteria were invited for the remainder of the study.

2.3.5 Hyperinsulinaemic Clamp Protocol

2.3.5.1 Euglycaemic Clamp Protocol

All the participants attended the Clinical Research Facility at 8 am having fasted from midnight. Diabetic patients were asked to omit their diabetic medications for 12 hours to check a four point capillary blood profile the day before the clamp. The clamp was then postponed if participants had a symptomatic hypoglycaemic episode or self-measured blood glucose of less than 3 mmol/L in the previous 24 hours as antecedent hypoglycaemia may modify counter-regulatory responses. Participants were advised to avoid smoking and caffeine on the day of the study and to avoid vigorous exercise for 24 hours before. Study participants were blinded to blood glucose levels throughout the duration of the clamp studies.

Baseline cardiovascular and biochemical measurements were performed. In the case of diabetic participants, blood glucose was initially stabilised via a variable insulin infusion (Human Actrapid, NovoNordisk Pharmaceuticals LT, Crawley, West Sussex, UK) for 40 minutes to 1 hour, aiming for a target glucose level of between 6-7 mmol/L. 50 units of Human Actrapid was added to 50 mL 0.9% sodium chloride (Baxter, Baxter Healthcare Ltd, Norfolk, UK) and infused via a syringe driver (Alaris ® GH Guardrails, Cardinal Health, Rolle, Switzerland) into an intravenous cannula in the non-dominant arm, and capillary blood glucose was monitored every 15 minutes. Nondiabetic volunteers did not require stabilisation of their blood glucose through intravenous insulin, and capillary blood glucose was

monitored every 20 minutes for the first 40 minutes to ensure it was in the euglycaemic range.

A second cannula was inserted into the opposite antecubital fossa for sampling of venous blood for biochemical, inflammatory and thrombotic measurements. These included urea and electrolytes, cortisol and growth hormone, and samples for platelet aggregation, fibrin clot properties and inflammatory cytokines.

A retrograde cannula was inserted following the application of local anaesthetic cream (EMLA, Astra Zeneca, UK) to the dorsal hand or wrist vein of the non-dominant hand. The hand was placed in a heated chamber at 55^{o}C to allow arterialisation of venous blood for glucose sampling and catecholamine sampling. A slow infusion of 0.9% sodium chloride was infused to keep the line patent.

Due to insulin resistance, T2DM patients require a higher insulin infusion rate compared with published studies in T1DM patients. A primed continuous infusion of insulin (Human Actrapid, Novo Nordisk Pharmaceuticals Ltd, Crawley, West Sussex, UK) was administered at a rate of 120 mU/m^2/minute, calculated according to the Dubois formula:

$$\text{Body surface area } (m^2) = 0.007184 \times (\text{patient height in cm})^{0.725} \times (\text{patient weight in kg})^{0.425}$$

In this study, the same rates of infusion were used for diabetic and nondiabetic participants, so that insulin levels would be comparable. This was infused into the cannula in the antecubital fossa of the non-dominant

arm via a syringe driver (Alaris® GH Guardrails, Cardinal Health, Rolle Switzerland). A variable rate 20% dextrose infusion (Baxter, Baxter Healthcare Ltd., Norfolk UK) was administered to the same cannula via an infusion pump (Alaris® GP plus, Cardinal Health, 1180 Rolle, Switzerland), and the glucose infusion rate was adjusted according to arterialised whole blood glucose concentrations sampled from the retrograde cannula every 5 minutes, which was analysed using a bedside glucose analyser (Yellow Spring Instruments, Ohio, USA). Glucose infusion rates were adjusted in order to reach the target blood glucose concentration of 6 mmol/L over half an hour. Blood glucose was then maintained at the target level of 6 mmol/L for 60 minutes.

At the end of the euglycaemic clamp period, the insulin was switched off and the 20% dextrose infusion was continued for at least 30 minutes, with the aim of achieving a stable glucose concentration in the euglycaemic range of 6-8 mmol/L during the recovery period. Blood glucose was monitored every 5 minutes during the first half an hour of recovery and every 15 minutes thereafter. In diabetic participants, a variable rate insulin infusion (50 units Human Actrapid in 50 mL 0.9 sodium chloride) was started if blood glucose was greater than 9 mmol/L. Participants also had a carbohydrate free midday meal and a rest period of 1 hour before the clamp study was repeated in the afternoon.

In the afternoon euglycaemic clamp the Actrapid infusion rate was doubled at 240 mU/m^2/min with a variable 20% dextrose infusion. It was necessary

to double the insulin administered in the afternoon to counteract the effects of increased resistance from counter-regulatory hormones following the hypoglycaemic clamp in the morning. To ensure that the insulin levels were equivalent for both the euglycaemic and hypoglycaemic arms, the insulin was also increased in the afternoon euglycaemic clamp. The clamp procedure was otherwise identical to that in the morning. A target blood glucose of 6 mmol/L was maintained for 60 minutes. Heart rate and blood pressure was measured every 30 minutes and participants were attached to a 3 lead ECG for monitoring purposes only. No additional measurements were collected in the afternoon except arterialised blood glucose which was measured every 5 minutes.

At recovery, the insulin was switched off and 20% dextrose was infused for a further at least 30 minutes until a minimum of 6 mmol/L was reached. Participants also ate a snack. Blood glucose was monitored for a minimum of 1 hour following the end of the afternoon clamp until stable and within the euglycaemic range to ensure that the effects of insulin had worn off. Diabetic participants were advised to take their usual dose of medication and were also advised to monitor their capillary blood profile for the next 24 hours and to keep a record of symptomatic hypoglycaemia over the following 7 days.

2.3.5.2 Hypoglycaemic Clamp Protocol

The protocol was identical to the euglycaemic visit except that blood glucose was lowered over 60 minutes to a target of 2.5 mmol/L. The

participants took part in morning and afternoon hypoglycaemic clamps where glucose was maintained at 2.5 mmol/L for 60 minutes. During the morning study they were commenced on a primed infusion of Human Actrapid insulin at the same rate as during the euglycaemic visit. A variable infusion of 20% dextrose was titrated to maintain a target glucose concentration of 2.5 mmol/L and the recovery procedure was identical. In the afternoon, Actrapid was administered at 240 mU/m2/min to overcome the increase in insulin resistance due to the hypoglycaemia in the morning. Diabetic participants were warned that their hypoglycaemic symptoms may be reduced in the following 48 hours. As the overall hypoglycaemic risk among the group of T2DM participants was low, doses of their usual diabetic medications were not reduced that evening.

2.3.6 Biochemical Analyses

2.3.6.1 Arterialised Whole Blood Glucose

Glucose was measured from 1 mL arterialised whole blood on a bedside glucose analyser (Yellow Springs Instrument 2300 STAT, Ohio, USA) every 5 minutes which employed the glucose oxidase method. Blood was sampled from a retrograde cannula inserted into the non-dominant hand vein and warmed at 55°C to aid the arterialisation of venous blood (Liu et al., 1992). Arterialised blood rather than venous blood glucose was measured, as venous glucose is variable depending on peripheral tissue consumption.

2.3.6.2 Free Insulin

Whole blood (3 mL) was collected into a 6 mL lithium heparin tube and immediately subjected to centrifugation at 4°C, 2400 rpm for 2 minutes. of The resulting plasma (0.5 mL) was added to a chilled plastic tube containing 0.5 mL polyethylene glycol (PEG) for precipitation of immune complexes and mixed, and the centrifugation step repeated for 30 minutes. The product was then stored at -80°C until analysed via an immunometric assay (Invitron Insulin ELISA, Invitron Ltd, Monmouth, UK), which had 100% cross-reactivity with Actrapid insulin. Briefly 100 µL labelled antibody solution and 25 µL sample was added to each well. Samples were incubated for 2 hours at 37^{o}C and washed 3 times before being read in a luminometer. The inter-assay coefficient of variation (CV) at a concentration of 14.9 mU/L was 7.1%, at 81.3 mU/L was 2.4%, and at 145.5 mU/l was 7.1%.

2.3.6.3 Catecholamines - Adrenaline and Noradrenaline

Whole blood (6 mL) was collected into chilled lithium heparin tubes containing 50 µL EGTA/glutathione as a preservative and subjected to centrifugation at 4^{o}C, 3000 rpm for 10 minutes. The resulting supernatant was stored at -80^{o}C until assayed via high performance liquid chromatography (HPLC). The inter-assay CV for noradrenaline (mean value 1.86 nmol/L) was 6.03% and for adrenaline (mean value 0.25 nmol/L) 15.85%.

2.3.6.4 Cortisol

Venous blood (4 mL) was collected into a serum separator tube and samples allowed to clot for 30 minutes. The sample was subjected to centrifugation and the serum frozen at <-20^{o}C until quantified using a commercial electrochemilluminescence assay (Cobas system, Roche Diagnostics, UK) at Sheffield Teaching Hospitals.

2.3.6.5 Urea and Electrolytes

Blood (2 mL) was collected into tubes with a clot accelerator and separation gel (BD Vacutainer® SST™ II Advance). Samples subjected to centrifugation at 3000 ×*g* at room temperature for 10 minutes. Assays on the resulting serum were performed at Sheffield Teaching Hospitals laboratories using an automated system (Cobas system, Roche Diagnostics, UK).

2.3.7 Cardiac Autonomic Function

2.3.7.1 Cardiovascular Autonomic Reflex Tests

These tests were performed at screening, days 1 and 7 on participants in both the euglycaemic and hypoglycaemia arms, as described in section 2.2.4.

2.3.7.2 Heart Rate Variability

The principles of HRV were described in section 2.2.8. Short term HRV recording and analysis were performed according to the recommendations of the 1996). Five minutes of ECG recording was performed with the

subject lying supine, followed by recordings performed with free breathing and paced breathing at 12 breaths per minute according to a timed visual display to control for the effects of respiratory frequency on HF components. Data from a 12 lead ECG was acquired and amplified (g®.USB amp, g.tec Medical Engineering, GmbH, Austria) using a 24 bit resolution and a sampling frequency of 1200 Hz, connected to a laptop computer (hp6730b) with ECG signals recorded using g.Recorder software (version 2007-2009, g.tec medical engineering GmbH Austria). Analyses were performed with g®.BSanalyze software (version 3.09a, g.tec medical engineering, GmBH, Austria) and MATLAB® software (version 7.8.0.347, R2009a, The MathWorks, Inc.). QRS complexes were detected using an automatic algorithm as described elsewhere (Schreier et al., 2003). Manual editing of R-R intervals was performed together with a visual inspection of QRS complexes to exclude any ectopic beats (1996). Time and domain HRV analysis was performed and the LF band was defined as 0.04-0.15 Hz and the HF band as 0.15-0.4 Hz.

2.3.7.3 Spontaneous Baroreceptor Sensitivity

BRS may be measured by bolus injections of vasoactive drugs, neck suction/pressure, or by spontaneous fluctuations in heart rate and blood pressure. Unlike the first two methods, spontaneous BRS allows the continuous non-invasive assessment of BRS and does not perturb the reflex it is trying to measure, and has been validated against intra-arterial and pharmacological techniques (Omboni et al., 1993; Parati et al., 1989). In

this study, 12 lead ECG data was simultaneously acquired using an Ivy Cardiac Trigger Monitor 3000 (Ivy Biomedical systems Inc, USA) as described previously. Non-invasive blood pressure monitoring was measured via a Portapres device (TNO Biomedical Instrumentation Amsterdam, Netherlands), which employs infrared plethysmography to detect pulsation in the digital artery and uses a volume clamp technique to detect changes in blood pressure. An appropriately sized finger cuff was applied to the middle finger of the right hand and a frontend unit attached to the wrist. The height correction unit was nulled and the transducer was attached to the upper arm at the level of the heart. When the Portapres device was started, the finger cuff automatically inflated, and physiocal, an internal calibration was switched on until a steady state was achieved, whereupon it was switched off. Analogue blood pressure data was digitised with a sampling frequency of 1000 Hz and together with ECG data was synchronously recorded using WR TestWorks™ software for 5 minutes with the subject lying supine.

Beat by beat SBP, DBP and R-R interval data was imported into the dedicated BRS analysis software (Nevrokard version 5.1.3, Intellectual Services, Slovenia) and BRS was calculated using the sequence method (Omboni et al., 1993; Parati et al., 1989). This method considers spontaneous increasing or decreasing ramps of blood pressure and its relationship to subsequent R-R intervals. The 'UP' sequence was generated where there was an increase in SBP of at least 0.5 mmHg associated with

an increase in the R-R interval of at least 5 ms for three or more consecutive beats. A 'DOWN' sequence was identified where there was a reduction in SBP of at least 0.5 mmHg with a corresponding decrease in the R-R interval of at least 5 ms for three or more consecutive beats. Total BRS, measured in ms/mmHg, was calculated using the average of the regression coefficients for UP and DOWN sequences.

2.3.8 Platelet Function

Platelet activated thrombus develops in three stages: 1) initial tethering involving vWF, collagen and glycoproteins 1b/V/IX integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_{1;}$ ii) an extension phase involving platelet activation, the release of granules, the additional recruitment of platelets and aggregation via GpIIb/IIIa, the integrin receptor; and iii) a perpetuation phase characterised by platelet stimulation and stabilisation of clots in a fibrin mesh (Kakouros et al., 2011). The integrin receptor $\alpha_{IIb}\beta_3$ is normally present in the resting state and agonists are able to activate a conformational change leading to high ligand binding affinity via 'inside-out signalling'.

2.3.8.1 Platelet Activation

Platelet activation may be quantified by factors such as a change in shape and a tendency to aggregate, and also by measuring the levels of platelet metabolic products. Platelet metabolites, such as beta thromboglobulin and platelet factor 4, have been used as soluble activation markers; however, these suffer from a number of limitations. Beta thromboglobulin is dependent on the venous platelet count and is raised during renal

failure, whilst platelet factor 4 has a short plasma half-life and is rapidly bound to endothelial cells (Kamath et al., 2001). P-selectin may also be derived from the endothelium, thus measurement of soluble P-selectin may not be completely specific to the state of platelet activation. Flow cytometry can be used with multiple fluorescent staining of platelets or in combination with monoclonal antibodies to measure circulating activated platelets. This technique involves incubation of whole blood with antibodies directed against platelet glycoproteins, such as P-selectin, which are only expressed on activated platelets. These platelet-bound antibodies are then detected using streptavidin conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Platelets may be differentiated from other blood cells using antibodies to detect membrane proteins specific to platelets, such as GP Ib or by their light-scatter profile. The advantage of flow cytometry is its high sensitivity and capability for detecting very low levels of platelet activation (2010). However, it needs to be processed rapidly to avoid stasis and subsequent platelet activation.

2.3.8.2 Platelet Reactivity

Platelet reactivity is defined as the level of aggregation response to a given platelet agonist. Platelet hyperreactivity has been shown to predict a 3- to 5-fold increase in major cardiovascular events over five years (Gorog and Fuster, 2013). Platelet reactivity has traditionally been measured using light transmission aggregometry. The extent of aggregation of platelets is detected and quantified by the amount of light that passes through

platelet-rich plasma in comparison to platelet free plasma in response to the addition of agonists. The disadvantage of this technique, apart from being laborious, is the requirement of plasma rich plasma which can cause artefactual distortion of the sample.

Impedance aggregometry was developed by Cardinal and Flower (1980) and detects platelet aggregation in whole blood. This technique measures the change in electrical resistance on a sensor wire due to platelet aggregation and has advantages over light transmission aggregometry in that it measures platelet aggregation in the fluid phase. The technique is rapid and can be used in a point-of-care setting. Impedance aggregometry relies on constant smooth stirring of the platelet sample and can be affected by cellular composition, such as the haematocrit (2010). However, impedance aggregometry is insensitive to small platelet aggregates as they cause minimal changes in electrical impedance. Therefore, impedance aggregometry is primarily suited for conditions in which there are high levels of platelet activation where it is able to detect variation in platelet macroaggregation to different agonists.

Platelet microaggregation, the formation of small aggregates of 2-4 platelets, is better detected with particle counting aggregometry methods, such as whole blood single platelet counting (2010). Platelet microaggregation may be particularly important in T2DM patients as it has been shown to be the predominant type of platelet aggregation event (Matsuno et al., 2004) .

When considering the choice of agonists, hypoglycaemia has been shown to potentiate aggregation as a result of a wide range of agonists, such as ADP, thrombin and platelet activating factor *in viv*o, that are likely to be mediated through alpha-2 adrenoreceptors (Trovati et al., 1986). Agonists all signal via G protein coupled receptors (GPCR) that converge on common intracellular signalling pathways, such as phosphlipase C and intracellular calcium. Several GPCR subfamilies are expressed in platelets (Gq, Gi, $G_{12/13}$), and it is thought that co-stimulation of at least two subfamilies of GPCR are necessary for full platelet activation and aggregation. With current knowledge of receptor signalling, one mechanism by which adrenaline can potentiate responses to other agonists is through co-stimulation of different GPCR pathways (Figure 2-5). The alpha-2-adrenoreceptor is coupled to G_z which is part of the G_i subfamily. G_i supplements other GPCR signalling pathways, such as G_q, which alone is insufficient to stimulate full platelet reaction. In this study, the effect of hypoglycaemia on 5-hydroxytriptamine (5HT) induced platelet aggregation, which acts exclusively through the G_q coupled $5HT2_A$ receptor, was investigated, as it has been shown that there are synergistic effects of 5HT and epinephrine co-stimulation that may occur through the PLC/Ca^{2+}signalling cascade (Shah et al., 1999). ADP acts via both the G_i coupled P2Y12 receptor and the G_q coupled P2Y1 receptor. To investigate whether potential activation of alpha-2 adrenoceptors during hypoglycaemia can substitute the requirement for P2Y12 Gi signalling

during ADP-induced aggregation, cangrelor, a P2Y12 antagonist was applied.

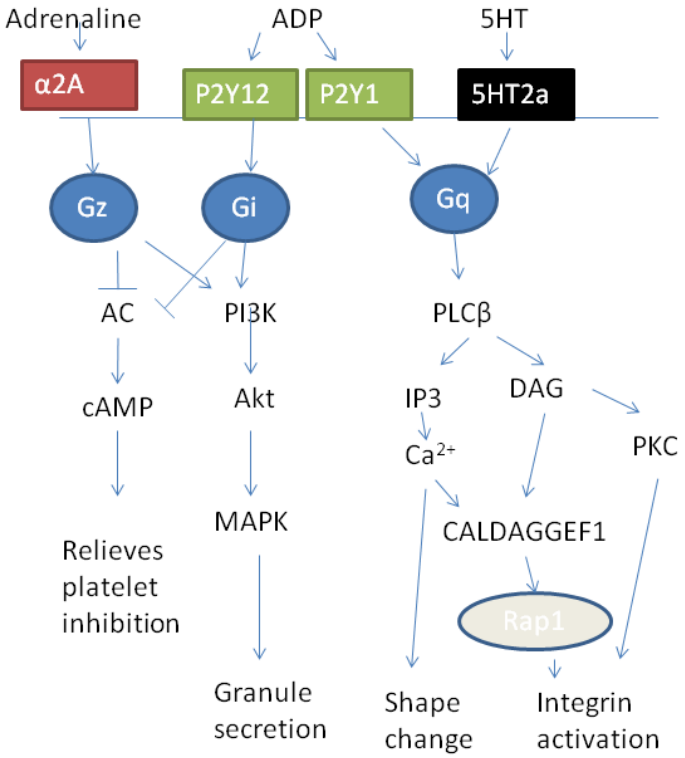

Figure 2-5: Potential pathways by which adrenaline can potentiate platelet responses to agonists

AC-adenyl cyclase, PI3K-phosphoinositide 3 kinase, MAPK-Mitogen-activated protein kinase, IP3-inositol triphosphate, DAG-diacylglycerol, PKC-protein kinase C, CALDEGGEF1-calcium and DAG regulated GEF1

Hypoglycaemia may also potentiate signalling through major platelet adhesion receptors, such as collagen. Collagen is regarded as a strong agonist, which in itself is sufficient to lead to platelet degranulation and aggregation. Collagen interacts with the GpVI receptor of the immunoglobulin family and activates PLC gamma and downstream thromboxane A2 synthesis, granule secretion and integrin activation (Li et al., 2010). Hypoglycaemia may potentiate a platelet response to collagen

via potentiation of cytosolic calcium release due to adrenaline (Ardlie et al., 1987).

In summary, previous literature has suggested that hypoglycaemia can lead to various alterations in platelet activation and aggregation which require investigation using complementary platelet function assays. Platelet activation was investigated by P-selectin expression using flow cytometry. The effect of hypoglycaemia on platelet macroaggregation was investigated by impedance aggregometry using ADP and collagen as agonists, while the effect of hypoglycaemia on platelet microaggregation to ADP was investigated using whole blood single platelet counting. Platelet reactivity to 5HT and ADP (with and without P2Y12 inhibition) was investigated by P-selectin expression via flow cytometry.

2.3.8.3 Impedance Aggregometry

Materials:

1 x 3 mL Multiplate hirudin tube

31 x 1 µM ADP

31 x 1 ug/mL collagen (Kollagenreagens Horm® Takeda Austria GmH, Austria)

Platelet aggregation was measured using impedance aggregometry (Multiplate®, Verum Diagnostica GmBH, Munich, Germany). Venous blood (2.7 mL) was collected and carefully transferred into a 3 mL hirudin tube (Multiplate®) avoiding any agitation of the sample. Recombinant hirudin anticoagulates the blood by direct thrombin inhibition, such that platelet

function was assessed under physiological calcium conditions. The samples were transported to the Cardiovascular Biomedical Research laboratory, Northern General Hospital, immediately after the venepuncture was performed in order to maximise the chances of observing a catecholamine effect. Aliquots of 300 µL saline and 300 µL hirudin-anticoagulated blood were added to a cuvette and incubated at 37°C for 3 minutes. Agonists (20 µL in total, ADP 1 µM and collagen 1 µM) were added and the assay commenced. The area under the curve (AUC) was measured which represents the level of platelet aggregation.

2.3.8.4 Whole Blood Single Platelet Count

Materials:

1 x 4.5 mL Citrate tube (BD Vacutainer)

25 x 1 µmol/L ADP

25 x 3 µmol/L ADP

Solution A (60 mL 0.077 M EDTA, 100 mL 4% Formalin, 40 mL PBS, 200 mL distilled water, 600 mL saline)

Cell Pack (Sysmex)

Whole blood single platelet counting (WBSPC) was used to determine platelet microaggregation responses to agonists. Citrated blood venous blood (4.5 mL) was taken and incubated at 37^{o}C. Aliquots of whole blood (480 µL) were placed in polystyrene tubes with a magnetic stirrer bar and 20 µL ADP at 1 or 3 µM as agonists. Samples were stirred at 1000 rpm for 4 minutes at 37°C prior to fixing in saline with 1 mL of Solution A [4.6 mM

sodium EDTA, 4.5 mM Na_2HPO_4, 1.6 mM KH_2PO_4 and 0.16% (w/v) formaldehyde, pH7.4] and counting using a Sysmex KX21 haematology analyser (Sysmex corporation, Illinois, USA). To a 460 µL cell pack, 60 uL of the fixed sample was added and single platelet and red cell counting performed using the haematology analyser in the pre-diluted mode. Total cell counts were obtained using 4 mM EDTA instead of ADP. The adjusted platelet count was then determined as:

$$\text{Adjusted platelet count ADP platelet count} \times \left(\frac{\text{Red cell count ADP}}{\text{Red cell count EDTA}}\right)$$

Data are presented as percentage platelet aggregation which was calculated as follows:

$$\text{Percentage platelet aggregation} = \frac{(\text{EDTA count} - \text{adjusted ADP count})}{\text{EDTA count}} \times 100$$

2.3.8.5 P-selectin Expression

Materials:

25 x 1 µmol/L ADP

25 x 3 µmol/L 5-hydroxytryptamine (5HT)

25 x 1 µmol/L cangrelor (AR-C69931MX mwt952)

PE Cy 5™ Mouse IgG1k isotype control (BD Pharmigen)

PECy ™Mouse Antihuman CD62P (BD Pharmigen)

PE Mouse Anti-Human CD41a (BD Pharmigen)

FACSfix (1.25 mL formaldehyde (38% v/v) in 100ml PBS)

HT buffer (1 L Millipore high grade water, 7.56 g NaCl, 0.75 g $NaHCO_3$, 0.209 g KCl, 0.109 KH_2PO_4, 1.02 g dextrose, 2.39 g HEPES)

1 x 3 mL Multiplate hirudin tube

Blood flow cytometry was used to determine the activation state of circulating platelets binding to the activation dependent monoclonal antibody CD62P in order to assess the reactivity of circulating platelets *in vitro* to ADP and 5HT. Cangrelor was used to assess the effect of P2Y12 blockade on platelet reactivity.

Hirudinised blood (192 µL) was added to 8 µL saline or 8 µL 25x1 µmol/L cangrelor for 2 minutes at room temperature. Aliquots of blood (5 µL) were then added to 33 µL HT buffer, 5 µL CD6 PECY5 for P-selectin, 5 µL CD41PE as a platelet marker, and either 2 µL 1 µmol ADP or 3 µmol 5HT as agonists or 2 µL saline (unstimulated). PE Cy 5™ Mouse IgG1k (5 µL) was added instead of CD62P PECY5 as an isotype control. Samples were incubated at room temperature in the dark for 20 minutes followed by the addition of 0.5 mL FACSfix. Flow cytometry was performed and forward light scatter, side scatter, and FITC fluorescence was measured (BD Accuri C6, BD Biosciences, UK). A gate was positioned around the platelet region to exclude any red cells. P-selectin expression was quantified as: 1) the percentage of cells with CD62P fluorescence greater than the fluorescence of the mouse IgG control (% positive events); and 2) the median CD62P fluorescence intensity of the platelet population.

2.3.9 Fibrin Polymerisation Characteristics

Although platelets are important in the initial formation of a platelet plug, subsequent stabilisation of a clot is due to the activation of a complex network of coagulation proteins that normally exist in the fluid phase. A summary of the coagulation and fibrinolysis pathways are shown in Figure 2-6. Of these, fibrinogen is of critical importance, as when converted by thrombin into fibrin, it is a key structural component of the fibrin clot. Increased plasma fibrinogen levels are associated with CVD (Kannel et al., 1987; Kaptoge et al., 2007), and there is evidence in mouse models that artificially elevated levels of fibrinogen shortened the time to carotid artery occlusion in response to injury, increased clot density and also resistance to clot lysis (Ariëns et al., 2014). Fibrinogen is an acute phase protein whose production is stimulated by IL-6 and other cytokines.

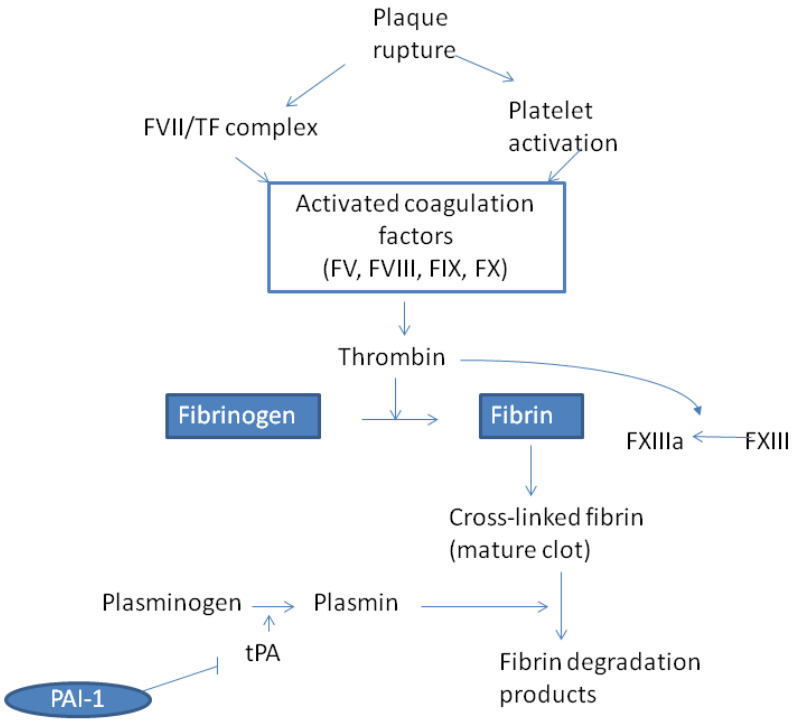

Figure 2-6: Summary of coagulation and fibrinolysis pathways
TF-tissue factor, PAI-1-plasminogen activator inhibitor-1, Tpa-tissue plasminogen activator

Although there is a strong association between fibrinogen and thrombotic risk, there is good evidence that apart from quantitative levels of the coagulation protein, the final product of the coagulation pathway, the fibrin clot structure itself, is also associated with an increased CVD risk (Fatah et al., 1996). Two aspects of the fibrin clot structure, its viscoelastic properties and resistance to lysis, may be linked to the overall propensity for thrombosis in an individual (Ariëns et al., 2014). With regards to the latter, the ability of fibrinolytic proteins to diffuse through the fibrin network, the incorporation of inhibitors of fibrinolysis to the fibrin clot, and differences in the way the fibrin matrix supports the conversion of plasminogen to plasmin, can influence resistance to clot lysis (Ariëns et al., 2014). Multiple factors influence the structure and function of a fibrin clot, as shown in Figure 2-7. In the diabetic individual, increased glycation fibrinogen itself can alter clot structure, independent of absolute levels of fibrinogen (Ariëns et al., 2014). Moreover, complement component C3 has recently been identified amongst other inflammatory proteins to influence clot structure in individuals with diabetes. Increased amounts of C3 were shown to be incorporated into clots of individuals with T1DM which prolonged clot lysis times (Hess et al., 2012a), and C3 was found to be independently associated with prolonged clot lysis time in the Edinburgh T2DM cohort, and operating via different pathways to PAI-1 (Hess et al.,

2014). C3 was also associated with a 3-fold increase in coronary artery disease after adjustment for traditional risk factors (Ajjan et al., 2005).

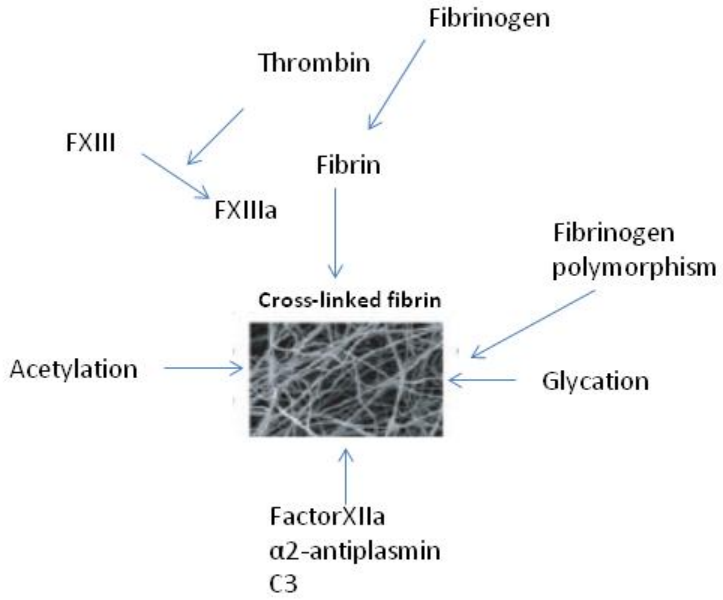

Figure 2-7: Factors influencing fibrin clot structure and function
Fibrin clot structure is influenced by fibrinogen and thrombin concentrations. Factor XIIIa is responsible for cross-linking of fibrin, and genetic factors, such as fibrinogen polymorphisms and acquired factors, such as glycation and acetylation can modulate fibrin clot structure. Proteins incorporated into the clot, such as of factor XIIa, α2-antiplasmin and C3 may increase resistance to lysis. Modified from (Ariëns et al., 2014).

The overall thrombotic potential is also influenced by the fibrinolytic pathway. PAI-1 is the main inhibitor of fibrinolysis and acts by binding to tPA and forming the PAI-1/tPA complex. PAI-1 is secreted by a large number of cells, including endothelial cells, vascular smooth muscle cells, adipocytes and the liver. As discussed earlier, elevated levels of PAI-1 are present in T2DM, and elevated levels are strongly implicated in an increased thrombotic risk (Alzahrani and Ajjan, 2010).

In the present study, plasma levels of key coagulation factors, fibrinogen and fibrinolysis inhibitor PAI-1, were measured. However, as global fibrin

clot properties are not dictated by absolute levels of these factors alone, a turbidity and lysis assay was performed on *ex vivo* plama clots to provide an indicator of the fibrin network density and fibrinolysis potential. Using this method, the maximum clot absorbance, an indirect measure of clot density, and the time to 50% clot lysis have both been shown to predict cardiovascular risk (Fatah et al., 1996). To complement these methods, scanning electron microscopy of *ex vivo* clots made from purified fibrinogen may elucidate changes in fibrin diameter and the fibrin network density (Alzahrani et al., 2012). C3 levels were also measured as this could potentially be elevated secondary to proinflammatory effects of hypoglycaemia, and be a mechanism of increased thrombosis.

2.3.9.1 Fibrinogen Assay: Clauss Method

Materials:

Veronal buffer pH 7.35 (9.714 g sodium acetate $3H_2O$, 14.714 g sodium 5,5 diethylbarbiturate, HCl)

Thrombin (Sigma) 10 U/mL diluted in imidazole buffered saline (IBS)

Fibrinogen reference plasma (Biomereux)

venous blood (4.5 mL) was collected in a citrated tube (BD Vacutainer Glass Citrate Tube containing 3.2% buffered sodium citrate solution), subjected to centrifugation at 2770 ×*g* for 30 minutes at 4^{o}C and the resultant plasma stored at -80^{o}C. Samples were diluted using Veronal buffer at a 1:10 dilution and incubated at 37°C. Samples (100 µL) were added to coagulometer cuvettes (KC 10, Amelung GmbH, Lemco, Germany) in

duplicate and incubated for 30 seconds. of Thrombin (100 µL) was added and the time for clot formation measured; if less than 7 seconds, samples were repeated at a 1:20 dilution. A standard calibration curve was generated using 1:6 to 1:24 dilutions of the reference plasma. The time taken for the clot to form was compared to the calibration curve and the fibrinogen concentration (g/L) deduced.

2.3.9.2 Plasminogen-Activator 1

Materials:

PAI1 (SERPINE1) Human SimpleStep ELISA™kit (Abcam ab184863)

- 10 x PAI1 Capture Antibody
- 10 x PAI 1 Detector Antibody
- PAI1 Lyophilized Recomibinant Protein
- Antibody Diluent 4I
- 10 x Wash Buffer PT
- TMB Subtrate
- Stop Solution
- Sample Diluent NS
- Sample Diluent 25BP
- SimpleStep Pre-Coated 96 Well microplate
- Plate seal
- Phosphate Buffered Saline (1.4 M KH_2PO_4, 8 M Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, pH7.4)

Venous blood (4.5 mL) was collected in a citrated tube (BD Vacutainer Glass Citrate Tube 4.5 mL containing 3.2% buffered sodium citrate solution), subjected to centrifugation at 2770 ×*g* for 30 minutes at 4°C and the resultant plasma stored at -80 ^{o}C. PAI-1 lyophilised recombinant protein standard was reconstituted in 500 µL sample diluent 25BP and serially diluted to final concentrations of 94, 188, 375, 750, 1500, 300 and 6000 pg/mL respectively. To each well 50 µL of the sample or standard was added, followed by 50 µL of an antibody cocktail consisting of 300 µL 10x capture body, 30 uL 10x detector antibody with 2.4 mL antibody diluent 4BI. The plate was sealed and incubated for 1 hour at room temperature on a plate shaker set to 400 rpm. Each well was washed three times with 350 µL of 1x wash buffer before adding 100 µL TMB Substrate to each well and incubating for 10 minutes in the dark on a plate shaker set to 400 rpm. 100 µL of Stop Solution was added to each well and the plate replaced on a plate shaker for 1 minute to mix. The optical density of each well was recorded at 450 nm and a standard curve was constructed and the protein concentration of the samples interpolated. The overall assay CV was 0.92%.

2.3.9.3 Complement C3

Materials:

Complement C3 ELISA kit (Biosource, San Diego, USA)

Assay plate

Standard

Antibody

HRP-conjugate

Wash Buffer

Substrate A

Substrate B

Stop solution

Plasma was collected in EDTA tube, subjected to centrifugation for 20 minute at 3000 ×*g* at room temperature, and samples stored at -80^{o} C prior to being assayed. To each well 50 µL of the sample or standard (0, 12.5, 37.5, 115, 250 or 500 µg/mL) was added, then 50 µL of HRP-conjugate, followed by 50 µL of antibody. The plate was mixed and incubated for one hour at 37°C. The well was washed three times using 20 µL wash buffer and 50 µL of substrate A and 50 µL of substrate B was then added to each well and mixed before incubating the plate at 37°C for 15 minutes. Stop solution (50 µL) was then added to each well and the optical density at 450 nm measured using a microplate reader. The concentrations of the samples were interpolated from the standard curve constructed. The overall assay CV was 1.33%.

2.3.9.4 Turbidimetric and Lysis Assay

Materials:

$CaCl_2$

Thrombin (Calbiochem, Merck Millipore, USA)

tPA (Technoclone, Vienna, Austria)

Permeation Buffer (12.12 g Tris, 11.68 g NaCl dissolved in distilled water, pH 7.4)

Venous blood (4.5 mL) was collected in citrated tube (BD Vacutainer Glass Citrate Tube 4.5 mL containing 3.2% buffered sodium citrate solution), subjected to centrifugation at 2770 ×*g* for 30 minutes at 4° C and the resultant plasma stored at -80^{o}C. Citrated plasma (25 µL) was added to 75 µL assay buffer (0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4) and 50 µL of activation mix (final concentrations: 0.03 U/mL thrombin and 7.5 mmol/L calcium in assay buffer) was added to each column of a 96-well plate using a multichannel pipette at 10 sec intervals. The time of addition of the activation mix was recorded to enable the plate reader times to be adjusted to the start of clot initiation. Plates were shaken and read at 340 nm every 12 seconds for 1 hour in a BIO-TEK ELx-808 microplate reader. The turbidimetric lysis assay was carried out as above but with the addition of 12.5 ng of tPA (Technoclone) to the 75 µL assay buffer (83 ng/mL final concentration) before the addition of the activation mix; this concentration of tPA resulted in complete lysis of pooled normal plasma within 1 hour. Plates were read at 340 nm every 12 seconds for 1 hour and subsequently every 2 minutes for up to 9 hours.

Maximum absorbance (MA) was taken as the maximum optical density during the clotting assay at which three consecutive readings were identical. Clot lysis time (LT) was calculated as the time from the initiation of clot formation to the time at which a 50% fall in absorbance from

maximum absorbance occurred. The inter-assay CVs for MA was 8% and LT 7%.

2.3.9.5 Scanning Electron Microscopy

Materials:

Recombinant fibrinogen

Thrombin

$CaCl_2$

Sodium cacodylate buffer

2% glutaraldehyde

Venous blood (4.5 mL) was collected in a citrated tube (BD Vacutainer Glass Citrate Tube 4.5 mL containing 3.2% buffered sodium citrate solution), subjected to centrifugation at 2770 ×*g* for 30 minutes at 4°C, and the resultant plasma stored at -80^{o}C. Recombinant fibrinogen (1.3 µM) was clotted upon addition of 0.5 U/mL thrombin and 2.5 mM $CaCl_2$ in specially devised small, perforated plastic vessels. Samples were incubated at room temperature in a moist chamber for 2 hours then washed with sodium cacodylate buffer and subsequently fixed overnight in 2% glutaraldehyde. Clots were recovered and further processed by a stepwise dehydration technique using an acetone gradient and sputter coated with platinum palladium. Samples were viewed and photographed using a field-emission scanning electron microscope (LEO1530 FEGSEM, Leo Electron Microscopy, Cambridge, United Kingdom) across ten different areas of each clot. Images were captured using Leo 32 version 03.0210 software (Leo Electron

Microscopy) and cropped using Paintshop Pro version 8.0 (Corel, Minneapolis, MN). Fibre diameters of all the clots were measured using the image analysis software package ImageJ 1,23y (National Institutes of Health, Bethesda, MD). In all, 160 fibrin fibres per sample were measured with the operator blinded to the sampling time point.

2.3.10 Inflammatory Cytokines

Inflammation is a critical component in the determination of plaque stability, and is orchestrated by multiple cell types, including endothelial cells, monocytes and macrophages that secrete cytokines and chemokines to amplify the inflammatory response. Among inflammatory biomarkers, hsCRP has the strongest evidence for predicting cardiovascular mortality in primary prevention cohorts and following ACS. CRP is produced by the liver in response to IL-6 and to a lesser extent by TNF-α and IL-1 at local sites of inflammation. IL-6 is a pleiotropic cytokine produced by multiple cell types, including the liver, fibroblasts, monocytes, lymphocytes and neural tissues (Stoner et al., 2013). IL-6 is important in the induction of the acute phase response, leading to the production of CRP and fibrinogen, the latter an important modulator of thrombosis. IL-6 is produced by human adipocytes and thus implicated in subclinical inflammation in obesity. IL-6 is a hormonally regulated cytokine that can be stimulated by catecholamines and insulin in adipocytes *in vitro* (Vicennati et al., 2002). The effect of hypoglycaemia on hsCRP and IL-6 has been investigated in T1DM and normal individuals but has yielded inconsistent results;

however, no studies have been performed in T2DM patients (Gogitidze Joy et al., 2010; Wright et al., 2010).

Acute hypoglycaemia has previously been shown to increase a number of proinflammatory molecules, including ICAM-1, VCAM, TNFα and CD40L in T1DM and normal individuals (Gogitidze Joy et al., 2010; Wright et al., 2010). Decreases in anti-inflammatory cytokines have also been shown to predict ACS (Stoner et al., 2013). However, few studies have looked at the effect of anti-inflammatory cytokines, such as interleukin 10 (IL-10), during hypoglycaemia. The effect of hypoglycaemia on monocytes has not been studied. MCP-1 is highly expressed in atherosclerotic plaques and is an important mediator for the migration of monocytes and macrophages to atherosclerotic lesions. Deletion of MCP-1 in $ApoB^{-/-}$ mice protects against leukocyte recruitment to plaques (Stoner et al., 2013), and MCP-1 has been shown to predict SCD and mortality following ACS (Gonzalez-Quesada and Frangogiannis, 2009).

IL-6, IL-1 and MCP-1 were assayed using a cytometric bead array (CBA). This array detects the analyte using immobilised antibody capture and uses fluorescence detection by flow cytometry for reporting and quantification. The advantages of CBA over ELISA include fewer sample dilutions, smaller sample volumes and that multiple analytes can be assayed at the same time, thereby increasing efficiency (Elshal and McCoy, 2006). Although there is a risk of cross-reactivity between analytes, this can generally be avoided by adhering to cytokine combinations as specified by the

manufacturer. In published studies, cytokines assayed by CBA generally show good correlations of >90% when compared to ELISA (Elshal and McCoy, 2006).

2.3.10.1 High Sensitivity C Reactive Protein

Whole blood (1 mL) was collected into a serum gel tube (Vacutainer ® BD SST™ Advance) and analysed using an immunoturbidimetric assay (Cardiac CRP (latex) high sensitivity, Roche Diagnostics, Indianopolis, USA) in Sheffield Teaching Hospitals Immunology laboratories. The within-run CV at 2.5 mg/L was 6%.

2.3.10.2 IL-6, IL-10 and MCP-1

Materials:

Human IL-6, IL-10 and MCP-1 Flex Sets (BD Biosciences, UK) containing

- Capture beads
- PE detection reagent
- Standards

PE Positive Control Detector

FITC positive Control Detector

Buffer reagents

Wash Buffer

Assay Diluent

Serum Enhancement buffer

Venous blood (5 mL) was collected in EDTA tubes (BD Vacutainer™ K3E), subjected to centrifugation at 3000 ×*g* for 20 minutes and the resulting

supernatant stored at -80^{o}C. During the assay the cytokine standards were serially diluted from 1:2 to 1:256 for use as controls. Wash buffer (100 µL) was added to each well of the filter plate and then the well was aspirated and dried before adding 50 µL of mixed capture beads and 50 µL of the sample or standard to each well. The plate was incubated for 1 hour at room temperature on a non-absorbent dry surface. Mixed PE detection reagent (50 µL) was added to each well followed by a further incubation period of 2 hours, before adding 150 µL of wash buffer and mixing to resuspend the beads. Samples were then acquired on a BD FACSarray flow cytometer (BD Biosciences, Oxford, UK) and the data analysed using FACSarray software (BD Biosciences, Oxfordm UK). Standard curves for each analyte was constructed using five parameter logistic equations from which median fluorescence values were converted into cytokine concentrations in pg/mL for each sample.

2.3.11 Statistical Analysis

Power calculations were performed based on change in high frequency HRV as the primary outcome measure The sample size calculation took into account the repeated measure design, assuming a correlation of 0.5 between repeated measures (Frison and Pocock, 1992). A sample size of 12 had 80% power to detect a clinically important difference of 35% (1.38 ms^2) in overall log high frequency power between glycaemic arms with a type 1 error rate of 0.05. This assumes a SD of 1.28 ms^2, based on a previous study of T2DM patients (Sacre et al., 2012).

The statistical analyses for study 2 are described individually for each sub-study in the respective chapters which describe the studies in detail.

Chapter 3 - Electrophysiological Response to Spontaneous Hypoglycaemia in Type 2 Diabetes Patients with Cardiovascular Risk

3.1 Summary

Hypoglycaemia might cause arrhythmias through effects on cardiac repolarisation and changes in cardiac autonomic activity. The aim of this study was to examine the risk of arrhythmias during spontaneous hypoglycaemia in T2DM patients with cardiovascular risk.

Twenty-five insulin treated patients with T2DM and a history of CVD or two or more risk factors underwent simultaneous continuous IG and ambulatory ECG monitoring. The frequency of arrhythmias, HRV and markers of cardiac repolarisation were compared between hypoglycaemia and euglycaemia, and between hyperglycaemia and euglycaemia matched for time of day.

In total, there were 134 hours of recording at hypoglycaemia, 65 hours at hyperglycaemic, and 1258 hours at euglycaemia. Bradycardia, atrial and ventricular ectopic counts were significantly higher during nocturnal hypoglycaemia compared to euglycaemia, and arrhythmias were more frequent during nocturnal versus daytime hypoglycaemia. Excessive compensatory vagal activation following the counter-regulatory phase may account for bradycardia and associated arrhythmias. QTc >500 ms and

abnormal T wave morphologies were observed during hypoglycaemia in some participants.

Hypoglycaemia, which is frequently asymptomatic and prolonged, may increase the risk of arrhythmias in patients with T2DM and high cardiovascular risk. This is a plausible mechanism that could contribute to increased cardiovascular mortality during intensive glycaemic therapy.

3.2 Introduction

Hypoglycaemia has been strongly associated with an increased downstream risk of vascular events and death but evidence of a direct causal link is lacking (Zoungas et al., 2010). Hypoglycaemia, including nocturnal episodes, was associated with increased risk of arrhythmias in the ORIGIN trial where patients with T2DM were randomised to treatment with insulin glargine (Mellbin et al., 2013) versus conventional therapy. There have been sporadic case reports of supraventricular and ventricular arrhythmias associated with hypoglycaemia. Among supraventricular arrhythmias, transient atrial fibrillation is the most frequently reported (Celebi et al., 2011), and there have also been a number of reports of bradycardia during severe hypoglycaemia in diabetic and nondiabetic patients (Bolognesi et al., 2011). In a study of experimental hypoglycaemia in six patients with T2DM and no cardiac disease, one patient developed a severe bradyarrhythmia and another developed frequent VPB (Lindström et al., 1992). Reports of ventricular arrhythmias associated with

hypoglycaemia are rare, perhaps because events are generally fatal if uncorrected.

Hypoglycaemia may be proarrhythmic via a number of mechanisms (Nordin, 2014) including a direct effect of low glucose on the hERG ion channel (Zhang et al., 2003b), hypokalaemia and catecholamine release prolong cardiac repolarisation, increasing the risk of early afterdepolarisations and ventricular arrhythmias (Robinson et al., 2003). Experimental hypoglycaemia has been previously shown to prolong the QT interval in individuals with T1DM and T2DM (Marques et al., 1997). Since arrhythmias can be triggered by a transient change in the sympathovagal balance, it seems worthwhile exploring the effect of hypoglycaemia on autonomic tone. Previous studies have reported inconsistent effects of acute experimental hypoglycaemia on cardiac autonomic activity (Koivikko et al., 2005; Laitinen et al., 2008), and our group has previously shown that CAN may modify the relationship between hypoglycaemia and cardiac repolarisation (Lee et al., 2004).

The aim of this study was to examine the frequency of arrhythmias during spontaneous hypoglycaemia and hyperglycaemic compared to euglycaemia in patients with T2DM at high cardiovascular risk. The effect of glucose on cardiac autonomic tone and repolarisation were further explored as potential mechanisms.

3.3 Research Design and Methods

The research methods have been described in detail in Section 2.2 and are outlined in brief below.

Twenty-five individuals with T2DM on insulin treatment for at least 4 years were recruited from Sheffield Teaching Hospitals diabetes outpatient clinics. All had a history of CVD (ischemic heart disease, peripheral vascular or cerebrovascular disease) and/or two additional cardiovascular risk factors: hypertension, dyslipidaemia (both defined as requiring medication), current smoker and obesity. Those on QT prolonging drugs were excluded from the study, as were all patients with permanent atrial fibrillation or a bundle branch block on baseline ECG.

3.3.1 Baseline Assessment

Cardiovascular autonomic reflex tests were performed as previously described (O'Brien et al., 1991) in accordance with the latest consensus for the diagnosis of CAN. Patients were classified as definite CAN if two or more cardioreflex tests were below the age adjusted reference range (O'Brien et al., 1986; Tesfaye et al., 2010). HbA1c was measured using ion exchange HLPC.

3.3.2 Monitoring

All patients underwent five days of simultaneous 12 lead Holter and CGM. Patients carried on with their usual daily activities and diabetes treatments. Twelve lead ambulatory ECGs (Lifecard 12, Spacelabs Healthcare, Hertford,

UK) were recorded at a sampling rate of 128 Hz with electrodes in a Mason-Likar configuration. Patients also had a time-synchronised CGM attached (Freestyle Navigator Continuous Glucose Monitoring System, Abbott Diabetes Care, Maidenhead, UK). Calibrations were performed at least four times during the study week according to manufacturer's instructions. Patients were also asked to keep a record of any symptomatic hypoglycaemia. An episode of low IG < 3.5 mmol/L on CGM without simultaneous self-reporting of symptoms was regarded as asymptomatic.

3.3.3 Continuous Glucose Monitoring Analysis

Hypoglycaemia was defined as IG ≤ 3.5 mmol/L in accordance with previously published studies (Gill et al., 2009) and hyperglycaemia was defined as IG ≥ 15 mmol/L. A valid hypoglycaemic episode consisted of an IG below the threshold for ≥ 20 minutes (2007). The first reading of IG ≤ 3.5 mmol/L marked the start of the hypoglycaemia and the first reading of IG ≥ 3.5 mmol/L signified the end of the episode. The lowest IG within the hypoglycaemic episode was designated the glucose nadir. Each episode was matched with a euglycaemic period, equivalent in duration, at the same time of day (within 20 minutes) on a different day. Hyperglycaemic episodes were identified an IG above the 15 mmol/L threshold for more than 20 minutes. The highest IG within the episode was designated as the maxima and matched euglycaemic periods on a different day were identified.

3.3.4 Arrhythmia Analysis

The 12 lead ambulatory ECG data was analysed with a Pathfinder Ambulatory ECG analysis system (v. 8.701, Delmar Reynolds Medical Ltd, Edinburgh & Hertford, UK). Leads I, II and V5 were used for analysis as they represented the orthogonal leads. Arrhythmic events, including atrial ectopic beats, bradycardia (defined as four or more consecutive beats at less than 45 beats per minute), VPB and complex VPB (bigeminy, trigeminy, couplet, Salvos and VT) were identified by the software and manually verified for accuracy. Hourly counts for each type of arrhythmia were paired against the hourly mean IG, which was categorised as hypoglycaemia (IG ≤ 3.5 mmol/L), hyperglycaemic (IG ≥ 15 mmol/L) or euglycaemia (5 mmol/L < IG < 10 mmol/L). Analyses were separated into day and night (2300-0700 hours) to take into account diurnal variation.

3.3.5 Hate Rate Variability Analysis

R-R intervals were extracted from annotated normal beats (NN intervals) using the Pathfinder Ambulatory ECG analysis system. A 5 minute segment of successive NN intervals was selected around each reported IG value, and spectral analysis was performed on each segment using the Fourier transformation. The LF band was defined as 0.04-0.15 Hz and the HF bandas 0.15-0.4 Hz. The ratio between the LF power and total power was calculated (LFnorm), as this has previously been suggested to indicate the level of sympathetic modulation in HRV (Bernardi et al., 2011; Montano et al., 1994).

3.3.6 Repolarisation Analysis

Analysis of QT intervals was performed using custom-built, semiautomatic software based on a selective beat averaging approach (Badilini et al., 1999). Annotated normal ECG beats were identified using the Pathfinder system (v.8.701, Delmar Reynolds Medical Ltd, Edinburgh and Hertford, UK), and following pre-processing, a composite wave was then calculated from averaged beats derived from leads I, II and V5. On the composite wave, the onset of the Q wave was marked as the first positive deflection from the isoelectric line above 10 mV, and the end of the T wave was determined using the tangent method. QT intervals were corrected for heart rate (QTcS) using subject-specific regression formulae generated from QT/R-R values during euglycaemia (Christensen et al., 2010).

3.3.7 Statistical Analysis

This was an observational study hence no formal power calculations were performed. Demographic data was compared between patients who experienced at least one hypoglycaemic episode versus those who experienced none using an independent t-test, Mann-Whitney U test, or Fisher's exact test for the effect of the insulin regimen and insulin type on hypoglycaemia. The IRR of arrhythmias during hypoglycaemia and hyperglycaemic compared with euglycaemia were calculated using generalised estimated equations (Hanley et al., 2003). HRV parameters and QTc were compared at the glucose nadir of the hypoglycaemic or glucose maxima of the hyperglycaemic episode against an equivalent euglycaemic

time point on a different day. Where there was more than one matching hypoglycaemic-euglycaemic episode in an individual participant over the course of the recording period, the mean from all daytime and nocturnal episodes for that individual were taken respectively. Data were analysed using a paired t-test. Statistical analysis was performed using SPSS (version 20.0, IBM, Chicago, Illinois), and a p-value ≤ 0.05 was deemed statistically significant.

3.4 Results

3.4.1 Participant Characteristics

A total of 2323 hours of valid simultaneous ECG and glucose recordings were obtained from 25 patients. Participants were similar in age, BMI and the prevalence of cardiovascular risk to the population in the ACCORD trial, with a history of CVD in one third of the participants (Gerstein et al., 2008) (Table 3-1). Two out of the 25 patients were on cardioselective beta-blockade. Out of the 25 patients, 14 experienced at least one episode of hypoglycaemia and 12 experienced at least one episode of hyperglycaemia. Demographic data, duration of diabetes, and prevalence of CVD were similar between patients who experienced hypoglycaemia versus those who did not (Table 3-1); however, a higher proportion of patients who experienced hypoglycaemia were on biphasic and human insulins. The duration of insulin therapy and baseline QTc were slightly longer in those who experienced hypoglycaemia but the difference did not reach statistical significance.

Table 3-1: Comparison of participant characteristics between those who experienced hypoglycaemia versus those who did not

Data are mean ± SD except for age, duration of diabetes and duration on insulin which are expressed as medians (interquartile range). The p-value indicates the comparison of participant characteristics between HYPO and no HYPO groups via parametric or nonparametric testing (see text). HYPO – hypoglycaemia, HbA1c – glycated hemoglobin, SBP – systolic blood pressure, DBP – diastolic blood pressure, CVD – cardiovascular disease, CAN – cardiac autonomic neuropathy.

	TOTAL n=25	**HYPO** n=14	**NO HYPO** n=11	**p-value**
Age (years)	64 (61-71)	68 (59-74)	64 (60-66)	0.26
Male n (%)	13 (52%)	8	5	0.82
Duration of diabetes (years)	17 ± 6	18 (12-21)	17 (15-21)	0.76
Duration on insulin (years)	9 ± 5	10 (7-16)	7 (5-11)	0.14
Insulin regimen				0.12
Twice daily biphasic n (%)	14 (56%)	10	4	
Basal-prandial n (%)	11 (44%)	4	7	
Insulin type				0.02
Human n (%)	18 (72%)	13	5	
Analogue n (%)	7 (28%)	1	6	
BMI (kg/m^2)	32 ± 5	33 ± 6	32 ± 4	0.49
HbA1c (%)	7.5 ± 1.0	7.4 ± 1.2	8.1 ± 1.0	0.15
(mmol/mol IFCC)	58.0 ± 10.9	57.0 ± 13.1	65.0 ± 10.9	
SBP (mmHg)	142 ± 23	141 ± 26	143 ± 20	0.83
DBP (mmHg)	72 ± 9	72 ± 10	72 ± 9	0.99
Heart rate (bpm)	74 ± 11	76 ± 12	73 ± 11	0.51
Baseline QTc (ms)	434 ± 23	437 ± 25	425 ± 16	0.20
CVD n (%)	8 (32%)	5	3	0.65
CAN n (%)	4 (16%)	3	1	0.40

3.4.2 Glucose Profiles during Hypoglycaemia

In total, there were 134 hours of recording at hypoglycaemia and 1258 hours at euglycaemia with the remainder outside this range. Twenty matched day hypoglycaemic episodes in 11 participants and 14 matched nocturnal episodes from 10 participants were analysed. There were diurnal differences in the duration and depth of hypoglycaemia. The mean±SD duration of daytime episodes was 62 ± 42 minutes, whereas the mean duration of nocturnal episodes was prolonged at 170 ± 112 minutes with a

lower glucose nadir (1.9 ± 0.7 mmol/L versus 2.8 ± 0.5 mmol/L). At night, glucose exhibited an undulating profile in contrast to daytime hypoglycaemia, where there was a single descent to the nadir followed by a brisk counterregulation (Figure 3-1). Of 34 hypoglycaemic episodes that were identified by CGM, three were symptomatic

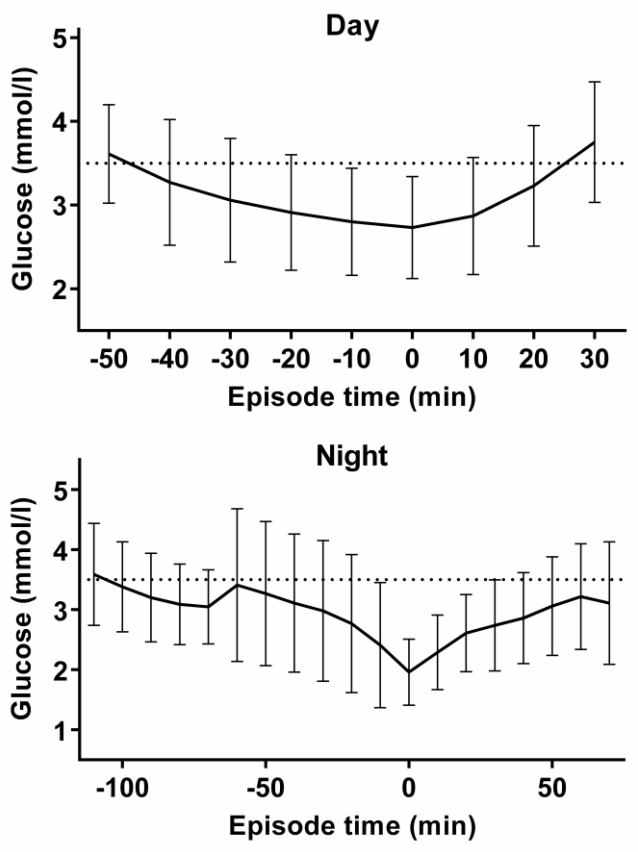

Figure 3-1: Mean interstitial glucose values during day versus night hypoglycaemic episodes

Mean IG profiles are shown for 20 day-time episodes from 11 participants and 14 nocturnal episodes from 10 participants. The mean duration of daytime hypoglycaemia was 62 ± 42 minutes with a mean IG at the nadir of 2.8 ± 0.5 mmol/L. The mean duration of nocturnal hypoglycaemia was 170 ± 112 minutes with a mean IG at the nadir of 1.9 ± 0.7 mmol/L. The hypoglycaemic nadir is shown as episode time 0, with negative time values indicating a change from the beginning of the hypoglycaemic episode and positive values from the nadir to recovery from hypoglycaemia. Data are mean ± SD.

3.4.3 Arrhythmias

The incident rate of arrhythmias during hypoglycaemia versus euglycaemia was compared. The minimum heart rate observed during nocturnal hypoglycaemia was 34 beats per minute, with the longest bradycardic period being 156 consecutive beats. Bradycardia was 8-fold higher during nocturnal hypoglycaemia when compared with euglycaemia (IRR 8.42; 95% CI 1.40 to 51.0) (Table 3-2). Bradycardia did not occur during the day under either glycaemic condition. Atrial ectopic activity was nearly 4-fold higher during nocturnal hypoglycaemia (IRR 3.98; 95% CI 1.10 to 14.4), but was not significantly different during daytime episodes. VPB were more frequent during hypoglycaemia for both day (IRR 1.31; 95% CI 1.10 to 1.57) and night (IRR 3.06; 2.11 to 4.44), but there were no differences in the incidence of complex VPB. Some examples of arrhythmias include sinus bradycardia with ventricular bigeminy at IG 2.7 mmol/L and atrial bigeminy at IG 2.8 mmol/L (Figure 3-2). The data was also analysed with the two patients on beta blockers excluded. The risk of bradycardia, atrial ectopics and VPB during nocturnal hypoglycaemia remained significantly elevated (data not shown).

Table 3-2: Incident rate ratios of arrhythmias during hypoglycaemia compared with euglycaemia during daytime and nocturnal periods

IRR and 95% CI of arrhythmias during hypoglycaemia versus euglycaemia as analysed using generalised estimated equations. VPB – ventricular premature beat.

	DAY			NIGHT		
	IRR	95% CI	p-value	IRR	95% CI	p-value
Bradycardia	-	-	-	8.42	(1.40 to 51.0)	0.02
Atrial ectopic	1.35	(0.92 to 1.98)	0.13	3.98	(1.10 to 14.40)	0.04
VPB	1.31	(1.10 to 1.57)	< 0.01	3.06	(2.11 to 4.44)	< 0.01
Complex VPB	1.13	(0.78 to 1.65)	0.52	0.79	(0.22 to 2.86)	0.72

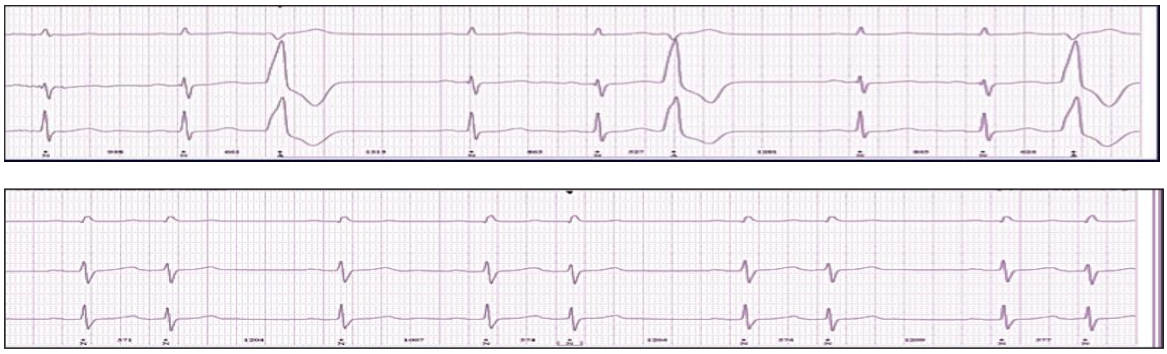

Figure 3-2: Examples of arrhythmias during hypoglycaemia

Sinus bradycardia with ventricular bigeminy at IG 2.7 mmol/L (above) and atrial bigeminy at IG 2.8 mmol/L (below)

3.4.4 Heart Rate Variability

The changes in HRV during hypoglycaemia were explored compared to matched euglycaemia. During the day, it was found that there was cardioacceleration (decrease in NN interval Δ -66 ± 55 ms, p = 0.001) at the glucose nadir accompanied by a decrease in total power (Δ -0.59 ± 0.83, p = 0.02) and HF power (Δ -0.62 ± 0.59, p = 0.003). Normalised LF was not significantly different (Table 3-3). During the night, analysis of all the episodes showed no significant differences in NN interval or any of the HRV

indices at the glucose nadir (Figure 3-2). However, the glucose response was more heterogeneous (Figure 3-1).

Table 3-3: Differences in heart rate variability parameters at the glucose nadir between hypoglycaemia and matched euglycaemia during daytime and nocturnal periods

HRV parameters (Mean ± SD) during the hypoglycaemic nadir (HYPO) versus euglycaemia (EU). Data from 11 participants recorded during the day (DAY) and 10 participants recorded during the night (NIGHT) are presented. Paired differences (Mean ± SD) and p-values from paired t-tests are shown. Total spectral power, high frequency power (HF) and low frequency power (LF) of HRV series are expressed as natural logarithms (ln). NN – normal to normal beat R-R interval, LFnorm – LF / Total power ratio.

		HYPO	EU	Paired difference	p-value
DAY	NN (ms)	775 ± 167	841 ± 185	-66 ± 55	0.001
(n = 11)	Total power	4.98 ± 1.10	5.57 ± 0.59	-0.59 ± 0.83	0.02
	HF	3.45 ± 1.01	4.08 ± 0.77	-0.62 ± 0.59	0.003
	LF	4.56 ± 1.42	5.18 ± 0.85	-0.61 ± 0.92	0.03
	LFnorm	0.73 ± 0.21	0.73 ± 0.20	-0.00 ± 0.08	0.95
	LF/HF	4.41 ± 2.86	4.05 ± 2.28	-0.36 ± 2.94	0.66
NIGHT	NN (ms)	937 ± 171	929 ± 139	8 ± 71	0.72
(n = 10)	Total power	6.01 ± 1.40	6.34 ± 1.32	-0.33 ± 0.68	0.16
	HF	4.94 ± 1.16	5.03 ± 1.43	-0.09 ± 0.84	0.75
	LF	5.36 ± 1.78	5.76 ± 1.56	-0.40 ± 0.66	0.09
	LFnorm	0.59 ± 0.23	0.64 ± 0.26	-0.06 ± 0.09	0.08
	LF/HF	2.53 ± 2.20	3.85 ± 3.30	-1.32 ± 2.00	0.07

Closer examination of individual episodes revealed a pattern, whereby counter-regulatory responses were accompanied by transient vagal withdrawal, with a concomitant rise in normalized LF (LFnorm) reflecting increased sympathetic contribution (Figure 3-3). This was followed by a compensatory increase in vagal activity and a relative decrease in normalised LF. Bradycardia was observed in this period of heightened vagal activity. Further examples of this phenomenon are shown in the Appendix 5. The data was again reanalysed with the patients on beta-blockers

excluded, and it was shown that the NN interval and HRV indices decreased to a similar extent under hypoglycaemic and euglycaemic conditions, therefore this made no significant difference to the conclusions.

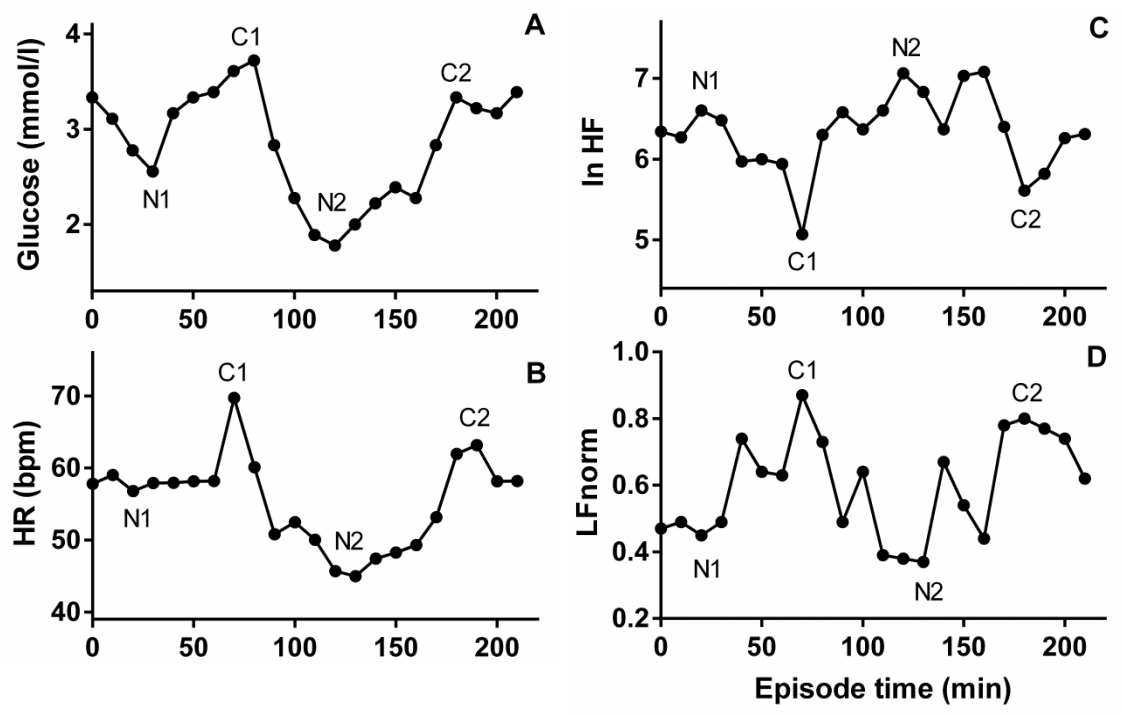

Figure 3-3: Phasic changes in HRV during a prolonged nocturnal hypoglycaemic episode

Glucose, heart rate, high frequency HRV power (HF) and normalised low frequency power (LFnorm) across a nocturnal hypoglycaemic episode in a single patient are illustrated. The initial fall in glucose to 2.5 mmol/L (N1) was followed by a brief counter-regulatory response (C1), panel A. There was transient cardioacceleration (panel B), withdrawal in HF power (panel C) and increase in LFnorm (panel D) at C1. This was followed by a subsequent increase in vagal activity (HF) and relative decrease in LFnorm, leading to bradycardia (45 bpm) up to fifty minutes later. Glucose further decreased to 1.8 mmol/L at N2. Glucose counter-regulation occurred (C2) which again was accompanied by a transient increase in heart rate and LFnorm with HF withdrawal. HF power is expresses as the natural logarithm (ln).

3.4.5 Cardiac Repolarisation

QTcS was compared between hypoglycaemia and euglycaemia for 20 matched day episodes in 11 participants and 14 nocturnal episodes in 10 participants. During the day, mean QTcS was longer during hypoglycaemia

(402 ± 49 ms) compared with euglycaemia (384 ± 36 ms, mean paired difference 18 ± 27 ms, p = 0.05), whilst at night, mean QTcS was 440 ± 43 ms during hypoglycaemia and 432 ± 16 ms during euglycaemia, and a paired comparison showed no significant difference between the groups (8 ± 44 ms, p = 0.60). Two individuals had a QTcS > 500 ms during hypoglycaemia. There was maximal prolongation of 100 ms at the glucose nadir, which was accompanied by flattening of the T wave (Figure 3-4) and emergence of U waves.

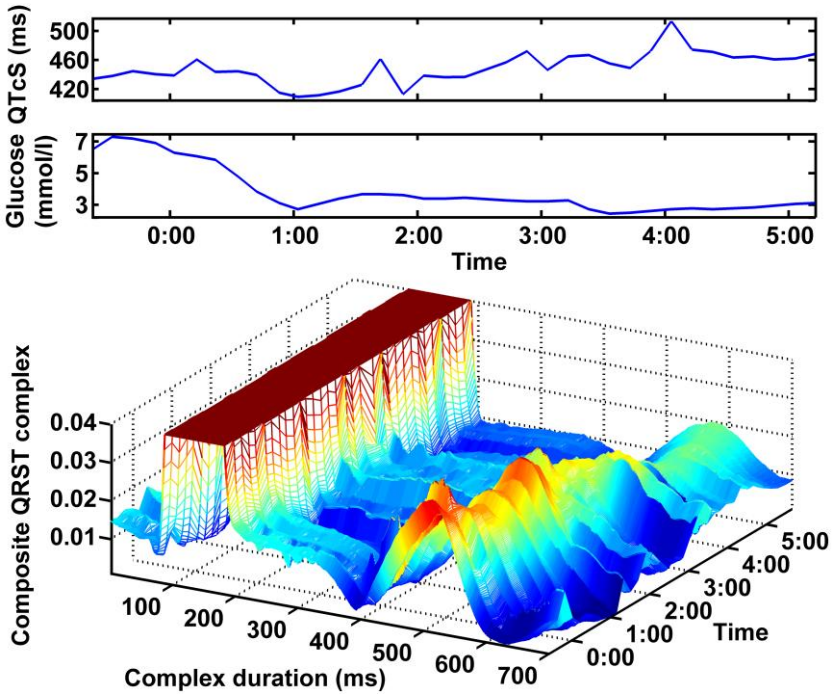

Figure 3-4: Abnormal QT prolongation and T wave morphology during hypoglycaemia in a single patient

Glucose and QTc change over the course of a hypoglycaemic episode are shown in the top two panels. In this participant, QTcS was prolonged from 456 ms at euglycaemia to 547 ms at the glucose nadir of 2.51 mmol/L. The change in complex morphology with absolute time is shown in the bottom panel. There is progressive flattening of the T wave with the fall in glucose.

3.4.6 Symptomatic versus Asymptomatic Hypoglycaemia

There were only three symptomatic episodes in three participants out of the total of 34 matched episodes identified, two of which occurred in the day. No arrhythmias were observed during the symptomatic episodes. The mean HRV indices at the glucose nadir between symptomatic versus asymptomatic episodes were also compared. There was a trend towards higher heart rates during symptomatic episodes, as shown by decrease in the NN interval (ΔNN -32 ± 37 ms vs 1 ± 87 ms). There was also a trend towards greater vagal withdrawal during symptomatic episodes (ΔlnHF -0.53 ± 0.79 vs -0.19 ± 0.79 and Δln [Total power] -0.64 ± 0.67 vs -0.27 ± 0.77), although due to the small numbers the uncertainty is large.

3.4.7 Effect of Depth and Duration of Hypoglycaemia

The role of the depth and duration of hypoglycaemia was explored by analysing HRV and QT changes in episodes with glucose nadirs < 2.5 mmol/L vs ≥ 2.5 mmol/L and episodes < 120 minutes versus ≥ 120 minutes. Episodes with a glucose nadir above 2.5 mmol/L were associated with a greater degree of QT prolongation when compared with episodes with a lower glucose nadir (42 ± 69 ms versus 2 ± 14 ms, p = 0.04), whilst prolonged episodes were associated with a smaller degree of QT change, although this did not reach statistical significance (16 ± 42 ms vs 30 ± 62 ms, p = 0.51). It is of note that prolonged episodes also had lower glucose nadirs. Similarly, longer and deeper episodes tended to be associated with a trend of a slowing of heart rate (increase in NN) and decreased

sympathetic contribution manifested as decreased ΔLFnorm and ΔLF/HF values.

3.4.8 Hyperglycaemia versus Euglycaemia

Hyperglycaemic episodes were observed predominantly during the day; there were a total of 22 daytime and 3 nocturnal hyperglycaemic events in 12 patients, 5 of whom were in the 'no hypoglycaemia' group. No significant arrhythmias were observed during nocturnal hyperglycaemic episodes, of which there were very few. Daytime hyperglycaemia was associated with lower rates of atrial ectopics (IRR = 0.41, $p < 0.01$) and complex VPB (IRR = 0.28, $p < 0.01$) when compared to euglycaemia, although there appeared to be a higher risk of VPB (IRR = 1.44, $p < 0.01$) (Table 3-4). There were no significant differences in HRV parameters during hyperglycaemia compared with matched euglycaemia and no significant differences in QTcS between hyperglycaemia and euglycaemia (paired difference 0.65 ± 21.0 ms, p = 0.92, two tailed paired t-test).

Table 3-4: Incident rate ratios of arrhythmias during daytime hyperglycaemia compared with matched euglycaemia

IRR and 95% CI of arrhythmias during hyperglycaemia versus euglycaemia as analysed using generalised estimated equations. VPB – ventricular premature beat.

	IRR	95% CI	p-value
Bradycardia	-	-	-
Atrial ectopic	0.41	0.33 to 0.50	< 0.01
VPB	1.44	1.15 to 1.80	< 0.01
Complex VPB	0.28	0.17 to 0.46	< 0.01

3.5 Discussion

This study demonstrates that hypoglycaemia is associated with an increased risk of cardiac arrhythmia in patients with T2DM and a history of CVD or at high cardiovascular risk. In this study, participant characteristics and CVD status were similar between patients who experienced hypoglycaemia versus none. Yet this observational data showed an increase in bradycardia, atrial and ventricular ectopic activity that was coincident with hypoglycaemic periods. The patterns of arrhythmia were consistent with those from anecdotal case reports (Bolognesi et al., 2011), previous studies of experimental hypoglycaemia in T2DM (Lindström et al., 1992) and ambulatory data in T1DM (Gill et al., 2009).

Potential mechanisms were explored by studying the effect of hypoglycaemia on heart rate, which is linked to cardiac autonomic activity, and repolarisation, which determines the propensity for non-driven cardiac action potentials and arrhythmogenesis. The analysis was separated into day and night due to diurnal differences in glucose counterregulation, autonomic fuction and QTc. In this study there was transient cardio-acceleration at the glucose nadir and vagal withdrawal during daytime episodes, consistent with studies of hypoglycaemia in patients with T1DM (Koivikko et al., 2012; Russell et al., 2001). However, what is striking is the difference in heart rate response and an 8-fold higher incidence of bradycardia during nocturnal hypoglycaemia. Defective counter-regulation during nocturnal hypoglycaemia is well known, and is characterised by

blunted and delayed adrenaline responses (Banarer and Cryer, 2003). Nocturnal hypoglycaemia is often prolonged with an undulating glucose profile with multiple nadirs that may occur as a result of repeated counterregulatory attempts to resist the glucose lowering effects of injected insulin.

During nocturnal episodes there was a pattern of transient cardioacceleration with each glucose nadir, followed by a phase of heightened vagal counteraction up to 40-50 minutes later that was associated with bradycardia. It was therefore speculated that the occurrence of bradycardia may be linked to increased vagal counteraction following sympathetic neural activation. This view is supported by studies of experimental hypoglycaemia with autonomic blockade (Fisher et al., 1990; Lloyd-Mostyn and Oram, 1975). When hypoglycaemia was induced with concomitant beta-adrenergic blockade, a late bradycardia was observed 45 minutes after the acute sympathetic reaction at the glucose nadir (Fisher et al., 1990) with heart rates below that on propranolol alone. This decrease in heart rate was abolished by atropine, confirming the role of heightened vagal tone (Fisher et al., 1990). The dampened nocturnal sympathoadrenal response in nocturnal hours may be analogous to the effect of adrenergic blockade in these experimental studies, such that while heart rate may be restored to the normal range during the day, the rate slowing effects would be more profound at night, when sympathetic responses are weakened and circadian vagal tone is high. Furthermore,

nocturnal hypoglycaemia is likely to be driven by basal insulin, where the relatively slow decline in glucose may be linked with a weaker sympathetic response, in contrast with a faster decline secondary to rapid acting insulin during the day. There may also be an additional contribution due to impaired sympathetic responses in those who experience repeated episodes of hypoglycaemia (8 of 14 patients in this study).

The effect of hypoglycaemia on cardiac repolarisation with QT as a marker of action potential duration length was also investigated. The degree of QT prolongation was smaller than that reported in previous experimental studies and ambulatory studies in T1DM, only achieving significance during the day (Gill et al., 2009; Landstedt-Hallin et al., 1999; Marques et al., 1997). This may reflect attenuated sympathoadrenal responses in this advanced T2DM group, who were older and had a relatively long disease duration (Segel et al., 2002). In this study, QTc was prolonged to over 500 ms with abnormal T wave morphology in two individuals during hypoglycaemia, a level that can strongly predispose to Torsade de pointe and ventricular tachyarrhythmias. These two individuals also had the longest baseline QTc in the group (456 ms in a male patient, 484 ms in a female patient). It is possible that individuals with a decreased repolarisation reserve, related to coronary heart disease or structural abnormalities, may be particularly predisposed to arrhythmias during a hypoglycaemic challenge.

It has been suggested that abnormal repolarisation associated with hypoglycaemia may be mediated by catecholamines, hypokalaemia (Robinson et al., 2003) or low glucose (Zhang et al., 2003b). However, in the absence of simultaneous biochemical measurements, the mechanism involved can only be speculated upon. Hypoglycaemic episodes which reached a higher glucose nadir (over 2.5 mmol/L) were associated with a greater QT prolongation in contrast to episodes with nadirs under 2.5 mmol/L. This can be interpreted to suggest that the degree of QT prolongation may be linked to the sympathoadrenal response, rather than absolute glucose *per se*. During clinical episodes of spontaneous hypoglycaemia (in contrast to hypoglycaemia induced experimentally), episodes of hypoglycaemia with a higher glucose nadir might generally reflect a stronger sympathoadrenal response. Conversely, in individuals with impaired counter-regulation, glucose may fall to a lower nadir due to reduced sympathoadrenal activation. Thus, one explanation of the data is that greater QT prolongation occurred in episodes that were less deep due to greater sympathoadrenal activation. Table 3-4 illustrates a hypoglycaemic episode with a higher glucose nadir (> 2.5 mmol/L), where it was possible that the combination of sympathoadrenal activation and inherent repolarisation abnormalities resulted in significant QT lengthening. This remains speculative and also an additional role for hypokalaemia cannot be excluded as this is also linked to adrenaline release.

In the present study, only 3 out of 34 hypoglycaemic episodes were accompanied by symptoms and no arrhythmias were observed during these episodes. The high prevalence of asymptomatic hypoglycaemia is striking, probably reflecting diminished counter-regulatory responses associated with long disease duration and nocturnal episodes where hypoglycaemia occurred during sleep in a supine posture. Asymptomatic hypoglycaemia can contribute to an excess of arrhythmic risk, and these episodes may be frequent and go unnoticed. The data from this study indicates a trend for a lower heart rate and greater vagal tone (especially at night) during asymptomatic compared with symptomatic hypoglycaemia. However, it is difficult to draw any firm conclusions from such small numbers.

This study suggests that hypoglycaemia is a proarrhythmic condition with a risk higher than during euglycaemia or hyperglycaemic. The electrophysiological conditions during hypoglycaemia could contribute to the initiation of ventricular tachyarrhythmias in a number of manners. During the day, QT prolongation may lead to increased triggered activity in the form of early afterdepolarisations. Sympathetic activation and an increase in cytosolic calcium can similarly lead to delayed afterdepolarisation and premature beats (Nordin, 2014). Both mechanisms can contribute to increased ventricular ectopic activity, which was observed during daytime hypoglycaemia. However, rates of arrhythmia were even higher at night, where sympathoadrenal responses are

attenuated. Arrhythmias during spontaneous nocturnal hypoglycaemia may be explained by alternate mechanisms, in contrast to strong sympathoadrenal activation and QT prolongation that is typically observed during experimental hypoglycaemia. During a state of vagal dominance, a slow sinus rate may reveal latent pacemakers, particularly under conditions of enhanced automaticity (Verrier and Josephson, 2009). This may explain the excess atrial and ventricular ectopic activity that was observed during nocturnal hypoglycaemia. Ventricular bigeminy during bradycardia occurred at low glucose levels in this study and the consequent long-short activation intervals are a common trigger for Torsade de pointe (El-Sherif and Turitto, 2004; Farkas et al., 2008). QT prolongation during bradycardia can also increase the frequency or risk of long-short intervals. Although this combination did not occur in patients during this study, it has previously been reported in a patient with T2DM during a hypoglycaemic coma (Bolognesi et al., 2011). Thus, hypoglycaemia has the potential to trigger a fatal arrhythmic event through more than one mechanism during spontaneous hypoglycaemia in patients at high cardiovascular risk.

Most of the arrhythmias that were observed in this study may be well tolerated by young healthy individuals, but could be clinically relevant in those with T2DM. A previous study has examined symptoms of ischemic heart disease in adults with T2DM undergoing CGM and reported a higher frequency of chest pain during hypoglycaemic episodes (Desouza et al., 2003). The authors did not separate their periods of recording into night

and day but it is conceivable that if their patients were generally reporting symptoms during the day (as is most likely), this might reflect the transient cardioacceleration during hypoglycaemia (causing subsequent angina), as observed in this study. Ischemia may further enhance hypoglycaemia-associated repolarisation abnormalities.

There are limitations to CGM concerning its accuracy at low glucose values and a time lag due to diffusion of glucose between the blood and interstitial compartments. Although there are reports that CGM glucose may be falsely low during hypoglycaemia in T1DM (McGowan et al., 2002), in T2DM CGM glucose has been reported to be falsely high due to differences in glucose distribution in the interstitial space (Choudhary et al., 2011). Therefore, there can be reasonable confidence that IG ≤ 3.5 mmol/L as detected by CGM in this study represents true biochemical hypoglycaemia. Since it would be unethical to perform experimental hypoglycaemia studies in these patients at high cardiovascular risk, CGM remains, at the moment, the best available tool to study clinical hypoglycaemia in an ambulatory setting.

3.6 Conclusions

In conclusion, I have shown that hypoglycaemia is associated with an increased susceptibility to cardiac arrhythmias in patients with T2DM at cardiovascular risk. Changes in cardiac autonomic tone and abnormal repolarisation are potential contributory mechanisms. These findings may also be relevant to the 'dead-in-bed' syndrome in T1DM, which also occurs

at night, typically in individuals with recurrent asymptomatic hypoglycaemia, where an arrhythmic mode of death has been suggested (Heller, 2002). This study confirms that clinical hypoglycaemia is common and is frequently unrecognised in patients with advanced T2DM, even when glycaemic control is not as aggressive as that in recent interventional trials. Hypoglycaemia may contribute towards increased cardiovascular mortality through arrhythmic mechanisms in this vulnerable group.

Chapter 4 - Effect of Acute Hypoglycaemia on Cardiac Autonomic Regulation in Type 2 Diabetes

4.1 Summary

Spontaneous hypoglycaemia has been associated with an increased risk of arrhythmias in T2DM patients. These changes could reflect differential changes in autonomic tone, which may differ according to the duration of hypoglycaemia. The aim of this study was to measure changes in cardiac autonomic function during sustained experimental hypoglycaemia.

Twelve adults with T2DM and normal cardiac autonomic function tests and eleven age and BMI-matched nondiabetic controls underwent paired hyperinsulinaemia clamps separated by four weeks. Glucose was maintained at 6.0 mmol/L for 2 hours during euglycaemia, while during hypoglycaemia glucose was lowered to 2.5 mmol/L over 1 hour and

maintained for a further hour. Heart rate, blood pressure and HRV and BRS were assessed at 30 minute intervals.

In subjects with diabetes, the heart rate increased maximally from 72±10 to 77±10 bpm after 30 minutes at hypoglycaemia (T90), but then fell to 71±8 bpm despite continued hypoglycaemia at 1 hour (T120). HF power decreased transiently but returned towards baseline at T120, suggesting vagal reactivation. In nondiabetic controls, there was an earlier increase in heart rate at T60 (from 62±9 to 66±13 bpm) which remained elevated throughout hypoglycaemia. HF power was persistently lower indicating continued vagal inhibition.

Cardiac autonomic regulation during hypoglycaemia appears time-dependent and different in T2DM and nondiabetic subjects. In individuals with T2DM, the initial heart rate increment to hypoglycaemia is delayed and there is reactivation of vagal activity during more sustained hypoglycaemia. These mechanisms might contribute to tachy- and bradyarrhythmias during clinical hypoglycaemic episodes.

4.2 Introduction

Recent studies of intensive insulin therapy in T2DM have shown a higher risk of fatal arrhythmic death associated with severe hypoglycaemia (Gerstein et al., 2012). One mechanism by which hypoglycaemia could promote arrhythmias is through changes in cardiac autonomic activity. During insulin-induced hypoglycaemia in nondiabetic and T1DM

individuals, studies have reported a transient beta-adrenergic mediated increase in heart rate (Fisher et al., 1990), while the role of cardiac vagal activity is less clear (Koivikko et al., 2005; Laitinen et al., 2008; Schachinger et al., 2005). Another study has reported increased stroke volume with a fall in total peripheral resistance secondary to beta and alpha adrenergic stimulation (Fisher et al., 1990). To date, few studies have examined the cardiac autonomic responses to acute hypoglycaemia in individuals with T2DM.

During ambulatory monitoring, a transient increase in heart rate was observed coinciding with vagal withdrawal during daytime hypoglycaemia in T2DM subjects as described in Chapter 3 (Chow et al., 2014). However, during nocturnal hypoglycaemic episodes, where glucose was generally lower and episodes more prolonged, there was a phasic response, whereby initial increases in heart rates were followed by a relative bradycardia. It was hypothesised that this may be due to diurnal differences in the depth and duration of hypoglycaemia, which result in differential sympathetic and parasympathetic stimulation over time. However, in spontaneous clinical episodes neither the depth nor duration can be controlled, and it is not possible to measure levels of circulating catecholamines or electrolytes. Therefore the aim of this study was to examine in detail cardiac autonomic regulation using an experimental model of hypoglycaemia in patients with T2DM and to compare this to a nondiabetic control group.

4.3 Research Design and Methods

These were described in detail in Section 2.3 and are outlined in brief below.

4.3.1 Participants

Twelve individuals with T2DM and no known CVD were recruited from Sheffield Teaching Hospitals diabetes outpatient clinics. Patients were on one or more oral hypoglycaemic agents and/or a GLP-1 analogue or insulin <2 years. Eleven nondiabetic age and BMI matched controls were recruited from staff at the University of Sheffield and Sheffield Teaching Hospitals. Patients taking beta-blocking agents were excluded. Written informed consent was obtained from all participants and the study received local ethics approval.

4.3.2 Baseline Assessment

Cardiovascular autonomic reflex tests were performed as previously described (O'Brien et al., 1986) according to the recent consensus statement on the diagnosis of CAN (Tesfaye et al., 2010). All patients were euglycaemic at the time of autonomic function testing. Results were compared against age-adjusted normal reference ranges (O'Brien et al., 1986) and those below the 95% CI were considered abnormal. Participants with established CAN, as defined by two or more abnormal cardiovagal tests, were excluded. All participants had a normal 12 lead ECG at baseline,

and the control group had normal fasting serum glucose (<7 mmol/L) and HbA1c <6.5% (< 48 mmol/mol) at baseline.

4.3.3 Hyperinsulinaemic Clamp Protocol

All subjects participated in paired hyperinsulinaemic euglycaemic and hypoglycaemic studies separated by at least four weeks. Details of the hyperinsulinaemic clamp protocol are described in Section 2.3.5. During the clamp, a primed continuous intravenous insulin infusion was administered at 120 mU/m^2/min, along with 20% dextrose at a variable rate and adjusted according to arterialised blood glucose concentrations, which were measured every five minutes. In the hypoglycaemic clamp, glucose was lowered from euglycaemia to 2.5 mmol/L over 60 minutes (T0 to T60), and thereafter maintained at 2.5 mmol/L for a further 60 minutes (T60-T120). In the euglycaemic clamp, arterialised whole blood glucose was maintained at 6 mmol/L for the duration of the study (T0-T120).

4.3.4 Heart Rate Variability and Blood Pressure

ECG recordings were performed at 30 minute intervals during the clamp study (Baseline, T30, T60, T90, T120). HRV was determined from five minute resting recordings with the participant supine and free breathing. Normal R-R intervals (NN) were extracted for HRV analysis performed in accordance with the recommendations of the 1996). The time domain measures the SDNN and the RMSSD of NN were analysed. Fast Fourier transformation was applied to R-R intervals for spectral analysis. The LF band was defined as 0.04-0.15 Hz and the HF band was defined as 0.15-0.4

Hz. The ratio between the LF power and total power was calculated (LFnorm), which is believed to indicate the level of sympathetic modulation in HRV (Bernardi et al., 2011; Montano et al., 1994). The HF power is an indicator of cardiac vagal activity.

Blood pressure was measured every 30 minutes using an automatic oscillometric sphygmomanometer (DINAMAP ®GE Medical Systems Information technologies, Inc.) after lying supine for five minutes. Pulse pressure was defined as the SBP minus the DBP, whilst mean arterial pressure (MAP) was calculated as DBP + 1/3 (SBP-DBP) in mmHg.

4.3.5 Spontaneous Baroreceptor Sensitivity

Spontaneous cardiovagal BRS was measured using a Portapres (TNO Biomedical Instrumentation, Amsterdam, Netherlands). BRS was recorded for five minutes following internal calibration once a steady state was achieved. BRS analysis was performed using dedicated software using the sequence method (Nevrokard V 5.1.3, Intellectual Services, Slovenia) (Parati et al., 1988). An 'UP' sequence was generated when there was an increase in SBP of at least 1 mmHg associated with an increase in R-R interval of at least 5 ms for three or more consecutive beats. A 'DOWN' sequence was generated where there was a reduction in SBP of at least 1 mmHg with an accompanying reduction in R-R interval of at least 5 ms for three or more consecutive beats. The total BRS, measured in ms/mmHg was calculated by averaging the regression coefficients of the RR/BP

relationship of the UP and DOWN sequences with correlation coefficients greater than 0.85.

4.3.6 Biochemical Analysis

Biochemical parameters were measured at baseline and after 120 minutes in the euglycaemic and hypoglycaemic arms in both groups. For catecholamines, 6 mL of whole blood was collected into a chilled lithium heparin tubes containing 50 µL of EGTA/glutathione preservative and subjected to centrifugation at 4^{o}C and 3000 rpm for 10 minutes. The resulting supernatant was stored at -80^{o}C until assayed by HPLC. Plasma free insulin was analysed by an immunometric assay (Invitron Insulin ELISA, Invitron Ltd, Monmouth, UK) following precipitation with PEG. Serum potassium was analysed using an automated system (Cobas® modular anlalyzer, Roche Diagnostics, West Sussex, UK).

4.3.7 Statistical Analysis

Data that followed an approximate normal distribution were summarised using the mean (SD) unless otherwise stated, whilst skewed data were summarised using the median (interquartile range). Baseline characteristics between the diabetic and control groups were compared using an independent t-test, Mann Whitney-U or Fisher's exact test. Catecholamines, glucose and potassium were compared at T120 under euglycaemic and hypoglycaemic conditions using a one way ANOVA with planned contrasts for the effect of the group (diabetes versus controls). The nonparametric Kruskal-Wallis test was used to compare free insulin

levels at T120 for euglycaemia versus hypoglycaemia and between the groups.

Spectral HRV parameters were logarithmically transformed to approximate a normal distribution. A two-way ANOVA with repeated measures was used to study the main effects of time, glucose, group, and interaction between factors on changes in heart rate, blood pressure, HRV and BRS parameters. Data were checked for sphericity using Mauchly's test, and where sphericity was violated the Greenhouse-Geisser correction was applied. Statistical analysis was performed using SPSS (version 20.0, IBM, Chicago, Illinois) and a p-value ≤ 0.05 was deemed statistically significant.

4.4 Results

4.4.1 Participant characteristics

Participant characteristics are shown in Table 4-1. T2DM patients had a median age of 54 (range 37-64) years with HbA1c 7.8±1.3% (62±14 mmol/mol). Five patients were taking oral hypoglycaemic agents only, five were taking a combination of oral hypoglycaemic agents and a GLP-1 analogue, and two had been taking oral hypoglycaemic agents and basal insulin for less than two years. Only two diabetic patients were on ACE inhibitors. Nondiabetic subjects were similar in demographics with median age 52 (range 34-63) years and BMI 31±8 kg/m^2.

The baseline measurements showed that diabetic subjects had higher baseline heart rates and blood pressure, but lower HRV and BRS. None of

the subjects had clinical CAN as defined by cardiovascular autonomic reflex tests below the normal reference range (Table 4-2).

Table 4-1: Participant characteristics

Data are mean ± SD except age which is the median value (range). p-value indicates a comparison of the two groups via parametric or nonparametric testing. HbA1c-glycated haemoglobin, SBP-systolic blood pressure, DBP-diastolic blood pressure, BMI-body mass index

	T2DM n=12	**Nondiabetic** n=11
Age (years)	54 (37-64)	52 (34-63)
Male (M/F)	9/3	5/6
BMI (kg/m^2)	34 ± 5	31 ± 8
Duration of diabetes (years)	11 ± 7	n/a
HbA1c (%)	7.8 ± 1.3	5.5 ±0.3
(mmol/mol IFCC)	62 ± 14	34±10
SBP (mmHg)	135 ± 15	131 ± 15
DBP (mmHg)	82 ± 10	72 ± 11
Heart Rate (bpm)	78 ± 8	64 ±6

Table 4-2: Baseline cardiac autonomic function in T2DM patients and nondiabetic subjects

Data mean ±SD. Independent t-test of baseline variables was used to compare the T2DM and nondiabetic groups. HRV- heart rate variability, HF-high frequency, BRS-baroreceptor sensitivity, E:I -expiration:inspiration ratio

	T2DM n=12	**Nondiabetic** n=11	**p-value**
Cardiovascular autonomic reflex tests			
E:I ratio	1.24±0.20	1.27±0.23	0.74
Valsava ratio	1.51±0.29	1.81±0.41	0.05
Standing ratio	1.28±0.17	1.58±0.28	0.005
Heart rate variability			
SDNN	27.4±11.8	48.2±14.6	0.001
RMSSD	15.5±7.94	32.9±15.3	0.002
Log Total power	2.34 ± 0.36	2.90±0.36	0.001
Log HF	1.85± 0.37	2.32±0.48	0.02
LFnorm	0.65±0.14	0.70±0.14	0.40
BRS	8.59±4.08	11.51±7.65	0.26

4.4.2 Blood Glucose and Insulin

Target arterialised blood glucose levels were achieved and are shown in Figure 4-1. Blood glucose concentrations at 120 minutes were similar between diabetic and nondiabetic groups in the euglycaemic arm (5.81± 0.29 mmol/L versus 5.96± 0.18 mmol/L, p=0.15, respectively) and in the hypoglycaemic arm (2.56± 0.22 mmol/L versus 2.56± 0.09 mmol/L, p>0.99, respectively).

Free insulin levels at 120 minutes were median (IQR) 576 (468-627) pmol/L during euglycaemia and 689(477-1076) pmol/L during hypoglycaemia in the diabetic group. In the nondiabetic group they were 865 (509-952) pmol/L in the euglycaemic and 665(468-967) pmol/L in the hypoglycaemic arms. A comparison of insulin levels between the groups under both euglycaemic and hypoglycaemic conditions were found to be not statistically different (p = 0.23).

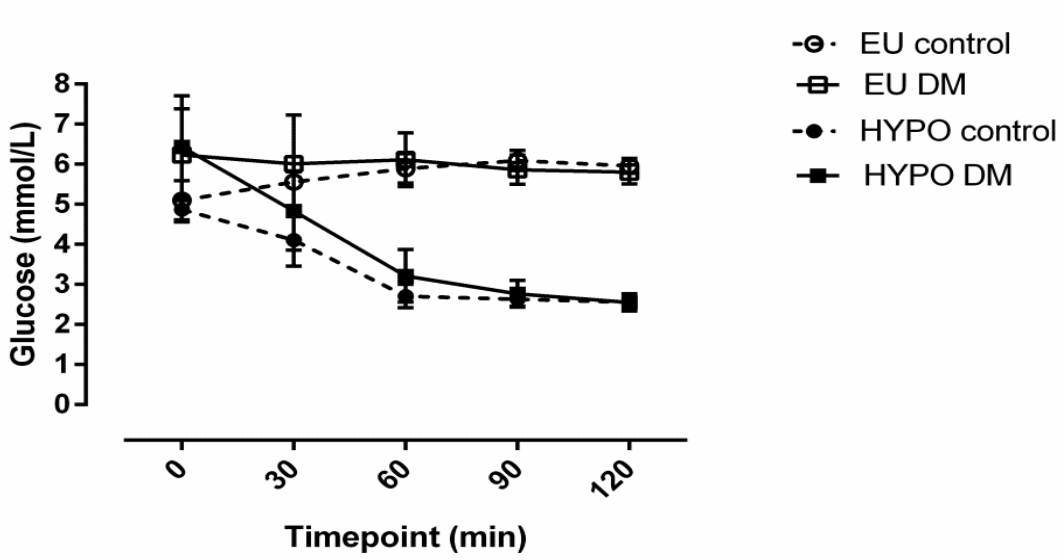

Figure 4-1: Arterialised blood glucose during hyperinsulinaemic euglycaemic and hypoglycaemic clamps

Open circle: euglycaemia (EU) nondiabetic control group; Open square: hypoglycaemia (HYPO) in nondiabetic control group; Closed circle: EU arm in diabetic group; Closed square: HYPO in diabetic group. Data are Mean (SE)

4.4.3 Heart Rate

During euglycaemia there were no significant changes in heart rate within the diabetic group nor in the nondiabetic subjects (p=0.19 and p=0.11, respectively for time by one way ANOVA) (Figure 4-2a). Conversely, the heart rate changed significantly during hypoglycaemia in the diabetic group (p = 0.03 for time by one way ANOVA). In the diabetic group, there was an initial maximal increase in heart rate from 72±10 to 77±10 bpm (Δ5±2 bpm, p=0.03 versus baseline) after 30 minutes of hypoglycaemia (T90) (Fig. 4-2a); however, this subsequently returned towards baseline 71±8 bpm (Δ-1±2 bpm) after an hour (T120) despite maintained hypoglycaemia (Figure 4-2a). In the nondiabetic group, there was a trend for an increase in heart rate during hypoglycaemia (p=0.20 by one way ANOVA), but the maximum

increase in heart rate occurred earlier at T60 (from 62±9 to 66±13 bpm, Δ4±5 bpm) and remained higher than baseline after 1 hour (T120, 64±10 bpm). The response was different to that during euglycaemia, as shown by a significant interaction between time and glucose (p=0.04). The absolute maximum increment in heart rate was similar between the groups but the time to maximum occurred later in diabetic subjects (Table 4-3).

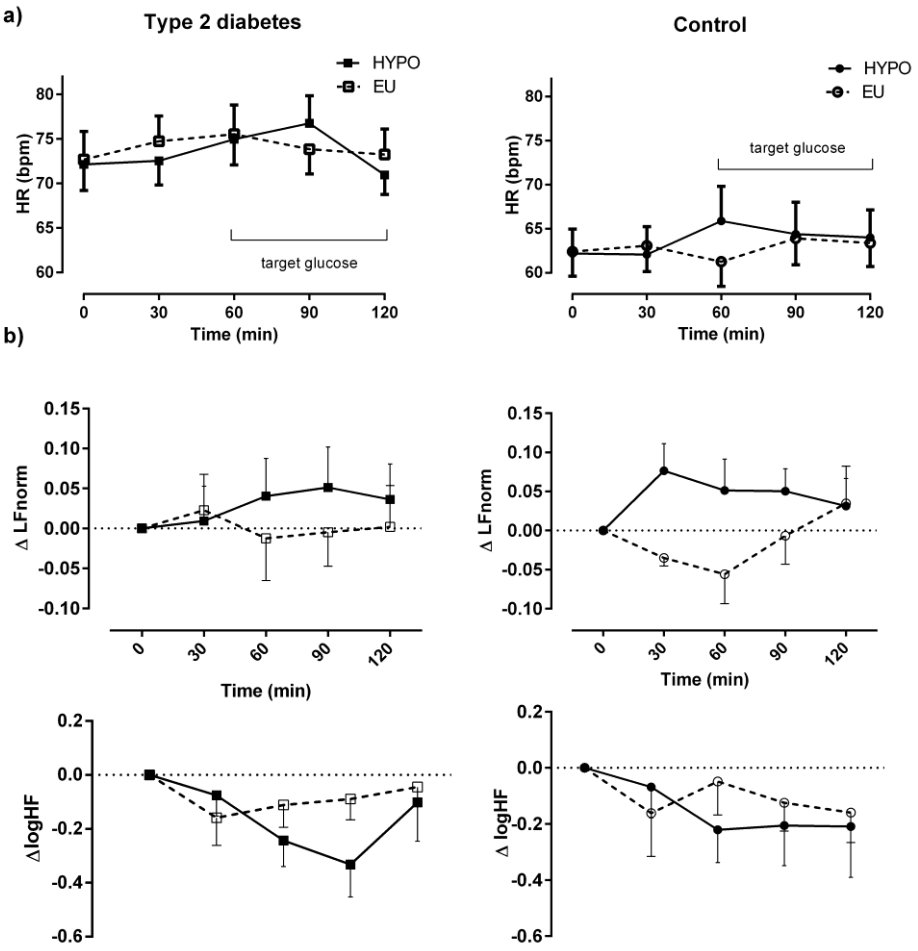

Figure 4-2: Heart rate and change in heart rate variability during hypoglycaemia in T2DM and nondiabetic subjects

(a) shows the heart rate during hypoglycaemia versus euglycaemia in diabetic and nondiabetic groups. The period at which glucose was maintained at the target levels during euglycaemia (6 mmol/L) and hypoglycaemia (2.5 mmol/L) are shown. (b) shows the hanges in normalised low frequency (LFnorm) and log HF (high frequency) power during hypoglycaemia in diabetic patients and nondiabetic controls. Data are mean (SE).

Table 4-3: Maximum change and time to maximum for cardiovascular parameters during hypoglycaemia in diabetic and nondiabetic subjects
Maximum change (Mean ±SD); time to maximum change; median (interquartile range), log HF high frequency heart rate variability, LFnorm-normalised low frequency HRV, SBP-systolic blood pressure, DBP-diastolic blood pressure, PP-pulse pressure

	Nondiabetic (n=11)		**Diabetes** (n=12)	
	Max change	**Time to max change**	**Max change**	**Time to max change**
Heart Rate	5.26±4.65 bpm	**60** (60-90) min	5.69±6.66 bpm	**90** (60-90) min
logHF	-0.45±0.21 ms^2	**60** (60-90) min	-0.45±0.34 ms^2	**90** (60-120) min
LFnorm	0.13±0.10 ms^2	**60** (30-90) min	0.13±0.13 ms^2	**75** (52.5-97.5) min
SBP	13±20 mmHg	**60** (30-90) min	14±15 mmHg	**90** (60-90) min
DBP	-13 ±7 mmHg	**120** (105-120) min	-11 ±7 mmHg	**90** (82.5-90) min
PP	21±15 mmHg	**90** (60-105)min	19 ±8 mmHg	**75** (60-90) min

4.4.4 Heart Rate Variability

4.4.4.1 Spectral Analysis

High frequency power

Log HF did not change significantly during euglycaemia in diabetic and nondiabetic groups (Figure 4-2b). During hypoglycaemia in diabetic

subjects there was an overall change in log HF (p=0.05 for time). There was a decrease in HF power (logHF) maximally at T90, suggesting vagal withdrawal, coincident with the maximal increase in heart rate during hypoglycaemia (Figure 4-2b). However, after one hour (T120), logHF returned towards baseline levels suggesting restoration of vagal tone. In the control group, logHF decreased during hypoglycaemia maximally at T60 and remained decreased up to T120. This was significantly different from euglycaemia (p=0.02 for glycaemic arm). The maximum decrease in logHF occurred earlier in the nondiabetic group but the magnitude of change was similar in both groups (Table 4-3).

Total power

Total power did not change significantly during euglycaemia in diabetic subjects. However, there was a trend towards a decrease in total power up to T90 during hypoglycaemia (p=0.10 for time). There was no significant difference between the glycaemic arms among diabetes subjects and in nondiabetic subjects there was no significant change during euglycaemia or hypoglycaemia.

Normalised low frequency power

LFnorm did not change significantly during euglycaemia within the diabetic group (Figure 4-2c), and similarly, there were no significant changes in LFnorm for during hypoglycaemia. In the nondiabetic group there was a trend towards a decrease at T60 during euglycaemia which then returned towards baseline (p=0.06 for time). LFnorm increased during

hypoglycaemia (p=0.04 for time) to a maximum at T30; however, the difference compared with euglycaemia did not reach statistical significance (p=0.23 for glucose, p=0.16 for interaction between time and glucose by two-way ANOVA) (Figure 4-2c).

Time domain analysis

RMSSD did not change during euglycaemia in either subject group. In diabetic subjects, RMSSD showed a trend towards a decrease during hypoglycaemia at T60 and T90, but returned to above the baseline level following hypoglycaemia at T120, exhibiting similar trends to HF power (p=0.11 for time) (Table 4-4). The pattern of change was different between hypoglycaemia and euglycaemia (p=0.03 for the interaction between glycaemic arms and time by two way repeated measures ANOVA). However, in nondiabetic subjects, RMSSD did not change during hypoglycaemia and there was no significant difference between the glycaemic arms (Table 4-4).

SDNN did not change during euglycaemia in diabetic subjects but tended to increase at T120 after hypoglycaemia (Table 4-4). There was a difference in change over time between glycaemic arms (p=0.04). There were no significant changes in SDNN in either glycaemic arm in the nondiabetic group.

Table 4-4: Time domain measures of Heart rate variability during hypoglycaemia and euglycaemia

EU-euglycaemia HYPO-hypoglycaemia, SDNN-standard deviation of NN intervals, RMSSD-root mean squared of successive standard deviations of NN; Data are mean ±SD.

	Time	0	30	60	90	120
Diabetic (n=12)						
RR (ms)	EU	844±183	816±164	811±189	826±161	830±152
	HYPO	849±171	835±145	815±167	794±163	855±126
SDNN (ms)	EU	33.0±12.5	29.1±12.6	29.1±13.0	29.4±14.5	29.2±12.9
	HYPO	31.0±14.9	33.9±16.8	30.3±14.7	34.0±18.1	40.4±26.9
RMSSD (ms)	EU	18.6±9.7	15.6±8.2	15.6±6.9	17.7±11.4	16.8±7.7
	HYPO	20.0±13.0	19.6±14.2	15.0±8.2	16.2±13.4	22.9±22.2
Nondiabetic (n=11)						
RR (ms)	EU	983±164	978±157	1008±164	968±161	976±136
	HYPO	982±148	1009±178	958±203	968±172	958±154
SDNN (ms)	EU	48.7±19.7	61.8±27.7	53.2±19.1	53.7±10.8	59.3±23.4
	HYPO	55.9±26.7	57.6±16.2	47.1+17.4	51.0±19.7	59.2±40.4
RMSSD (ms)	EU	30.6±18.2	33.3±16.0	32.0±15.8	28.9±10.8	30.9±12.4
	HYPO	35.0±18.9	34.1±17.3	28.7±17.4	33.7±21.2	36.0±36.2

4.4.5 Blood Pressure

4.4.5.1 Systolic Blood Pressure

SBP did not change significantly during euglycaemia in the diabetic group or nondiabetic group. In T2DM subjects there was a non-significant trend towards increased SBP maximally at T90, (Δ 8±20 mmHg) but this fell to

below baseline (Δ-2±13) during sustained hypoglycaemia at T120 (p=0.21 for time) (Figure 4-3). The response was not significantly different compared with that for euglycaemia (p=0.80 for glucose, p=0.54 for interaction between time and glucose). SBP did not change significantly during hypoglycaemia in the nondiabetic group.

4.4.5.2 Diastolic Blood Pressure

There were no changes in DBP during euglycaemia in diabetic or nondiabetic subjects. During hypoglycaemia, DBP tended to decrease from the baseline (77±7 mmHg up to 71±12 mmHg) at T90 in the diabetic group (Δ -6.0±10 mmHg) and then plateaued (Figure 4-3) (p =0.06 for time by one way ANOVA), and there was an interaction between time and the glycaemic arm (p=0.04).

In the nondiabetic group, DBP fell progressively from 74±9 mmHg to 62±10 mmHg (Δ-11.3±5.93 mmHg) up to T120 (Figure 4-3) (p<0.001 for time). There was a significant difference between the glycaemic arms (p=0.001) and the interaction between time and the glycaemic arm (p=0.006) by two way repeated measures ANOVA. The time to minimum DBP occurred later in the nondiabetic group (T120 (105-120) versus T90 (82.5-90) in the diabetic group (p=0.01).

4.4.5.3 Mean Arterial Pressure

MAP did not change during euglycaemia in either subject group. During hypoglycaemia, MAP remained steady between baseline to T90 minutes

but there was a trend towards a decrease at T120 in both diabetic and nondiabetic subjects (Fig 4-3). Overall, MAP was lower during hypoglycaemia compared to euglycaemia in the diabetic (p=0.021 for glycaemic arm) and nondiabetic groups (p=0.014 for glycaemic arm by two way repeated measures ANOVA).

4.4.5.4 Pulse Pressure

Pulse pressure did not change significantly during euglycaemia in diabetic subjects but increased significantly during hypoglycaemia (p<0.001 for time). Pulse pressure at T60 and T90 were significantly higher than at baseline (p=0.001 and p=0.002 respectively). There was a significant interaction between time and the glycaemic arm (p=0.02 by two way repeated measures ANOVA). In the nondiabetic group, pulse pressure did not change significantly during euglycaemia, but increased during hypoglycaemia (p=0.03 for time). The differences between the glycaemic arms did not reach statistical significance in nondiabetic subjects. The maximum change in pulse pressure occurred at a median of 75 minutes in the diabetic group, primarily due to a rise in SBP, whereas the maximum change occurred later at 90 minutes in the nondiabetic group mediated mainly by a decline in DBP (Figure 4-3).

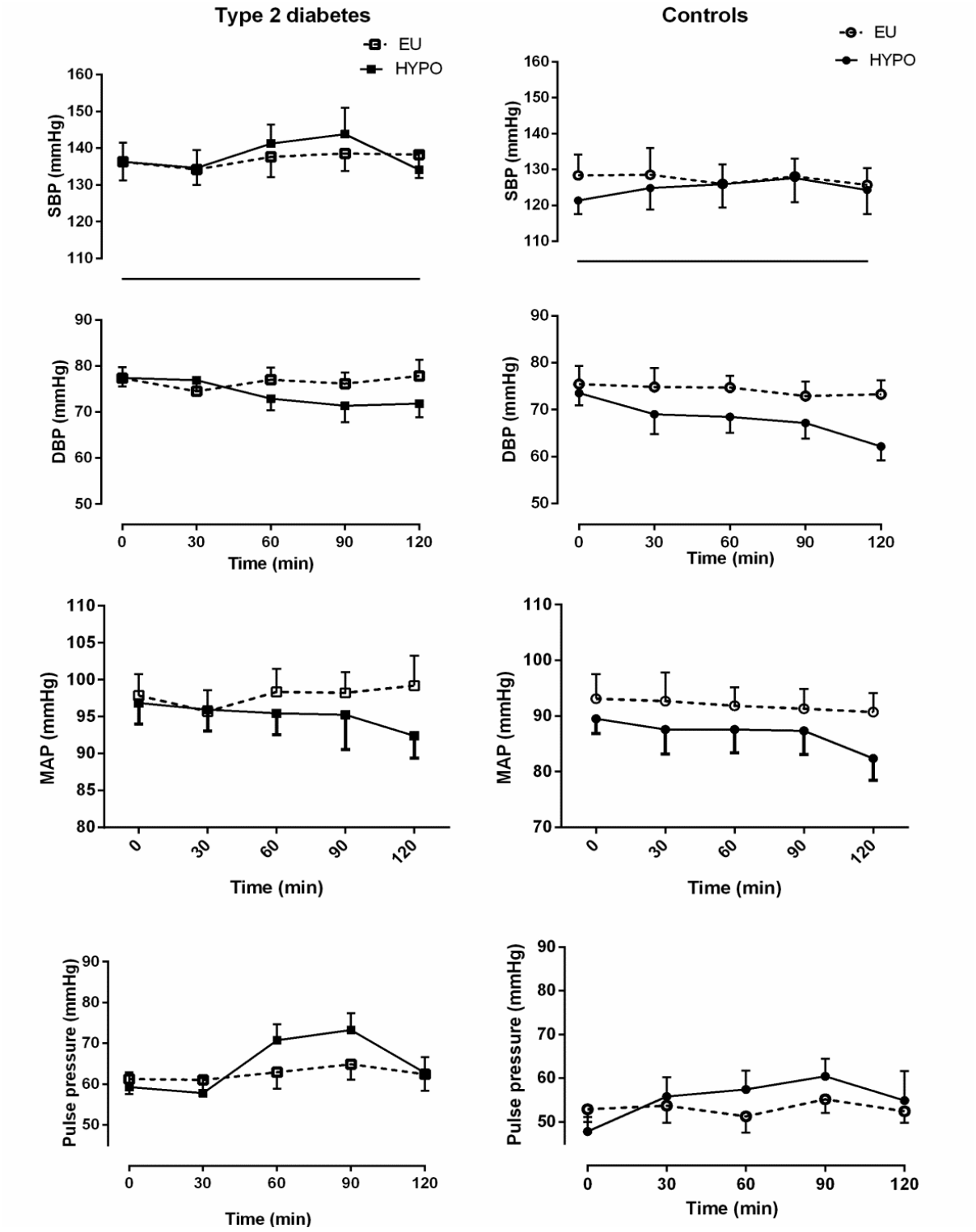

Figure 4-3: Blood pressure during hypoglycaemia and euglycaemia in T2DM and control subjects

Systolic blood pressure (SBP); diastolic blood pressure (DBP); mean arterial pressure (MAP) Data are mean (SE). Closed square – HYPO DM, open square - EU DM, Closed circle – HYPO control, Open circle – EU control

4.4.6 Baroreceptor Sensitivity

In the diabetic group during euglycaemia, BRS significantly decreased ($p<0.001$ for time) from within 30 minutes of the insulin infusion and remained lower at T120. However, there were no significant changes during hypoglycaemia (Figure 4-4). The difference between the glycaemic arms did not reach statistical significance ($p=0.29$ for glucose). In the nondiabetic group, there were no significant changes in BRS in both euglycaemic and hypoglycaemic arms.

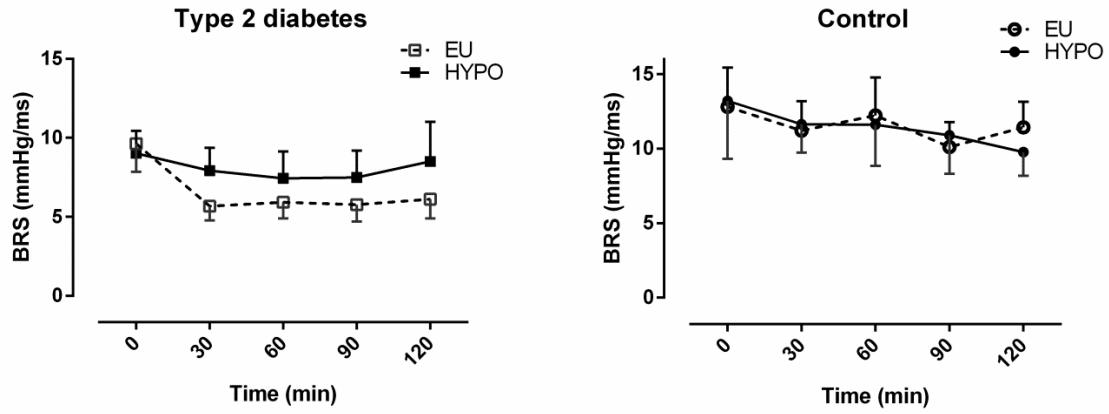

Figure 4-4: Baroreceptor sensitivity during hypoglycaemia and euglycaemia in T2DM and control subjects
Data are Mean (SE)

4.4.7 Biochemical Measurements

No significant changes in adrenaline and noradrenaline occurred during euglycaemia in both groups.

In the diabetic group, plasma adrenaline increased significantly from baseline, from 0.12±0.09 to 3.05±2.46 nmol/L at the end of the hypoglycaemic clamp ($p=0.002$). In the nondiabetic group, plasma adrenaline increased from 0.15±0.07 nmol/L to 3.83±2.83 nmol/L at the

end of hypoglycaemia (p=0.001 versus baseline). However, peak adrenaline levels were not significantly different between the groups during hypoglycaemia (p = 0.48).

During hypoglycaemia, plasma noradrenaline increased from 1.25±0.53 to 2.37±0.82 nmol/L in the diabetic group and 1.46±0.5 to 2.69±1.45 nmol/L in the nondiabetic group (both p<0.01 compared with baseline), but there was no significant difference between the groups (p=0.52).

Serum potassium decreased during both euglycaemia and hypoglycaemia, as expected due to the effects of insulin. In the diabetic group, potassium was significantly lower at the end of the hypoglycaemic clamp compared with euglycaemia (3.30±0.32 mmol/L versus 3.70±0.43 mmol/L, p=0.02). In the nondiabetic group levels of serum potassium during hypoglycaemia and euglycaemia were not statistically different (3.48±0.39 mmol/L versus 3.65±0.45 mmmol/L respectively, p = 0.36).

4.5 Discussion

The main findings of this study are as follows. 1) In T2DM subjects hypoglycaemia resulted in transient increases in heart rate with coincident vagal withdrawal, followed by a relative decrease in heart rate during more sustained hypoglycaemia of one hour accompanied by vagal reactivation, as shown by HF power and RMSSD. In nondiabetic subjects this did not occur and there was continued vagal inhibition during hypoglycaemia. 2) During hypoglycaemia, there was a decrease in DBP, a non-significant

change in SBP, a slight decrease in MAP and an increase in pulse pressure in both groups. There was a progressive decline in DBP maximally after 60 minutes of hypoglycaemia in nondiabetic subjects but this decline plateaued after 30 minutes in diabetic subjects.

In T2DM patients there was an initial increase in heart rate followed by a subsequent decrease during sustained hypoglycaemia after 1 hour. A similar phasic response was observed during spontaneous prolonged hypoglycaemia in T2DM patients during ambulatory monitoring (Chow et al., 2014) (see Chapter 3), and there was a transient cardioacceleration followed by subsequent decreases in heart rate up to fifty minutes later. It was hypothesised that this was secondary to a rebound in vagal activity, as shown by increased HF power, a phenomenon that has been observed in other studies of nondiabetic individuals ((Fisher et al., 1990; Lloyd-Mostyn and Oram, 1975). The extent of bradycardia was not as profound as that observed during spontaneous nocturnal hypoglycaemia, where episodes were generally more prolonged and at lower glucose values. However, since the experiments were conducted during the day, the differences in changes in heart rates may reflect circadian effects and a lower daytime vagal tone. Due to ethical reasons, the duration (60 minutes) and depth (2.5 mmol) of hypoglycaemia was limited to what can be induced in the laboratory safely, particularly in individuals with T2DM. It is conceivable that more prolonged hypoglycaemia of over two hours, as seen during

spontaneous episodes, would have led to continued vagal counteraction and similar decreases in heart rate.

There was a delayed increment in heart rate in diabetic patients compared with the nondiabetic subjects which only occurred after 30 minutes of hypoglycaemia. Russell et al. (2001) also reported a delayed increment in heart rate during hyperinsulinaemic hypoglycaemia in adults with T1DM compared to control subjects. In support of this finding, we also observed a delayed and lower magnitude of increase in normalised LF power in the nondiabetic group which is an indicator of the degree of sympathetic activity as a proportion of the overall autonomic balance. However, absolute noradrenaline and adrenaline levels during hypoglycaemia were similar in diabetic and nondiabetic subjects.

The decline in DBP during hypoglycaemia is consistent with previous studies (Fisher et al., 1990). Systemic or limb vascular resistance were not assessed due to the difficulties in performing additional invasive measurements, especially in diabetic subjects. Therefore we could only infer that the decrease in DBP during hypoglycaemia may be partly related to a decline in systemic vascular resistance secondary to predominant beta-2 adrenergic stimulation by adrenaline over noradrenaline (Fisher et al., 1990). DBP progressively declined up to the end of the hypoglycaemic period in the nondiabetic group, whereas in diabetic subjects, the declined plateaued after 30 minutes of hypoglycaemia. This is less likely to be explained by resistance to vasodilatory effects of insulin, as the changes in

DBP during hyperinsulinaemic euglycaemia were similar between the two groups. Adrenaline levels were slightly lower in diabetic subjects but not significantly different. Instread the attenuated decline in DBP in T2DM patients may reflect increased adrenergic sensitivity to noradrenaline-enhanced vasoconstriction in resistance arteries (Cipolla et al., 1996).

Insulin itself is sympathoexcitatory via central mechanisms, and intravenous administration is associated with vasodilatory effects. The cardiovascular changes that were observed during hyperinsulinaemic euglycaemia are consistent with those previously reported. These include an early increase in heart rate within 30 minutes of infusion, decreases in total power, and a sympathetic shift in autonomic balance (Emdin et al., 2001; Muscelli et al., 1998). Previous studies have shown that intravenous insulin infusions, at physiological doses, increased muscle and lumbar sympathetic nerve activity (Cassaglia et al., 2011) and also a gain of the baroreflex as measured by the relationship between DBP and muscle sympathetic nerve activity (Young et al., 2010). This effect is diminished in the presence of insulin resistance. However, the same study reported unchanged cardiovagal BRS during intravenous insulin infusions as measured by the relationship between spontaneous SBP/heart rate fluctuations.

In the present study, there were no changes in cardiovagal BRS during hyperinsulinaemic euglycaemia in nondiabetic subjects and a decrease in BRS in subjects with diabetes. The reasons for this decline in BRS during

euglycaemia in the diabetic group are unclear; however, the relationship was consistent across subjects. There were no changes in cardiovagal BRS during hypoglycaemia in diabetic and nondiabetic groups. Fagius and Berne (1991) measured BRS during insulin–induced hypoglycaemia in a small group of nondiabetic subjects, and similar to the findings here, they reported no change in BRS but a rapid resetting of the baroreflex working range.

One of the key findings of this study was that the decrease in heart rate and vagal reactivation at 1 hour (T120) only occurred in the diabetic group, whilst in contrast the nondiabetic group demonstrated continued vagal inhibition throughout the period of hypoglycaemia. Reasons for these differences are unclear but could conceivably relate to differential changes in blood pressure. A decline in mean arterial pressure is normally accompanied by decreases in baroreceptor firing and vagal inhibition. Hypoglycaemia was accompanied by more abrupt increases in pulse pressure between 60-90 minutes in diabetic subjects, whereas in nondiabetic individuals changes in pulse pressure were more gradual (Figure 4-3). Baroreceptors are also sensitive to the rate of change in arterial pressures. During hypoglycaemia, more abrupt rises in pulse pressure, even without significant changes in MAP, can increase baroreceptor firing and vagal activation (Bell, 2013). Furthermore, acute baroreceptor resetting is absent or blunted when the pressure is pulsatile and this can promote the reflex inhibition of sympathetic activity, both by

sensitising the baroreceptors and by centrally facilitating the reflex (Chapleau et al., 1988).

One of the strengths of this study is the inclusion of a hyperinsulinaemic euglyacemic control arm to account for the effects of insulin. In this study, changes in heart rate, HRV and blood pressure during hypoglycaemia have been demonstrated that are different when compared to those observed during hyperinsulinaemic euglycaemia. In addition, cardiovascular parameters have been measured at multiple time points during hypoglycaemia. Previous studies have yielded inconsistent results on the effect of hyperinsulinaemic hypoglycaemia on cardiac autonomic function (Koivikko et al., 2005; Laitinen et al., 2008; Schachinger et al., 2004), perhaps due to measurements at a single, uncontrolled time-point. Koivikko et al. (2005) induced hypoglycaemia in T1DM and healthy volunteers in a stepwise manner from euglycaemia to 2.0-2.5 mmol/L, and reported an increase in heart rate and a progressive decrease in HF power, indicating vagal withdrawal. In contrast, another study concluded that hypoglycaemia did not increase the heart rate and was associated with higher vagal power when measured after 65 minutes of hypoglycaemia (Schachinger et al., 2004). This study suggests that changes in cardiac autonomic regulation are phasic and are dependent on the duration of hypoglycaemia.

The depth and frequency of breathing during HRV recordings was not controlled for in this study, and this is known to affect the HF component

of HRV (Bernardi et al., 2011). In the original protocol, subjects were asked to perform paced breathing at 12 breaths/minute by following a timed visual display. However, subjects were unable to follow this reliably at hypoglycaemic levels due to impaired concentration. The respiratory frequencies were extracted from the ECG recordings during free breathing and it was found that the mean respiratory frequencies across the five minutes of recording were consistently situated in the middle of the HF frequency band, around 0.25 Hz. There was a slight increase in the respiratory frequency during both clamps but these increases were too small to significantly affect the HRV spectra (data shown in Appendix 6).Thus, it is believed that the HRV analyses as shown here are due to the effects of hypoglycaemia itself on heart rate rather than indirect influences via respiration.

A further limitation was the higher number of males in the diabetic group. This was an unintended consequence of the larger number of screen failures and withdrawals by female diabetic patients. Men have stronger sympathoadrenal responses compared to women in response to hypoglycaemia (Amiel et al., 1993). Thus smaller increments (and possibly a further delay) in heart rate and smaller changes in SBP and DBP compared to the nondiabetic subjects might have been expected, had the diabetic group been better gender balanced. Limitations of normalised LF HRV as an indicator of relative sympathetic contribution have been discussed in the methods section. However, other measurements of cardiac

sympathetic activity, such as noradrenaline spillover are invasive and difficult to perform during hyperinsulinaemic clamp studies. Spontaneous BRS does not evaluate the entire sigmoidal baroreceptor curve throughout its working range, and the number of spontaneously increasing or decreasing blood pressure ramps may limit the number of valid sequences suitable for analysis. BRS analysis from this study was also repeated using spectral methods, which showed largely similar results to those obtained using the sequence method (data not shown).

The current findings support the notion that hypoglycaemia may contribute to the risk of cardiac arrhythmias through alterations in cardiac autonomic responses. Thus, hypoglycaemia could induce arrhythmias by different mechanisms depending on the duration of hypoglycaemia and autonomic balance. Sympathetic activation can lead to ventricular tachyarrhythmias via the effect of catecholamines on the myocardial calcium influx, leading to delayed afterdepolarisations (Nordin, 2010). During continuous glucose and Holter monitoring in T2DM patients, a higher number of ventricular arrhythmias were seen in patients with severe hypoglycaemia (Stahn et al., 2014). In animal studies, ventricular arrhythmias during insulin induced hypoglycaemia were abolished by beta adrenergic blockade (Reno et al., 2013). Vagal reactivation during sustained hypoglycaemia might protect against sympathetic mediated ventricular tachyarrhythmias. Alternatively, excessive vagal counteraction could lead to bradycardia and the unmasking of ectopic foci under

conditions where background vagal tone is high, such as at night. This can predispose to Torsade de pointe, particularly in combination with hypokalaemia and abnormal repolarisation (Bolognesi et al., 2011; Nordin, 2014). In a previous study, a higher number of ventricular and atrial ectopic activities during spontaneous nocturnal hypoglycaemia were observed coinciding with conditions of high vagal tone (Chow et al., 2014).

An alteration in autonomic tone is not the only mechanism by which hypoglycaemia may be proarrhthmic. The risk of arrhythmias is further enhanced when combined with abnormalities such as hypokalaemia, QT prolongation, hypertension and hypothermia (Tsujimoto et al., 2014). Whilst it is impossible to study diabetes patients with manifest CVD for ethical reasons, it might be expected such patients may be at even greater risk of arrhythmia, whereby hypoglycaemia-related ischaemia may further predispose patients to fatal events.

In conclusion, it has been demonstrated that cardiac autonomic regulation during hypoglycaemia appears to be time-dependent. There were differences between those with T2DM and nondiabetic subjects which may be explained by differential haemodynamic effects and an impaired decline in DBP in response to hypoglycaemia. In T2DM, the initial heart rate increment to hypoglycaemia is delayed and there is reactivation of vagal activity during more sustained hypoglycaemia. These mechanisms could contribute to arrhythmias that have been reported in clinical hypoglycaemic episodes, and provides further evidence to support a

possible relationship between hypoglycaemia and increased cardiovascular mortality in T2DM.

Chapter 5 - Effect of Acute Hypoglycaemia on Cardiac Repolarisation in Type 2 Diabetes

5.1 Summary

Hypoglycaemia has been associated with increased cardiovascular mortality during trials of intensive glycaemic control. Increased risk of arrhythmias and QT prolongation during clinical hypoglycaemic episodes in patients with T2DM has previously been reported. The aim of this study was to explore the mechanisms underlying abnormal repolarisation using an experimental hypoglycaemic model.

Twelve T2DM subjects with no CVD and 11 age and BMI matched nondiabetic subjects underwent paired hyperinsulinaemic euglycaemic (glucose 6 mmol/L) and hypoglycaemic clamps (glucose 2.5 mmol/L) separated by at least four weeks. Identical insulin infusion rates were used across all the experiments. Twelve lead high resolution ECG recordings were made at baseline and after 60 minutes of euglycaemia or hypoglycaemia. Corrected QT interval and T wave symmetry were determined along with serum potassium and plasma catecholamines.

QTc increased by mean±SD Δ76±70 ms in the diabetic group during hypoglycaemia compared to Δ54±16 ms in the nondiabetic group. In contrast, T wave symmetry decreased to a greater extent during hypoglycaemia compared with euglycaemia in diabetic subjects (Δ -0.56±0.43 versus Δ -0.20±0.18, $p<0.001$) but were similar under both

conditions in nondiabetic subjects. Potassium was lower at the end of hypoglycaemia compared with euglycaemia in diabetic subjects (3.30±0.32 versus 3.70±0.43 mmol/L, p=0.02) but was similar between glycaemic arms in nondiabetic subjects. Peak adrenaline levels during hypoglycaemia were 3.05±2.46 and 3.83 ±2.83 nmol/L in diabetic and nondiabetic groups, respectively (p= 0.48).

Individuals with T2DM showed greater repolarisation abnormalities for a given hypoglycaemic stimulus despite comparable sympathoadrenal responses. This may reflect increased metabolic adrenergic sensitivity leading to lower potassium levels. This is a potential mechanism by which hypoglycaemia can predispose to cardiac arrhythmias during intensive glycaemic control.

5.2 Introduction

Abnormal cardiac repolarisation is strongly associated with an increase in cardiovascular mortality (Straus et al., 2006). Greater heterogeneity of repolarisation increases early afterdepolarisation and risk of ventricular arrhythmias (Nordin, 2010). In Chapter 3 we reported prolonged QT interval in acute spontaneous hypoglycaemia during the day. In selected individuals with a reduced repolarisation reserve, QT increased to over 500 ms during hypoglycaemia with concurrent abnormalities in T wave morphology. Studies from our group and others have also demonstrated repolarisation abnormalities during hypoglycaemia in patients with T1DM and nondiabetic individuals (Gill et al., 2009; Marques et al., 1997;

Robinson et al., 2003). However, few studies have examined the effect of hypoglycaemia in T2DM on repolarisation (Landstedt-Hallin et al., 1999; Marques et al., 1997) and these have not involved a nondiabetic control group.

It has been hypothesised that changes in QT during hypoglycaemia are secondary to increases in catecholamines. Robinson et al. (2003) demonstrated that the prolongation in QT during hypoglycaemia was abolished in the presence of beta-adrenoreceptor blockade, while maintenance of normokalaemia only partially attenuated QT prolongation. Thus it was concluded that changes in QT during hypoglycaemia are mediated by sympathoadrenal activation but not limited to adrenaline-mediated hypokalaemia. Simultaneous measurements of catecholamines and electrolytes at the time of the hypoglycaemic event could not been recorded in ambulatory monitoring as described in Chapter 3. Furthermore, the depth, duration and environmental conditions surrounding a hypoglycaemic episode could not be controlled. Thus the aim of this study was to examine in detail the mechanisms underlying abnormal cardiac repolarisation during experimental hypoglycaemia in T2DM individuals and to compare their responses to those of nondiabetic subjects. Although QT remains the most widely accepted measure of repolarisation, QT measurement is influenced by heart rate. Therefore other electrophysiological measures of cardiac repolarisation were also

investigated to capture T wave morphology, including T amplitude and T symmetry that are rate independent.

5.3 Research Design and Methods

These are described in detail in Section 2.3 and are outlined in brief below.

5.3.1 Participants

Twelve individuals with T2DM and no known CVD were recruited from Sheffield Teaching Hospitals diabetes outpatient clinics. Patients were on one or more oral hypoglycaemic agents and/or GLP-1 analogues or insulin < 2 years. Eleven nondiabetic age and BMI matched controls were recruited from staff at the University of Sheffield and Sheffield Teaching Hospitals. Patients taking beta-blocking agents were excluded. Written informed consent was obtained from all participants and the study received local ethics approval.

5.3.2 Baseline Assessment

Cardiovascular autonomic reflex tests were performed as previously described (O'Brien et al., 1986) according to a recent consensus statement on the diagnosis of CAN (Tesfaye et al., 2010). All patients were euglycaemic at the time of autonomic function testing. Results were compared against age-adjusted normal reference ranges (O'Brien et al., 1986) and those below the 95% CI were considered abnormal. Participants with established CAN, as defined by two or more abnormal cardiovagal tests, were excluded. All participants had a normal 12 lead ECG at baseline

and the nondiabetic group had normal fasting serum glucose (<7 mmol/L) and HbA1c <6.5% (< 48 mmol/mol) at baseline.

5.3.3 Hyperinsulinaemic Clamp Protocol

All subjects participated in paired hyperinsulinaemic euglycaemic and hypoglycaemic studies separated by at least four weeks. Details of the hyperinsulinaemic clamp protocol are described in Section 2.3.5. During the clamp study, a primed continuous intravenous insulin infusion was administered at 120 mU/m^2/min, along with 20% dextrose at a variable rate and adjusted according to arterialised blood glucose concentrations, which were measured every five minutes. In the hypoglycaemic clamp, glucose was lowered from euglycaemia to 2.5 mmol/L over 60 minutes (T0 to T60), and then maintained at 2.5 mmol/L for a further 60 minutes (T60-T120). In the euglycaemic clamp, arterialised whole blood glucose was maintained at 6 mmol/L for the duration of the study (T120).

5.3.4 High Resolution ECG Recording

High resolution 12 lead ECGs were recorded for five minutes with the subject lying supine in a Mason-Likar configuration. To minimise mains electrical interference, all non-essential electrical equipment were switched off during the recording period. Signals were acquired at 1200 Hz and amplified using g®.USBamp (g.tec Medical Engineering, GmbH, Austria) and connected to a hp 6730 laptop computer with g.®Recorder software (g.tec Medical Engineering, GmbH, Austria) as previously described. All pre-processing and data analysis was performed using g.®BSAnalyze software.

Recordings were made at baseline, T60, T120 minutes and during recovery (30 minutes after the end of the clamp).

5.3.5 Repolarisation Analysis

The ECG signal was pre-processed with bandpass filtering between 0.2 and 40 Hz. R peaks were detected using automated software and manually verified. Signal averaging was then performed using correlation methods to improve the signal to noise ratio. Beat selection was performed by template matching (correlation coefficient > 0.95) and the mean beat was calculated using the selected beats. The repolarisation analysis was based on a composite wave, calculated from leads I, II and V5. The QT interval, from Q onset to T end, was based on the tangent method and Bazett correction of the QT interval was applied. As the Bazzett correction may overcorrect at higher heart rates, QT was also corrected according to the nomogram method (Karjalainen et al., 1994) which has been validated for heart rates between 40 to 120 beats per minute. T wave amplitude was defined from the isoelectric line to maximum T wave amplitude. This was expressed as the normalised T wave amplitude defined as the ratio of the T wave amplitude at the end of the clamp relative to the baseline. T wave symmetry was defined as the area under the T wave from T onset to T peak divided by the T wave area between T peak to T end (Merri et al., 1989) (Figure 5-1).

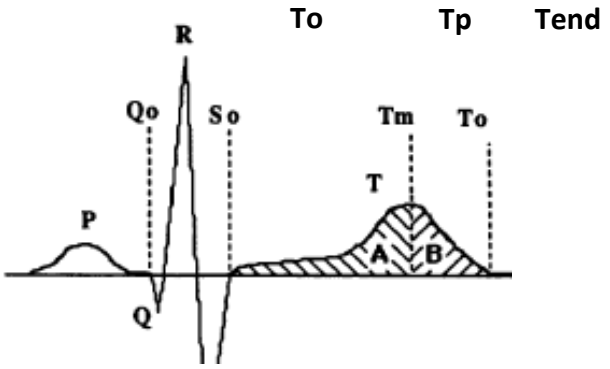

Figure 5-1: Description of T wave morphology measurements

Tp-T peak, T end, Qo-Q onset, To-Tonset; QT was defined as Q onset (first negative deflection from the isoelectric line) to T end defined using the tangent method. A tangent is fitted to the point of maximum gradient on the downward slope of the T wave and where it crosses the isoelectric line is defined as T end. T amplitude was defined from the isoelectric line to the maximum T amplitude at T peak in microvolts. T wave symmetry was defined as the area under the T wave from T onset to T peak divided (Area A) by the T wave area between T peak to T end (area B).

5.3.6 Biochemical Analysis

Biochemical parameters were measured at baseline and T120 in the euglycaemic and hypoglycaemic arms in both groups. For catecholamines, 6 mL whole blood was collected into chilled lithium heparin tubes containing 50 µL of EGTA/glutathione preservative and subjected to centrifugation at 4^{o}C, 3000 rpm for 10 minutes. The resulting supernatant was stored at -80^{o}C until assayed by HPLC. Plasma free insulin was analysed by an immunometric assay (Invitron Insulin ELISA, Invitron Ltd, Monmouth, UK) following precipitation with PEG. Serum potassium was analysed using an automated system (Cobas® system, Roche Diagnostics, West Sussex, UK).

5.3.7 Statistical Analysis

Data that followed an approximate normal distribution were summarised using mean±SD unless otherwise stated, whilst skewed data were summarised using the median (interquartile range). The effect of time and glycaemic arm on repolarisation measures were analysed using a two-way ANOVA with repeated measures within each subject group. Data were checked for sphericity using Mauchly's test, and where sphericity was violated the Greenhouse-Geisser correction was applied. Catecholamines, glucose and potassium at T120 were compared under euglycaemic and hypoglycaemic conditions using a one way ANOVA with planned contrasts for the effect of the group (diabetes versus nondiabetic). A nonparametric Kruskal-Wallis test was used to compare free insulin levels at T120 under euglycaemia versus hypoglycaemia and between groups. Statistical analysis was performed using SPSS (version 20.0, IBM, Chicago, Illinois) and a p-value ≤ 0.05 was deemed statistically significant.

5.4 Results

5.4.1 Baseline Characteristics

Participant characteristics are as previously described in Table 4-1. T2DM patients (9 male, 3 female) had a median age of 54 (range 37-64) years, with an HbA1c of 7.8±1.3% (62±14) mmol/mol. Nondiabetic subjects (5 male, 6 female) were similar in demographics with a median age 52 (34-63) years and BMI 31±8 kg/m^2. Baseline repolarisation measures are shown in Table 5-1. Baseline heart rates were higher and T wave amplitude lower

in the diabetic group at baseline compared with nondiabetic subjects; however, other measures, including QTc and T wave symmetry, were similar.

Table 5-1: Baseline repolarisation measures in diabetic and nondiabetic subjects

QTcB – corrected QT by Bazzett's formula; QTcN – corrected QT by nomogram method; Data are mean ± SD

	Diabetes n=12	**Nondiabetic** n=11
Heart Rate (bpm)	78 ± 8	64 ±6
QT (ms)	381±26	406±31
QTcB (ms)	417±6	411±7
QTcN (ms)	406±15	410±23
T wave amplitude (μV)	399±121	466±278
T wave symmetry	1.55±0.06	1.44±0.04

5.4.2 Blood Glucose and Insulin

Target blood glucoses were achieved (Figure 5-2). Blood glucose concentrations at 120 minutes were similar between the diabetic and control groups in both the euglycaemic arm (5.81± 0.29 mmol/L versus 5.96± 0.18 mmol/L, p=0.15) and hypoglycaemic arms (2.56± 0.22 mmol/L versus 2.56± 0.09 mmol/L, p>0.99).

Free insulin levels at 120 minutes were had a median value of 576 (IQR: 468-627) pmol/L during euglycaemia and 689 (IQR: 477-1076) pmol/L during hypoglycaemia in the diabetic group. In the nondiabetic group, levels were 865 (IQR: 509-952) pmol/L in the euglycaemic arm and 665 (IQR: 468-967) pmol/L in the hypoglycaemic arm. A comparison of the insulin levels between the groups under both euglycaemic and

hypoglycaemic conditions was found to not be statistically different (p = 0.23).

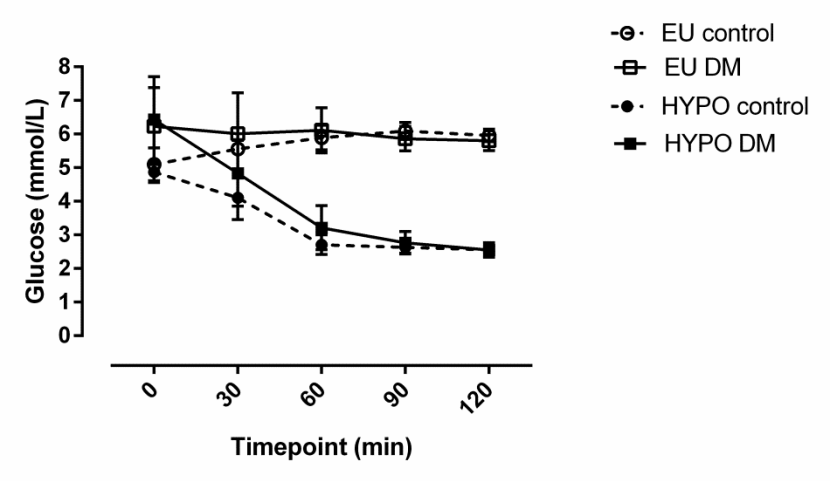

Figure 5-2: Arterialised blood glucose during hyperinsulinaemic euglycaemic and hypoglycaemic clamps
Closed circle EU arm in diabetic group, Closed triangle HYPO in diabetic group, Open circle EU in control group, Open triangle HYPO in control group. Data are mean (SE)

5.4.3 Heart Rate

During euglycaemia there were no significant changes in heart rate within the diabetic group nor in the nondiabetic subjects (p=0.19 and p=0.11, respectively for time by one way ANOVA). In the diabetic group, there was an initial maximal increase in heart rate from 72±10 to 75±10 bpm at T60, however, this subsequently returned towards baseline 71±8 bpm (Δ-1±2 bpm) after an 1 hour (T120). In the nondiabetic group, heart rate increased at T60 (from 62±9 to 66±13 bpm, Δ4±5 bpm) and remained higher than baseline after 1 hour (T120, 64±10 bpm).

5.4.4 QTc Interval

QTcB using Bazett's correction increased to a greater extent in the diabetic group during acute hypoglycaemia (Δ76±70 ms) at T120 compared to during euglycaemia (Δ15±15 ms, p=0.04 for glycaemic arm, p =0.002 for interaction between time and glycaemic arm). QTc also increased significantly in the nondiabetic group (Δ54±16 ms) during acute hypoglycaemia at end of clamp, but there was no significant change in the euglycaemic arm (p =0.002 for interaction between glycaemic arm and time) (Figure 5-3). The change in QTc during hypoglycaemia did not differ significantly between diabetic and nondiabetic groups (p = 0.32 by two group t-test).

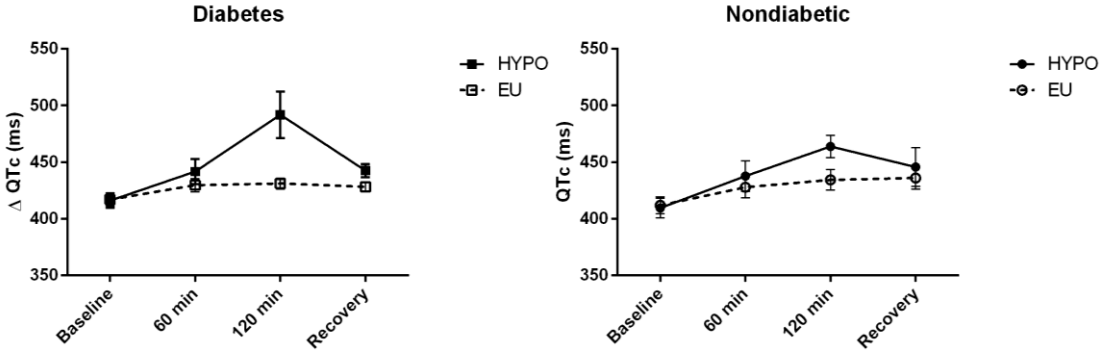

Figure 5-3: Changes in corrected QT interval (QTc) during hypoglycaemic and euglyacemic clamps in diabetic and nondiabetic subjects
Corrected QT using Bazzett's correction. Data mean (SE)

Changes in QTc using the nomogram method were also compared. QTcN did not change end of the euglycaemic clamp (Δ 9±10 ms) in T2DM subjects compared to an increase of Δ68±65 ms at the end of the hypoglycaemic clamp. In nondiabetic subjects, QTcN did not change during the

euglycaemic clamp but increased b7 Δ 56±18 ms at the end of hypoglycaemia.

Among diabetic subjects, QTc increased to >600 ms at the end of the hypoglycaemic clamp in two individuals, and the ECG waveform of one of these subjects is shown in Figure 5-4. This showed progressive T wave flattening, a decrease in T wave amplitude, a prolonged QT, and an increase in T wave symmetry. QTc returned to near baseline levels at recovery but T amplitude remained lower than at baseline.

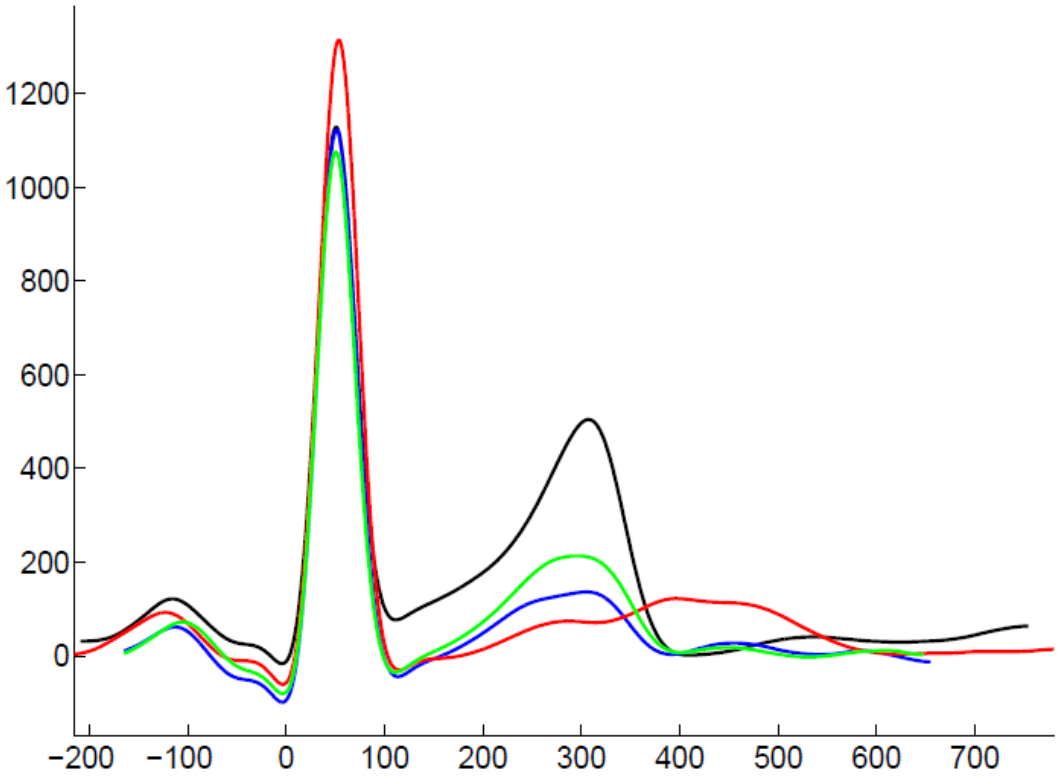

Figure 5-4: Composite ECG waveform showing abnormal repolarisation morphology with QTc >600 ms in one diabetic participant during hypoglycaemia

Black - baseline (QTc 397 ms), Blue (60 minutes) QTc 433 ms, Red (120 minutes) – QTc 605 ms, Green (Recovery) – QTc 444 ms

5.4.5 T Amplitude

The normalised T amplitude decreased during both hypoglycaemia and euglycaemic clamps from 60 minutes and persisted to recovery. In the diabetic group, the normalised T amplitude decreased to 46±18% of its baseline value during hypoglycaemia at T120. However, this was not significantly different compared to during euglycaemia, when it decreased to 60±12% of its baseline value (p= 0.11 for glycaemic group). In the nondiabetic group, the normalised T amplitude decreased to 66±50% of its baseline value at T120 during hypoglycaemia versus a decrease to 61±18% during euglycaemia (p=0.95 for glycaemic arm) (Figure 5-5).

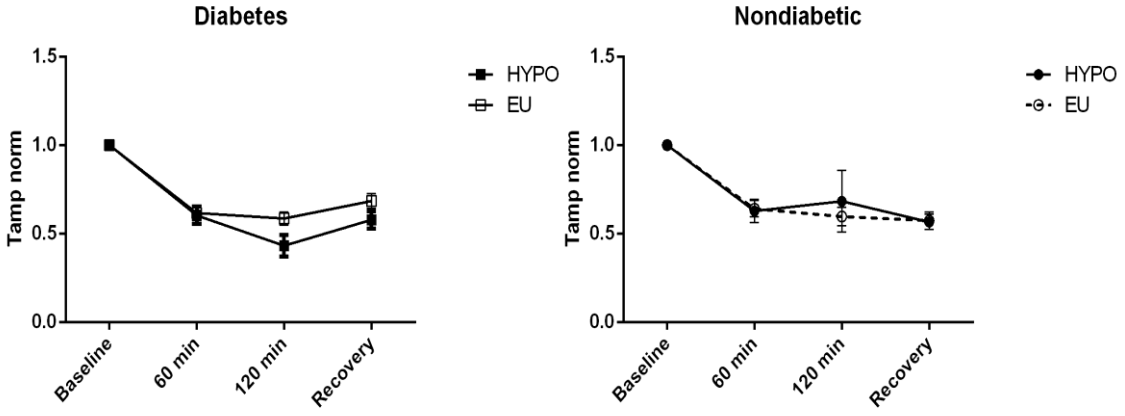

Figure 5-5: Normalised T amplitude during hypoglycaemia and euglycaemia in diabetic and nondiabetic subjects
Normalised T amplitude expressed as a ratio to baseline (1.0). Data mean (SE)

5.4.6 T Symmetry

The T symmetry decreased (Δ= -0.56±0.43) at the end of hypoglycaemic clamp, returning to baseline at recovery. This contrasts with no overall change during the euglycaemic clamp and a significant interaction between glycaemic arm and time (p=0.02) (Figure 5-6). In the nondiabetic group, T

symmetry decreased to a similar extent in both glycaemic arms from T60 and persisting to the recovery period (p =0.98 for glycaemic arm).

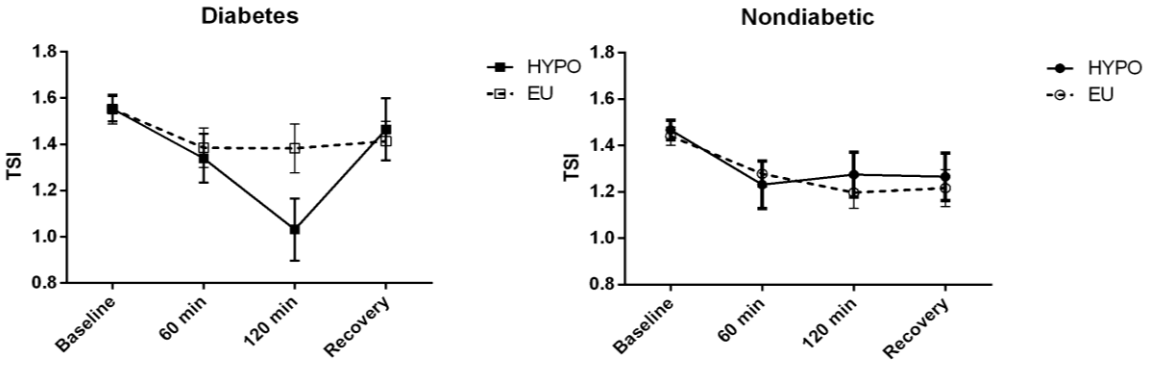

Figure 5-6: T wave symmetry during hypoglycaemia versus euglycaemia in diabetic and nondiabetic subjects
TSI-T wave symmetry; EU-euglycaemia; HYPO-hypoglycaemia; Data mean (SE)

5.4.7 Biochemical Measurements

No significant changes in adrenaline and noradrenaline occurred during euglycaemia in both groups. In the diabetic group, plasma adrenaline increased significantly from baseline, from 0.12±0.09 to 3.05±2.46 nmol/L at the end of the hypoglycaemic clamp (p=0.002), while in the nondiabetic group, plasma adrenaline increased from 0.15±0.07 nmol/L to 3.83±2.83 nmol/L at the end of hypoglycaemia (p=0.001 versus baseline). However, peak adrenaline levels were not significantly different between the groups during hypoglycaemia.

During hypoglycaemia, plasma noradrenaline increased from 1.25±0.53 to 2.37±0.82 nmol/L in the diabetic group and 1.46±0.5 to 2.69±1.45 nmol/L in the nondiabetic group (both p<0.01 compared with baseline). These as no significant difference between the groups (p=0.52).

Serum potassium decreased during both euglycaemia and hypoglycaemia as expected due to the effects of insulin. In the diabetic group, potassium was significantly lower at the end of the hypoglycaemic clamp compared with euglycaemia (3.30±0.32 mmol/L versus 3.70±0.43 mmol/L, p=0.02). In contrast, in the nondiabetic group the concentrations were not statistically different (3.48±0.39 mmol/L versus 3.65±0.45 mmmol/L) during hypoglycaemia and euglycaemia (p = 0.36). Serum potassium decreased to a greater extent in diabetic (Δ – 1.03±0.28 mmol/L) compared with nondiabetic subjects (Δ- 0.85±0.41 mmol/L) during hypoglycaemia, though this difference did not reach statistical significance.

There was a significant correlation between the change in serum potassium and the change in T symmetry during hypoglycaemia (r = -0.46, p =0.03) in diabetic and nondiabetic subjects (Table 5-2). However, there were no significant correlations between adrenaline and QTc, T amplitude and T symmetry.

Table 5-2: Correlation between change in biochemical parameters and repolarisation parameters

Pearson correlation coefficients r between change in adrenaline and potassium (K^+) versus change in corrected QT interval (QTc), normalised T amplitude (Tamp) and T symmetry (TS). Significant correlations are shown in italics.

	ΔQTc	**ΔTamp**	**ΔTS**
Δ Adrenaline	r = -0.10	r = 0.33	r =0.26
	p= 0.66	p = 0.13	p = 0.24
ΔK^+	r =0.27	r = -0.25	*r = -0.46*
	P = 0.66	p = 0.13	*p = 0.03*

5.5 Discussion

The main findings of this study are as follows. 1) QTc was prolonged to a greater extent during hypoglycaemia compared to euglycaemia in both groups but there were larger changes in QTc in subjects with T2DM. 2) T amplitude and symmetry decreased during hypoglycaemia compared with euglycaemia in diabetic subjects, but there were no differences between the glycaemic arms in nondiabetic subjects. Peak catecholamine levels were similar in both groups during hypoglycaemia, but potassium decreased to a greater extent during hypoglycaemia in diabetic compared with nondiabetic subjects.

These findings are similar those reported in previous studies of hyperinsulinaemic hypoglycaemia in subjects with T2DM. Landstedt-Hallin et al. (1999) performed hypoglycaemic clamps in T2DM subjects with blood glucose maintained at 2.5-3.0 mmol/L for 60 minutes and reported comparable increases in QTc of 61 ms. In a study by Marques et al. (1997), QTc increased by a median value of 128 (range: 16-166) ms. However these subjects were male, younger, and had a shorter disease duration compared with T2DM subjects in this study. Thus they may be expected to show stronger sympathoadrenal responses during hypoglycaemia and hence greater QTc changes. The change in QTc during spontaneous hypoglycaemia in the ambulatory study described in Chapter 3, were smaller (Δ18ms) compared to those observed in this clamp study. This is to

be expected, as experimental hypoglycaemia is associated with greater counterregulatory responses in addition to hypokalaemic effects of supraphysiological doses of insulin. Furthermore, subjects in the ambulatory study tended to be older and had a longer duration of diabetes compared with clamp participants, thus sympathoadrenal responses may be diminished.

Previous studies have shown that QTc changes were smaller during hypoglycaemia in T1DM compared with nondiabetic individuals, which was attributed to diminished sympathoadrenal responses (Koivikko et al., 2008). However, this study has shown larger increases in QTc in T2DM subjecst compared with nondiabetic individuals (Δ76 ms versus Δ54 ms). A stronger sympathoadrenal response is unlikely to be the primary explanation as the peak adrenaline levels were similar and if anything slightly lower in the diabetic group. Furthermore, there were no significant correlations between changes in catecholamines and changes in QTc. Prolonged QTc is unlikely to be explained by factors related to a correction in heart rate, and heart rates at the end of clamp were similar to baseline. QT correction has been explored via several methods and did not appear to make any major differences to the pattern of findings.

Our group has previously suggested that QT changes during hypoglycaemia are mediated by catecholamines that are independent of its potassium lowering effects in nondiabetic subjects (Robinson et al., 2003). No significant relationships could be identified between adrenaline and

changes in QT or T wave parameters observed in both groups when combined. However, as the numbers were small these observations should be interpreted with caution. In the present study, only T symmetry was found to be significantly correlated with changes in serum potassium. Potassium decreased to a greater extent during hypoglycaemia in diabetic compared to nondiabetic subjects. However, adrenaline levels tended to be similar between groups during hypoglycaemia. This may be explained by a metabolic adrenergic hypersensitivity leading to a greater beta-adrenoreceptor mediated fall in potassium, analogous to that observed in other processes, such blood glucose and FFA production (Hilsted et al., 1987). However, this relationship does not imply causation and mechanisms need to be confirmed in experimental studies involving potassium clamping or adrenergic blockade.

Spatial heterogeneity of repolarisation is an important determinant of risk of EAD and the risk of arrhythmogenesis (Antzelevitch, 2007). Earlier studies have shown that there is an increase in QTd during hypoglycaemia in T2DM subjects (Landstedt-Hallin et al., 1999). However, QTd is no longer widely used due to inaccuracies in determining the T end across multiple leads and poor correlation against experimental models in canine hearts (Laguna et al., 2009). Additional information can be obtained by examining the morphology of the T wave, which also describes the dispersion of repolarisation but is free of the rate induced methodological limitations of QT measurements. T wave symmetry, flatness and amplitude

have been shown to reliably discriminate between patients with LQTS and normal subjects (Struijk et al., 2006). Increased dispersion of repolarisation has been shown to correlate with increased symmetry of T waves in cell to ECG simulation models (Xue et al., 2009).

In the present study, T amplitude decreased to a similar extent during hypoglycaemia and euglycaemia in both T2DM and nondiabetic groups, which suggests that the effect is primarily due to insulin. Koivikko et al. (2008) also showed that T amplitude decreased by 50% during hyperinsulinaemic hypoglycaemia similarly in T1DM and nondiabetic subjects, which was correlated with potassium levels. Similarly, other authors have shown decreases in T amplitude during hyperinsulinaemic hypoglycaemia in healthy subjects (Laitinen et al., 2008). Fewer studies have examined the effect of hypoglycaemia on T symmetry. In this current study, T wave symmetry decreased to 1.00 in the diabetic group indicating a near symmetrical T wave at the end of hypoglycaemia, but there was no change during euglycaemia. This suggests that the dispersion of repolarisation is greatly enhanced during hypoglycaemia in T2DM subjects which may be proarrhythmic.

Ventricular bigeminy occurred during hypoglycaemia in one female subject that was likely to have a ectopic focus originating from the right ventricular outflow tract. This subject had no known CVD or risk factors and no history of palpitations. The subject subsequently underwent further evaluation, including a twenty four hour tape which showed rare ectopic activity, and

also exercise tolerance testing which did not provoke any arrhythmias. Benign VT originating from the right ventricular outflow tract is commonly associated with increases in sympathetic cardiac activity, such as exercise (Yoshida et al., 1998). These events suggest that hypoglycaemia may act as a transient stimulus to unmask occult arrhythmias. It is noteworthy that an arrhythmic event was observed in a nondiabetic rather than a diabetic subject, suggesting that this is mediated via the sympathoadrenal drive, although diabetic subjects on average, exhibited greater repolarisation abnormalities during hypoglycaemia.

This appears to be the first study comparing the effects of hypoglycaemia on cardiac repolarisation in T2DM patients against nondiabetic individuals. Euglycaemic and hypoglycaemic clamp studies were performed on separate occasions rather than serial euglycaemic-hypoglycaemic clamps. Insulin infusion rates were identical and thus potassium lowering effects were secondary to changes associated with hypoglycaemia rather than hyperinsulinaemia alone. One of the strengths of this study is that 12 lead ECG high resolution ECG (1200 Hz) were employed in contrast with previous studies that used a lower resolution (Koivikko et al., 2008). The higher signal to noise ratio allowed for a more robust determination of fudicial points and QT measurements. The semi-automatic method meant that all fudicial points were manually verified, which is particularly important in the presence of abnormal T wave morphology.

However, this study also had a number of limitations. The sample size was small and thus it may not have been relatively under-powered to demonstrate significant differences between diabetic and nondiabetic groups. Previous studies have shown that the spatial QRS-tangle and T wave loop measures were abnormal during hypoglycaemia in T1DM patients (Koivikko et al., 2008). Principle component based QT measurements and T wave morphologies may be superior as it improves the signal-to-noise ratio, although these are computationally more demanding (Lipponen et al., 2010) but may be worthy of exploration in future studies.

Two diabetic individuals in this study experienced QTc prolongation in excess of 600 ms during hypoglycaemia. The baseline QTcB was 433 and 415 ms, respectively, which was in the normal range, and catecholamine levels were not particularly high at the end of hypoglycaemia (2.97 and 1.45 nmol/L, respectively). However, they did have the lowest potassium (2.9 mmol/L) concentrations in the group. This level of QTc prolongation is strongly associated with an excess of ventricular arrhythmias. It is conceivable that a similar magnitude of QT change may be observed during severe hypoglycaemia in T2DM subjects. Tsujimoto et al. (2014) studied a retrospective cohort of 414 cases of T1DM and T2DM patients presenting to the emergency room with severe hypoglycaemia. In their study, 50% of T1DM patients and 60% of T2DM patients had a QTcB in excess of 440 ms at presentation, with 14% of T2DM patients presenting with a QTc >500

ms. The authors also found that T2DM patients with QTc >500 ms were significantly more likely to develop new onset atrial fibrillation. Interestingly, in this study, 42% of T1DM and 36% of T2DM patients also had hypokalaemia (<3.5 mmol/L) at presentation. This confirms the clinical relevance of our observations made using an experimental model.

In conclusion, patients with T2DM showed prolonged QTc and abnormal cardiac repolarisation in response to acute hypoglycaemia, which was more marked than in nondiabetic subjects. T2DM patients experienced greater decreases in potassium during hyperinsulinaemic hypoglycaemia. Thus, abnormal repolarisation in combination with autonomic changes during acute hypoglycaemia may be sufficient to precipitate arrhythmic events via mechanisms which include abnormal automaticity and triggered activity (Nordin, 2010). These observations add to the evidence linking increased cardiovascular mortality during intensive glycaemic control in T2DM patients with high cardiovascular risk.

Chapter 6 - Effect of Antecedent Hypoglycaemia on Cardiac Autonomic Function in Type 2 Diabetes

6.1 Summary

Intensive glycaemic therapy is associated with excess cardiovascular death in T2DM patients at high cardiovascular risk and severe hypoglycaemia predicts increased cardiovascular mortality in the weeks following an event. Decreased cardiac vagal function predisposes to arrhythmias and SCD.The aim was to investigate the effect of hypoglycaemia on cardiac autonomic function for up to 7 days following an experimental hypoglycaemic episode.

Twelve adults with T2DM and 11 age and BMI matched nondiabetic subjects underwent paired hyperinsulinaemic clamps at least one month apart. Glucose was maintained at euglycaemia (6 mmol/L) and hypoglycaemia (2.5 mmol/L) for two 60 minute periods in each arm. HRV, spontaneous BRS and cardiac autonomic reflex tests were measured at baseline, one and seven days after clamps in both glycaemic arms.

In the hypoglycaemic arm the heart rate increased Δ6±3 bpm at day 1 and remained increased at day 7 Δ5±2 bpm (p=0.03 for time) in diabetic subjects. RMSSD, a measure of vagal activity, decreased following hypoglycaemia at day 7 compared with no change following euglycaemia (p=0.04 for glycaemic arm). In nondiabetic subjects, log HF, an indicator of vagal activity, was decreased at day 1 following both euglycaemia (ΔlogHF -

0.21±0.12) and hypoglycaemia (Δ-0.18±0.09). However, this returned to baseline at day 7 in the euglycaemic arm but remained decreased following the hypoglycaemic clamp. There were no significant changes in normalised LF power, an indicator of relative sympathetic contribution, or BRS following either glycaemic condition in both groups.

Hypoglycaemia influenced cardiac vagal function, an effect that persisted beyond the episode for at least one week. This suggests a potential mechanism which could contribute to increased cardiovascular mortality following hypoglycaemia.

6.2 Introduction

Severe hypoglycaemia has been strongly associated with CV mortality with an elevated risk persisting at 90 days (Duckworth et al., 2009; Zoungas et al., 2010). Antecedent hypoglycaemia may impair cardiac autonomic function for at least 24 hours in nondiabetic individuals, whilst in healthy subjects HRV, BRS and sympathetic response to hypotensive stress were found to be attenuated one day after experimentally induced hypoglycaemia (Adler et al., 2009). In rats, antecedent hypoglycaemia similarly impaired sympathetic responses to hypotensive stress after experimentally induced hypoglycaemia (Herlein et al., 2006). Impaired HRV and BRS are predictors of increased cardiovascular mortality following myocardial infarction (Kleiger et al., 1987; La Rovere et al., 1998), and are linked to an increased risk of arrhythmias (La Rovere et al., 2001). No study to date has examined the effect of antecedent hypoglycaemia in humans

with T2DM, and these individuals may have cardiac autonomic dysfunction which might compound impaired autonomic responses.

The aim of this study was to examine the effect of antecedent hypoglycaemia on cardiac autonomic function in patients with T2DM versus nondiabetic controls. Given that an increased risk of cardiovascular mortality persists over the weeks and months following a severe hypoglycaemic episode, downstream changes in autonomic function at day 1 and 7 following experimental hypoglycaemia were investigated and compared to following hyperinsulinaemic euglycaemia.

6.3 Research Design and Methods

6.3.1 Participants

Twelve patients with T2DM aged between 18-65 and no history or signs or symptoms of CVD were recruited from Sheffield Teaching Hospitals outpatient clinics. All patients were on oral hypoglycaemic agents and/or GLP-1 analogues and/or insulin for ≤ 2 years. These patients were included as they were at low baseline risk of clinical hypoglycaemia. Eleven age and BMI matched nondiabetic individuals were recruited as a control group. Patients on beta blockers or QT prolonging drugs were excluded from the study. All participants gave their written informed consent and the study was approved by the local research ethics committee.

6.3.2 Baseline Assessment

All subjects had a normal full blood count, renal function and ECG at baseline. Subjects underwent cardiovascular autonomic reflex testing, including three tests of cardiovagal function (heart rate to deep breathing, the Valsava manoeuvre, heart rate response to standing) and blood pressure response to standing as previously described (see Section 2.2.5) (O'Brien et al., 1986) . Results were compared against age-adjusted normal ranges and values below 95% CI were regarded as abnormal (O'Brien et al., 1986). The presence of two or more abnormal cardiovagal tests were defined as definite CAN and these subjects were excluded (Tesfaye et al., 2010).

6.3.3 Hyperinsulinaemic Clamp Protocol

All diabetic and nondiabetic subjects participated in paired hyperinsulinaemic euglycaemic and hypoglycaemic studies separated by at least four weeks but no more than eight weeks. In the hypoglycaemic arm, glucose was maintained at 2.5 mmol/L for 60 minutes in the morning and 60 minutes in the afternoon. In the euglycaemic arm, arterialised blood glucose was maintained at the target level of 6 mmol/L for 60 minutes in the morning and 60 minutes in the afternoon. The hyperinsulinaemic clamp study protocol is as described in detail in Section 2.3.5.

Diabetic participants continued on their usual diabetic medications at stable doses following the clamp studies and were asked to report any spontaneous hypoglycaemia throughout the study period.

6.3.4 Cardiac Autonomic Testing

Autonomic measurements were conducted in the morning to control for diurnal variation. Blood glucose was concurrently measured to ensure that participants were euglycaemic during these tests. All participants were asked to avoid caffeine or smoking on the day of the tests and vigorous exertion for 24 hours prior. HRV, spontaneous BRS, and cardiac autonomic reflex tests were conducted with the subject supine following at least five minutes of rest in a quiet room. Blood pressure was measured using an automatic oscillometric sphygmomanometer (DINAMAP ®GE Medical Systems Information technologies, Inc.) after five minutes lying supine. Cardiac autonomic function was assessed at baseline (in the morning prior to euglycaemic and hypoglycaemic clamps), and then at days 1 and 7 in both arms.

6.3.5 Heart Rate Variability

HRV was determined from five minute resting recordings with the participant supine. Recordings were performed with subjects free breathing and also with paced breathing, where subjects followed a timed visual display breathing at 12 breaths/minute. QRS complexes were detected using an automatic algorithm. Manual editing of R-R intervals was performed together with a visual inspection of QRS complexes to exclude any ectopic beats and artefacts. Normal R-R intervals (NN) were extracted for HRV analysis performed in accordance with the recommendations of the 1996). The time domain measures SDNN and RMSSD of NN were

analysed and fast Fourier transformation was applied to R-R intervals for spectral analysis. The LF band was defined as 0.04-0.15 Hz and the HF band as 0.15-0.4 Hz. The ratio between the LF power and total power was also calculated (LFnorm).

6.3.6 Spontaneous Baroreceptor Sensitivity

Spontaneous cardiovagal BRS was measured using Poratpres (TNO Biomedical Instrumentation, Amsterdam, Netherlands) as described in section 2.3.7. BRS analysis was performed using dedicated software using the sequence method (Nevrokard V 5.1.3, Intellectual Services, Slovenia) (Parati et al., 1988). An 'UP' sequence was generated when there was an increase in SBP of at least 1 mmHg associated with an increase in R-R interval of at least 5 ms for three or more consecutive beats, while a 'DOWN' sequence was generated when there was a reduction in SBP of at least 1 mmHg with an accompanying reduction in R-R interval of at least 5 ms for three or more consecutive beats. Total BRS, measured in ms/mmHg was calculated by averaging the regression coefficients of RR/BP relations of the UP and DOWN sequences with correlation coefficients greater than 0.85.

6.3.7 Biochemical Measures

Catecholamines were assayed by HPLC. Whole blood (6 mL) was collected in chilled lithium heparin tubes containing 50 µL EGTA/glutathione preservative, subjected to centrifugation at 1000 ×*g* for 10 minutes, and the resultant plasma samples stored at -80^{o}C prior to assaying. Free insulin

was analysed by an immunometric assay (Invitron Insulin ELISA, Invitron Ltd, Monmouth, UK). Whole blood (3 mL) was collected in a 6 mL lithium heparin tube and subjected to centrifugation immediately at 4°C, 2400 rpm for 2 minutes. A 0.5 mL aliquot of the resulting plasma was added to a chilled plastic tube containing 0.5 mL PEG for the precipitation of immune complexes and mixed. The centrifuged step was then repeated for 30 minutes and the product at -80°C until analysed by the immunometric assay. Biochemical parameters were measured at baseline and at the end of the euglycaemic and hypoglycaemic arms in both groups, and then at days 1 and 7.

6.3.8 Statistical Analysis

Baseline demographic data is summarised as mean (SE) for parametric data unless otherwise stated, or median (interquartile range) for nonparametric data. Comparisons of baseline data between diabetic and control groups were made using an independent t-test for parametric data and Mann Whitney U test for nonparametric data. Blood glucose and hormone concentrations were compared between equivalent euglycaemic and hypoglycaemic time points using a paired t-test or Wilcoxson signed-rank test.

Autonomic function parameters were analysed using a linear mixed model with repeated measures. The mixed model allows for internally correlated measures from repeated measures (with respect to both the glycaemic arm and time) within an individual and also allows for missing values (Galbraith

et al., 2010). To investigate whether there was a significant change in autonomic parameters within euglycaemic or hypoglycaemic arms, time point was specified as a fixed effect in the linear mixed model. Changes in autonomic function between glycaemic arms were also compared using a linear mixed model, where glycaemic arm, time and interaction between the glycaemic arm and time were specified as fixed effects. In both analyses the glycaemic arm and time point were specified as repeated measures and fitted with an unstructured, compound symmetry or autoregressive 1 covariance structure. The model with the best fit (lowest Akaike's information criterion) was selected. p values were obtained by restricted maximum likelihood estimation. A p value of <0.05 was considered significant. Results were analysed using SPSS Statistics (v 20.0, IBM, Chicago, Ilinois).

6.4 Results

6.4.1 Participant Characteristics

Twelve patients with T2DM (9 male, 3 female) and 11 nondiabetic controls (5 male, 6 female) participated in the study. Participants with diabetes were similar in age 54 (50-58) years compared to the nondiabetic group, 52 (47-59) years. The mean BMI was also comparable, 34±5 kg/m^2 in the diabetic group versus 31±8 kg/m^2 in the nondiabetic group. Two diabetic patients were taking ACE inhibitors. Individuals with diabetes had higher baseline heart rates, blood pressure and lower HRV and BRS at baseline (Table 6-1).

Table 6-1: Baseline cardiac autonomic function in individuals with T2DM and nondiabetic subjects

Data mean±SD. An independent t-test of baseline variables was used to compare T2DM and nondiabetic groups. RV-heart rate variability, HF-high frequency, BRS- baroreceptor sensitivity, E:I- expiration:inspiration

	T2DM n=12	**Nondiabetic** n=11	**p-value**
Cardiovascular autonomic reflex tests			
E:I ratio	1.24±0.20	1.27±0.23	0.74
Valsava ratio	1.51±0.29	1.81±0.41	0.05
Standing ratio	1.28±0.17	1.58±0.28	0.005
Heart rate variability			
SDNN	27.4±11.8	48.2±14.6	0.001
RMSSD	15.5±7.94	32.9±15.3	0.002
Log Total power	2.34 ± 0.36	2.90±0.36	0.001
Log HF	1.85± 0.37	2.32±0.48	0.02
LFnorm	0.65±0.14	0.70±0.14	0.40
BRS	8.59±4.08	11.51±7.65	0.26

6.4.2 Heart Rate

In T2DM patients the heart rate was higher at recovery then returned to baseline at day 7 in the euglycaemic arm. In the hypoglycaemic arm, the heart rate increased from baseline by Δ6±3 bpm at day 1 and remained increased at day 7 Δ5±2 bpm (p=0.04 for time by linear mixed model) (Figure 6-1). There was a trend towards an interaction between the glycaemic arm and time (p =0.07).

In the nondiabetic group, heart rate increased significantly at day 1 following euglycaemia (Δ9±3 bpm) but then returned to baseline levels at day 7 (p =0.01 for time by linear mixed model). Conversely, there were no significant rises in heart rate following hypoglycaemia (p = 0.25 for time).

The differences between glycaemic arms did not reach statistical significance (p=0.18).

6.4.3 Heart Rate Variability

6.4.3.1 Frequency domain measures

HF power, an indicator of vagal activity, did not change significantly following euglycaemia in diabetic subjects. However, Log HF was slightly higher at recovery then progressively decreased up to day 7 following hypoglycaemia from a baseline of 1.85±0.16 to 1.75±0.16 ms^2 ($p < 0.001$ for time) (Figure 6-1). Log HF was lower at day 7 following hypoglycaemia compared to euglycaemia; however, the difference in glycaemic arms did not reach statistical significance (p=0.17).

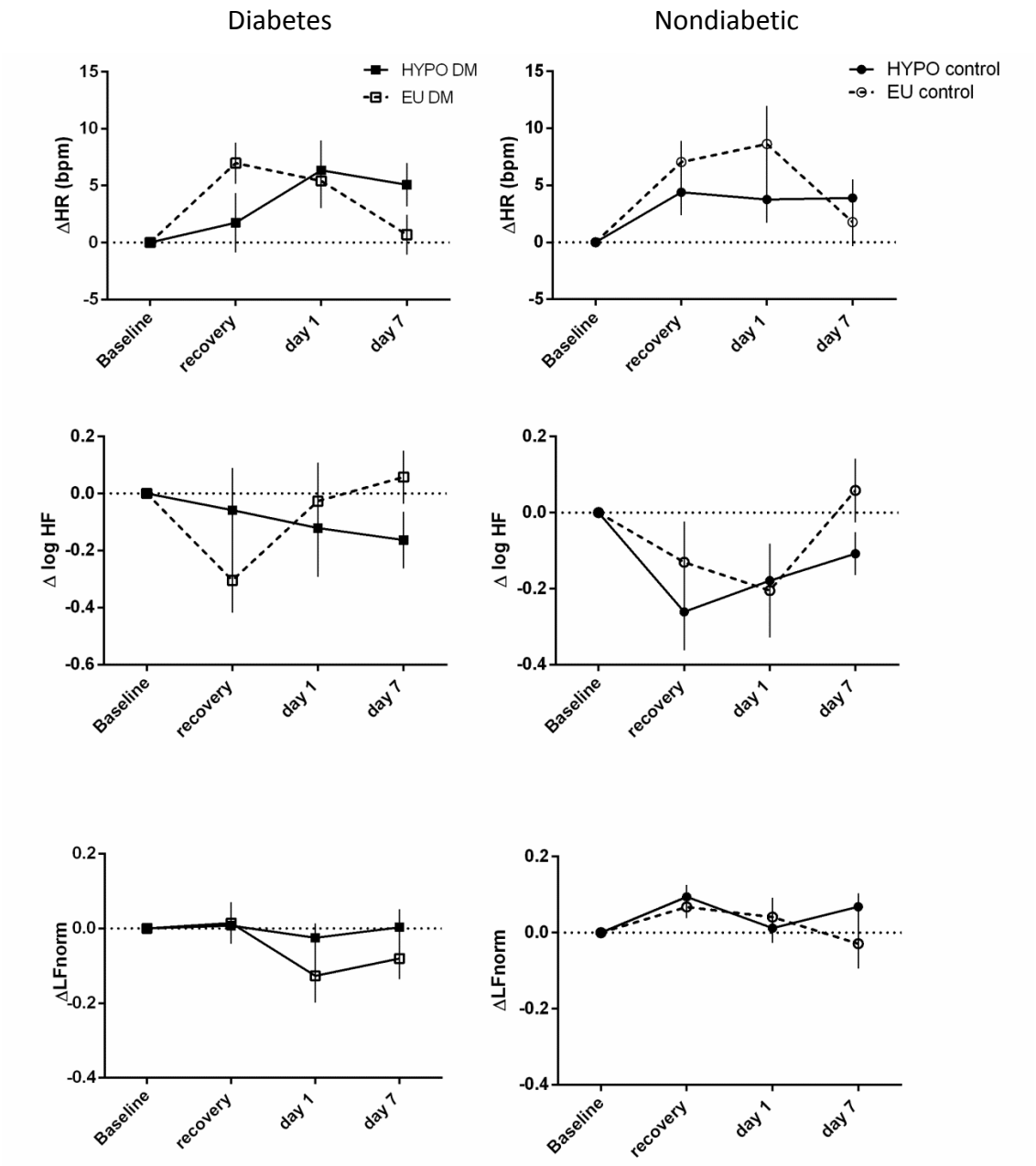

Figure 6-1: Change in heart rate and heart rate variability following hypoglycaemia versus euglycaemia in diabetic and nondiabetic subjects

Top panel - change in heart rate (beats per minute); middle panel – change in log high frequency (log HF) heart rate variability; bottom panel – change in normalised high frequency (LFnorm) heart rate variability. Data Mean (SE). Diabetic group – hypoglycaemia (closed square); euglycaemia (open square); nondiabetic group – hypoglycaemia (closed circle); euglycaemia (open circle)

In nondiabetic subjects, log HF was decreased at day 1 following both euglycaemia (ΔlogHF -0.21±0.12) and hypoglycaemia (Δ-0.18±0.09, both $p<0.001$ for time) (Figure 6-1). However, this returned to baseline at day 7

in the euglycaemic arm but remained decreased following the hypoglycaemic clamp, although this did not reach statistical significance between arms.

There were no significant changes in normalised LF power, an indicator of relative sympathetic contribution, following euglycaemia or hypoglycaemia in diabetic or nondiabetic subjects (Figure 6-1).

6.4.3.2 Time Domain Measures

Time domain HRV measures are presented in Figure 6-2. SDNN did not change significantly in the diabetic group in either glycaemic arm. However, in nondiabetic subjects, SDNN significantly decreased at day 1 following both euglycaemia and hypoglycaemia (both $p<0.05$). RMSSD exhibited a similar trend to log HF, a measure of vagal activity. In diabetic subjects, RMSSD was lower at day 7 following hypoglycaemia compared with euglycaemia and there was a significant interaction between time and glucose ($p=0.04$). In nondiabetic subjects, RMSSD decreased at day 1 following both glycaemic conditions but returned to baseline following euglycaemia while remaining lower following hypoglycaemia at day 7 (Figure 6-2).

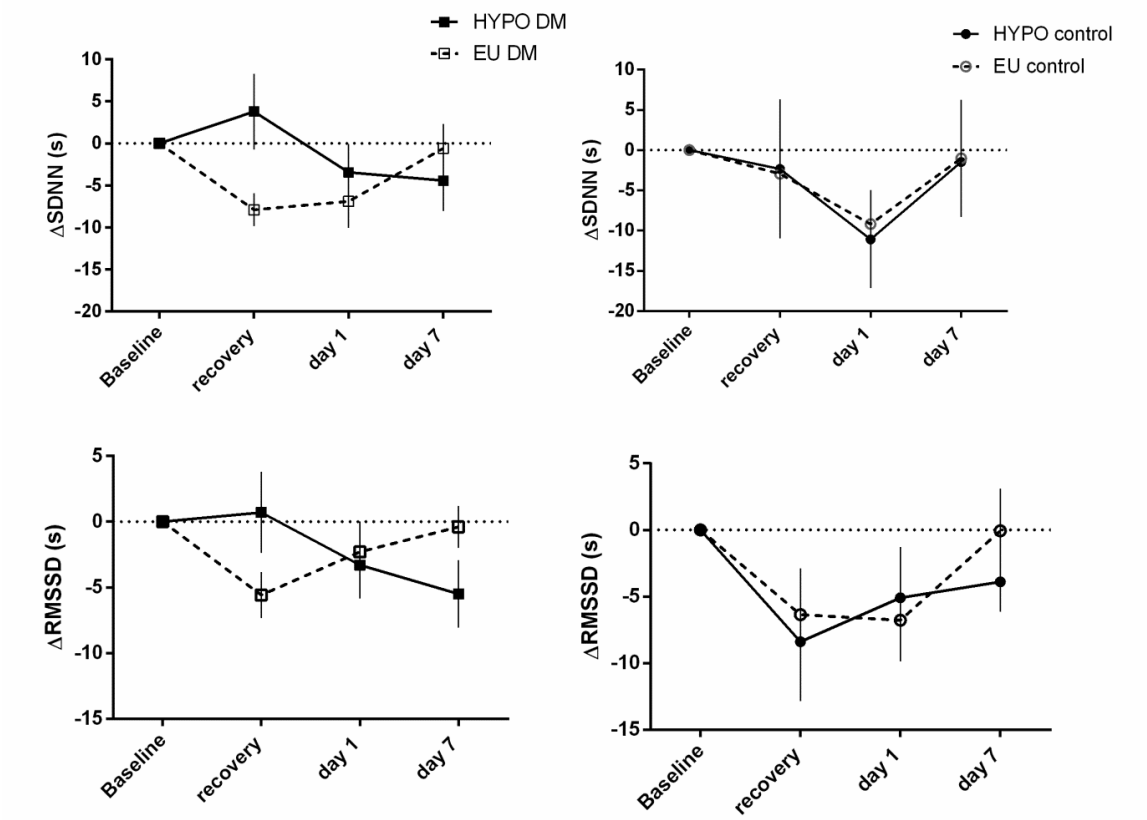

Figure 6-2: Changes in time domain measures of heart rate variability following hypoglycaemia versus euglyacemia in diabetic and nondiabetic subjects

Top: SDNN –standard deviation of NN intervals; Bottom: RMSSD root mean squared of standard deviation of NN intervals. HYPO-hypoglycaemia, EU-euglycaemia. Data Mean(SE)

6.4.3.3 Effect of Respiration

The heart rate and HRV results presented above are based on resting recordings with spontaneous breathing, and the mean respiratory frequencies during resting recordings are shown in Table 6-2. There was little difference between the glycaemic conditions on different days and no differences between the groups. HRV was also analysed with paced breathing (12/ breaths minute) and this showed similar trends to spontaneous breathing (data not shown).

Table 6-2: Respiratory frequencies during heart rate variability recordings at rest

Mean±SD; EU- euglycaemia; HYPO- hypoglycaemia

	Baseline	Recovery	Day 1	Day 7	Mean
Diabetes					
EU	0.25±0.05	0.29±0.06	0.24±0.05	0.24±0.05	0.24±0.05
HYPO	0.25±0.06	0.29±0.05	0.26±0.05	0.25±0.05	0.25±0.05
Nondiabetic					
EU	0.24±0.03	0.26±0.06	0.21±0.05	0.24±0.04	0.24±0.04
HYPO	0.24±0.04	0.26±0.06	0.26±0.06	0.24±0.04	0.25±0.05

6.4.4 Blood Pressure

In diabetic subjects, SBP tended to fall following both glycaemic conditions maximally at day 7 and there was no difference between the glycaemic arms (p=0.69) (Figure 6-3). Similarly DBP tended to decrease following both glycaemic conditions up to day 7. Both SBP and DBP did not change in the nondiabetic group following hypoglycaemia or euglycaemia (Figure 6-3).

6.4.5 Baroreceptor Sensitivity

There were no significant changes in BRS following hypoglycaemia or euglycaemia in either group (Figure 6-4).

6.4.6 Cardiovascular Autonomic Reflex Tests

The E:I ratio during deep breathing and 30:15 ratio following standing were similar between glycaemic arms in diabetic and nondiabetic subjects (Table 6-3). The Valsava ratio tended to be lower at day 1 in the hypoglycaemic arm compared to

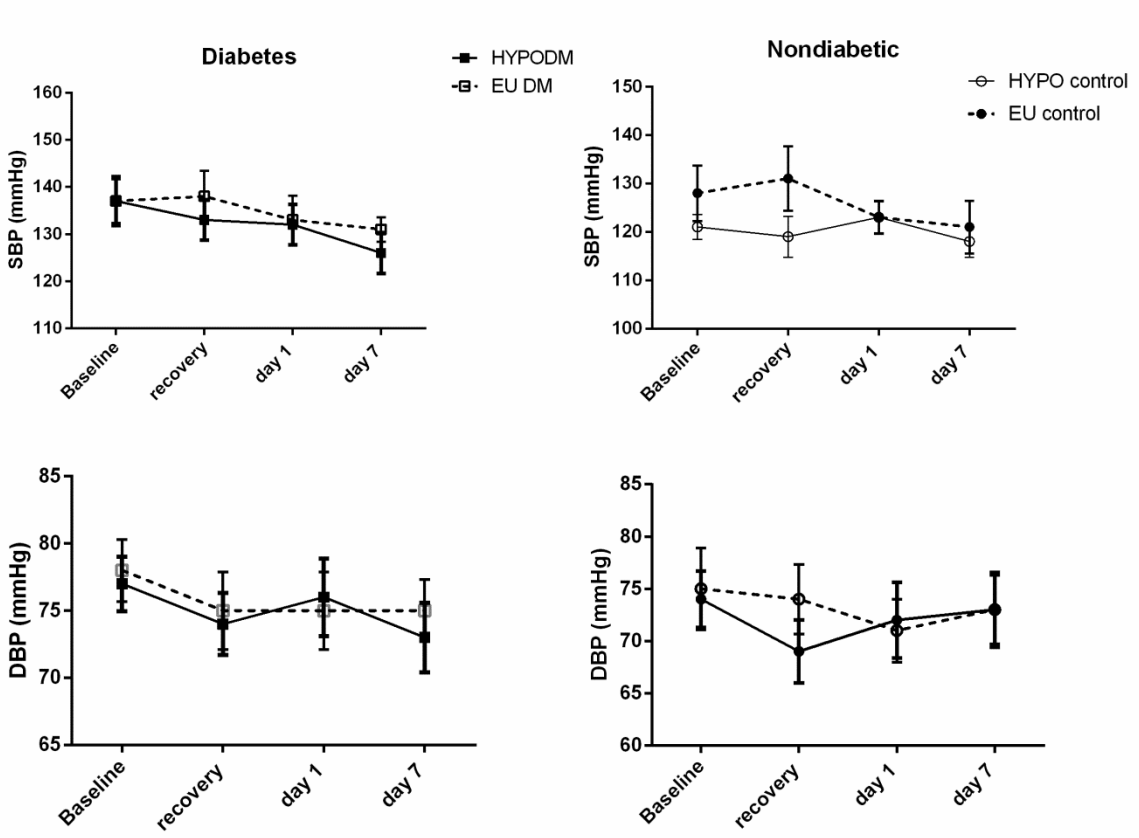

Figure 6-3: Blood pressure following euglycaemia or hypoglycaemia in diabetic and nondiabetic subjects

SBP-systolic blood pressure, DBP-diastolic blood pressure. Data mean (SE)

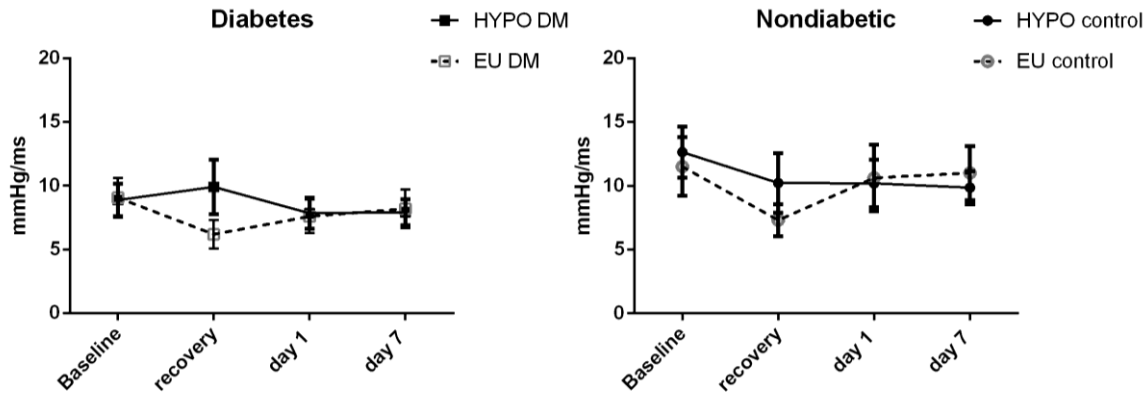

Figure 6-4: Spontaneous baroreceptor sensitivity following euglycaemia and hypoglycaemia in diabetic and nondiabetic participants

Baroreceptor sensitivity (BRS) in mmHg/ms. Data mean (SE)

euglycaemia in subjects with diabetes but did not reach statistical significance. In nondiabetic subjects, the Valsava ratio tended to be lower at day 1 and day 7 following hypoglycaemia compared to euglycaemia, but did not reach statistical significance.

Table 6-3: Cardiovascular autonomic reflex tests following euglycaemic or hypoglycaemic clamps in diabetic and nondiabetic individuals
E:I ratio expiration: inspiration ratio; 30:15 ratio to standing; Data mean(SE)

	Euglycaemia	Hypoglycaemia
E:I ratio		
Diabetes		
Day1	1.28±0.07	1.34±0.11
Day 7	1.28±0.08	1.27±0.08
Nondiabetic		
Day 1	1.18±0.04	1.22±0.04
Day 7	1.24±0.07	1.18±0.02
Valsava ratio		
Diabetes		
Day 1	1.61±0.13	1.51±0.08
Day 7	1.49+0.09	1.54±0.12
Nondiabetic		
Day 1	1.87±0.13	1.66±0.17
Day 7	1.79±0.16	1.56±0.11
30:15 ratio		
Diabetic		
Day 1	1.23±0.08	1.27±0.07
Day 7	1.23±0.05	1.27±0.08
Nondiabetic		
Day 1	1.42±0.05	1.55±0.04
Day 7	1.46±0.07	1.47±0.05

6.4.7 Blood Glucose and Insulin

Blood glucose concentrations at the end of the morning clamp were similar between diabetic and nondiabetic groups in the euglycaemic arm (5.81± 0.29 mmol/L versus 5.96± 0.18 mmol/L, p=0.15) and hypoglycaemic arm

(2.56± 0.22 mmol/L versus 2.56± 0.09 mmol/L, p>0.99). Target arterialised blood glucose levels were similarly achieved in the afternoon (Table 6-4). Blood glucose was not significantly different between glycaemic arms except at day 1, where it was higher following hypoglycaemia in the diabetic group.

Free insulin levels at the end of the clamp was median 576 (IQR: 468-627) pmol/L during euglycaemia and 689 (477-1076) pmol/L during hypoglycaemia in the diabetic group. In the nondiabetic group the median was 865 (509-952) pmol/L in the euglycaemic arm and 665(468-967) pmol/L in the hypoglycaemic arm.

6.4.8 Plasma Catecholamines

There were no significant changes in adrenaline following the euglycaemic clamp; however, adrenaline increased during hypoglycaemia clamp and then returned to baseline levels at day 1 and day 7 in both groups (Table 6-4).

In nondiabetic subjects noradrenaline increased following euglycaemia at day 1 then falling at day 7. Following hypoglycaemia at day 1, noradrenaline was similarly increased (Table 6-4). Noradrenaline levels at day 1 following the euglycaemic clamp were significantly higher in the nondiabetic group compared to T2DM individuals (3.08±1.48 versus 1.97±0.52 nmol/L, p <0.05).

Table 6-4: Blood glucose during the hyperinsulinaemic clamp and plasma catecholamines following euglycaemia versus hypoglycaemia

Data mean±SD *p<0.05, **p<0.01 Euglycaemia versus hypoglycaemia within each group at the equivalent time point †† p<0.01 denotes a significant difference between diabetic and control groups at equivalent euglycaemic or hypoglycaemic time points as determined by an independent t-test.

	Diabetic		Nondiabetic	
	EU	HYPO	EU	HYPO
Blood glucose (mmol/L)				
Baseline AM	6.24±1.15	6.44±1.27	5.10±0.49†	4.87±0.31†
End of clamp AM	5.81±0.30	2.55±0.22**	5.96±0.18	2.56±0.10**
Baseline PM	6.44±1.51	6.69±1.56	5.14±0.62	5.53±1.04
End of clamp PM	6.01±0.30	2.70±0.17	6.03±0.27	2.61±0.12
Recovery	6.98±1.14	6.63±1.90	6.37±0.63	8.40±1.96**
Day 1	7.88±2.52	11.4±3.76*	5.08±0.94†	5.00±0.90†
Day 7	9.30±2.70	10.3±0.94	5.09±0.37†	4.96±0.62†
Adrenaline (nmol/L)				
Baseline	0.15±0.14	0.12±0.09	0.17±0.12	0.15±0.07
End of clamp	0.16±0.16	3.05±2.46**	0.14±0.08	3.83±2.83**
Day 1	0.23±0.43	0.17±0.21	0.17±0.11	0.20±0.25
Day 7	0.10±0.08	0.16±0.27	0.22±0.20	0.11±0.07
Noradrenaline (nmol/L)				
Baseline	1.23±0.47	1.25±0.53	1.56±0.51	1.46±0.52
End of clamp	1.27±0.31	2.37±0.82**	1.56±0.40	2.69±1.45**
Day 1	1.97±0.52	1.93±0.35	3.08±1.48†	2.32±0.72
Day 7	2.07±0.58	2.52±1.20	2.56±0.78	2.78±1.18

6.5 Discussion

The main findings of this study are as follows. Hypoglycaemia was accompanied by increases in heart rate for up to seven days later in T2DM subjects compared to no change following hyperinsulinaemic euglycaemia.

This increase in heart rate was accompanied by decreases in cardiac vagal activity (as shown by HF HRV and RMSSD) with no apparent change in cardiovagal BRS or relative sympathetic contribution. In nondiabetic subjects, heart rates were increased and noradrenaline levels were higher at day 1 following hyperinsulinaemic euglycaemia but no significant changes in heart rate or HF HRV occurred at day seven following hypoglycaemia.

These results show that impairment in cardiac vagal activity, as shown by HF power and RMSSD, may persist for as long as seven days after hypoglycaemia in T2DM subjects. It is well known that hypoglycaemia impairs counter-regulatory responses to subsequent hypoglycaemic episodes, an effect that may persist for at least several days (George et al., 1995). Such an attenuation of sympathoadrenal responses to hypoglycaemia may extend to non-hypoglycaemic stimuli (Herlein et al., 2006). Although the exact mechanism by which sympathoadrenal responses are attenuated following hypoglycaemia is unclear, it is thought to occur at the level of the central nervous system (Cryer, 2013) and it is tempting to speculate that impairment of cardiac vagal activity following hypoglycaemia may occur by related mechanisms. Cardiac vagal neurones are located in the nucleus ambiguous and dorsal motor nucleus of the vagus, which receives input from the nucleus tractus solitariuss. It has recently been shown that a subpopulation of GLUT-2 expressing neurones in the nucleus tractus solitaries may stimulate vagal activity during

hypoglycaemia (Lamy et al., 2014). There is further evidence that recurrent insulin-induced hypoglycaemia in rats reduced orexigenic transmission in the dorsal vagal motor nucleus via receptor down-regulation (Paranjape et al., 2007).

There was a trend towards a decrease in the Valsava ratio following hypoglycaemia but not in the E:I or 30:15 ratios. Although these are also markers of cardiovagal function, reproducibility is lower compared with resting HRV as it is partly dependent on participant effort (Kowalewski and Urban, 2003). Thus HRV may be more sensitive in demonstrating smaller reductions in vagal activity compared with autonomic reflex testing.

Adler et al. (2009) showed that two episodes of antecedent hypoglycaemia reduced cardiac vagal activity in nondiabetic subjects after 24 hours. The study by Adler and colleagues also showed decreases in cardiovagal BRS one day after hypoglycaemia as measured by changes using the modified Oxford method with nitroprusside and phenylephirine. In this current study there was a slight reduction in spontaneous BRS at days 1 and 7 following both hypoglycaemia and euglycaemia in diabetic subjects but this did not reach statistical significance. No changes in BRS were shown following either glycaemic condition in the nondiabetic group. The reasons for these discrepancies are not clear but may relate to the method of BRS measurement. The measurement of BRS using the sequence method is limited by the number of spontaneously increasing or decreasing ramps in blood pressure and heart rate available for analysis. Furthermore,

spontaneous BRS does not evaluate the entire working range of the sigmoidal BRS curve, unlike pharmacological methods. However, pharmacological methods also have disadvantages in that they are more invasive and perturb the reflex that being measured.

In the present study there was an increase in heart rate, decrease in vagal activity and an increase in noradrenaline at day 1 following hyperinsulinaemic euglycaemia in nondiabetic subjects that are believed to be due to sympathoexcitatory effects of insulin. Studies have shown that insulin acts on the arcuate nucleus where POMC neurones communicate with the paraventricular nucleus to elicit its sympathoexcitatory effects. A number of human and animal studies have examined the effect of acute intravenous insulin infusions on heart rate and autonomic activity. In nondiabetic subjects, small increases in plasma insulin levels elicited large increases in sympathetic responses (Scherrer and Sartori, 1997). The reason why this did not occur in the diabetic group during hyperinsulinaemic euglycaemia may be due to resistance to the sympathoexcitatory effects of insulin. Sympathetic responsiveness to insulin infusion has been shown to be impaired in conditions associated with insulin resistance (Paolisso et al., 2000; Vollenweider et al., 1994).

It is less clear how long sympathoexcitatory effects of insulin may persist beyond the period of infusion. Anderssen and colleagues (1992) reported increases in muscle sympathetic nerve activity 1.5 hours after the end of the infusion, when plasma insulin levels have returned to basal levels.

Animal studies suggest that the sympathoexcitatory effects of insulin may persist for at least 24 hours and, rats subjected to four days of antecedent euglycaemic hyperinsulinaemia had increased basal norepinephrine levels for at least 24 hours (Shum et al., 2001). Rats that received intracerebroventricular injections of insulin with no change in plasma glucose exhibited an enhancement of reflex tachycardia that was maintained for 24 hours (Okada and Bunag, 1994).

There are a number of limitations to this study. Although HF power and RMSSD of HRV are widely accepted measures of vagal activity, there is no robust measure of cardiac sympathetic activity. Plasma noradrenaline levels can serve as a marker of systemic sympathetic activation but is not specific to cardiac sympathetic activity. Similarly, muscle sympathetic nerve activity reflects the state of activation in skeletal muscles which do not necessarily reflect the level of firing in the heart. Cardiac norepinpehrine spillover may be regarded as the gold standard for measuring cardiac sympathetic neuronal activity; however, this is relatively invasive and is difficult to perform in combination with hyperinsulinaemic clamps. LF power, expressed as a proportion of total HRV, has been shown empirically to reflect the degree of cardiac sympathetic contribution to overall heart rate control (Pagani et al., 1986). However, others have questioned these observations and argued that normalised LF power may be a more complex measure that also reflects the baroreflex function (Goldstein et al., 2011). It was impossible in this study to control for all the intervening factors

between the clamp visit and day 7 that may impact upon cardiac autonomic function, although no participant experienced severe spontaneous hypoglycaemia outside of that induced during clamps.

Clinically, depressed cardiac vagal function has been strongly associated with increased cardiovascular mortality. Low cardiac vagal activity, as shown by HRV on 24 hour monitoring, is associated with a 4-fold increased risk of SCD (Algra et al., 1993). In the ATRAMI study which examined 1071 post-myocardial infarction patients with a low ventricular ejection fraction, BRS and HRV independently predicted an increase in mortality (La Rovere et al., 1998). In the current study, RMSSD was decreased by 20% seven days after hypoglycaemia in diabetic subjects who already exhibited impaired cardiac vagal function at baseline. Thus hypoglycaemia may further predispose to arrhythmias and SCD. In the NICE-SUGAR study where hypoglycaemia was associated with increased mortality during intensive glycaemic control in critical care patients, the median time from hypoglycaemic event to death was seven days and this mirrors the time course of the maximal vagal impairment in this study. It is unclear whether autonomic impairment persists beyond seven days and future studies should investigate the time course of autonomic changes beyond seven days following a hypoglycaemic episode.

In conclusion, hypoglycaemia was associated with an increase in heart rate and impaired cardiac vagal function seven days later in T2DM subjects but no significant changes in spontaneous cardiovagal BRS. Impairment of

cardiac vagal function may be one mechanism that might predispose to cardiovascular mortality in the weeks following a hypoglycaemic event during intensive glycaemic control.

Chapter 7 - Effect of Hypoglycaemia on Platelet Function in Type 2 Diabetes

7.1 Summary

Increased platelet activation and reactivity are strong predictors of cardiovascular death in subjects with T2DM. Previous studies have suggested that acute hypoglycaemia can increase platelet aggregation via adrenergic mechanisms in T1DM and nondiabetic subjects but no study to date has examined the duration of these effects. The aim of this study was to investigate the effects of hypoglycaemia on platelet function acutely and downstream of the event in T2DM subjects.

Twelve patients with T2DM and eleven age and BMI matched nondiabetic subjects completed paired hyperinsulinaemic clamp studies separated by at least four weeks. Patients were on no antiplatelet drugs except for two on aspirin. Glucose was maintained during hypoglycaemia (2.5 mmol/L) or euglycaemia (6 mmol/L) for two 60 minute periods. Platelet aggregation was measured at the baseline, end of the clamp, at recovery, and 1 and 7 days later using whole blood impedance aggregometry (Multiplate), WBSPC, and P-selectin expression using flow cytometry.

Platelet activation as measured by unstimulated P-selectin increased following acute hypoglycaemia in both groups. Platelet macroaggregation to collagen by impedance aggregometry increased acutely during hypoglycaemia in diabetic (Mean±SE Δ 9±6 U) and nondiabetic subjects (Δ

14±4 U) compared with decreases during euglycaemia (Δ -15±7 and Δ -9±6 U respectively). 5HT-induced P-selectin expression increased during acute hypoglycaemia in both diabetic and nondiabetic subjects, returning to baseline at day 1 (both $p < 0.01$ vs. euglycaemia). The percentage aggregation to 3 μM ADP by WBSPC decreased during euglycaemia but did not change in the hypoglycaemic arm, suggesting no changes in microaggregation. Change in platelet reactivity following hypoglycaemia did not persist beyond day 1.

Moderate experimental hypoglycaemia, frequently observed clinically, can increase platelet reactivity in individuals with T2DM during acute hypoglycaemia, although effects did not persist beyond day 1. This is one potential mechanism by which acute hypoglycaemia can precipitate coronary thrombosis.

7.2 Introduction

Platelets are key contributors to the atherothrombotic process and when activated contribute to the activation of arterial thrombosis following plaque rupture. Platelets in T2DM patients are hyperactive and show greater aggregation responses (Kakouros et al., 2011). Clinical studies have shown that high platelet reactivity, despite chronic dual antiplatelet therapy, was the strongest independent predictor of major adverse cardiovascular events in cohorts of T2DM patients with coronary artery disease (Angiolillo et al., 2007).

Early work by Hutton et al. (1979) demonstrated enhanced platelet aggregation to ADP as measured by Born aggregometry during hypoglycaemia induced by an insulin bolus. Subsequent studies have also shown that platelet activation occurs in response to acute hypoglycaemia and leads to the release of beta-thromboglobulin from platelet granules (Trovati et al., 1986), as well as the expression of cell surface receptors, such as P-selectin (Gogitidze Joy et al., 2010). During hypoglycaemia, increased platelet activation is predominantly mediated via adrenaline and can be inhibited by alpha2-adrenoreceptor blockade (Kishikawa et al., 1987).

No study to date has examined the effect of hypoglycaemia on platelet reactivity in T2DM individuals. Furthermore, few studies have examined the effect of hypoglycaemia in the days after the event. A novel aspect of this work was to investigate differences in both platelet macroaggregation and microaggregation. Platelet microaggregation, the initial formation of few platelet conglomerates, has been shown to be the key platelet aggregation event in T2DM patients that can mediate hyperreactivity (Matsuno et al., 2004). Another novel aspect to this work is the effect of P2Y12 blockade on platelet reactivity during acute hypoglycaemia. The P2Y12 pathway amplifies aggregation responses to a wide range of agonists and P2Y12 inhibitors are used clinically as antiplatelet agents.

The aim of this study was to examine the effect of hypoglycaemia on platelet activation and platelet reactivity during and following an acute episode in patients with T2DM.

7.3 Research Design and Methods

7.3.1 Participants

The inclusion and exclusion criteria are as previously described in Section 2.3.2. Briefly, 12 patient with T2DM aged 18-65 and no history or signs or symptoms of CVD were recruited from Sheffield Teaching Hospitals outpatient clinics. Eleven age and BMI matched nondiabetic individuals were recruited as a control group. None were on antiplatelet agents or anticoagulants apart from two diabetic patients who were on aspirin. All subjects had a normal full blood count, renal function and cardiac autonomic function at baseline. All participants gave their written informed consent and the study was approved by the local research ethics committee.

7.3.2 Hyperinsulinaemic Clamp Protocol

All diabetic and nondiabetic subjects participated in paired hyperinsulinaemic euglycaemic and hypoglycaemic clamps separated by at least four weeks. In the hypoglycaemic arm, glucose was maintained at 2.5 mmol/L for 60 minutes in the morning and 60 minutes in the afternoon, whilst in the euglycaemic arm, arterialised blood glucose was maintained at a target level of 6 mmol/L for 60 minutes in the morning and 60 minutes

in the afternoon. Details of the hyperinsulinaemic clamp protocol are described in section 2.3.5. During the morning clamp a primed continuous intravenous insulin infusion was administered at 120 mU/m^2/min (body surface area), and in the afternoon clamp at 240 mU/m^2/min along with 20% dextrose at a variable rate, and adjusted according to arterialised blood glucose concentrations which were measured every five minutes. Equivalent amounts of insulin were administered in the euglycaemic and hypoglycaemic arms and in diabetic and nondiabetic subjects. Platelet function assays were performed at the end of the clamp, at recovery (30 minutes after the end of the clamp), and days 1 and 7 after the euglycaemic and hypoglycaemic clamps. Study visits were conducted in the morning to control for diurnal variation.

7.3.3 Platelet Function Assays

Detailed methods were described in section 2.3.8 but are outlined in brief below.

7.3.3.1 Impedance Aggregometry

Venous blood (2.7 mL) was collected from subjects and transferred to a 3 mL hirudin tube (Multiplate®) carefully avoiding any agitation of the sample. The samples were then transported to the Cardiovascular Biomedical Research laboratory, Northern General Hospital immediately following venepuncture to maximise the chances of observing a catecholamine effect. Aliquots of 300 µL saline and 300 µL hirudin-anticoagulated blood were added to the cuvette and incubated at 37°C for

3 minutes before adding 20 µL of the agonists ADP 1 µM and collagen 1 µM and commencing the assay. The AUC was measured and this represents the level of platelet aggregation.

7.3.3.2 Whole Blood Single Platelet Counting

Citrated blood venous blood (4.5 mL) was incubated at 37^{o}C and 480 µL aliquots of whole blood were placed in polystyrene tubes with a magnetic stirrer bar and 20 µL of ADP 1 or 3 µM as an agonist. Samples were stirred at 1000 rpm for 4 minutes at 37°C prior to fixing (saline with 4.6 mM sodium EDTA, 4.5 mM Na_2HPO_4, 1.6 mM KH_2PO_4 and 0.16% (w/v) formaldehyde, pH7.4) and counting using a Sysmex KX21 haematology analyser. The total single platelet count was obtained using EDTA 4 mM instead of ADP. The percentage aggregation was calculated as follows:

$$\text{Percentage platelet aggregation} = \frac{(\text{EDTA count} - \text{ADP count})}{\text{EDTA count}} \times 100$$

7.3.3.3 Platelet P-selectin Expression

Hirudin-anticoagulated blood was anticoagulated with saline or cangrelor 1 µM for 2 minutes at room temperature. Aliquots of blood were then added to tubes containing PE Cy5 mouse anti CD62P antibody and either ADP (1 µM) or 5HT (3 µM) as agonists, and after 20 minutes incubation at room temperature, fixing solution was added. Flow cytometry was performed and forward light scatter, side scatter and FITC fluorescence were measured. A gate was positioned around the platelet region to exclude any

red cells. P-selectin expression was quantified as the percentage of cells with CD62P fluorescence greater than the fluorescence of the mouse IgG control (% positive events) and the median CD62P fluorescence intensity of the platelet population.

7.3.4 Biochemical Parameters

For catecholamines, 6 mL whole blood was collected into chilled lithium heparin tubes containing 50 µL of EGTA/glutathione preservative, subjected to centrifugation at 4^{o}C, 3000 rpm for 10 minutes and the resulting supernatant stored at -80^{o}C until assayed by HPLC. Plasma free insulin was analysed by an immunometric assay (Invitron Insulin ELISA, Invitron Ltd, Monmouth, UK) following precipitation with PEG. The assay has 100% cross reactivity with Actrapid insulin. Serum potassium was analysed using an automated system (Cobas®system, Roche Diagnostics, UK). Biochemical parameters were measured at baseline and 120 minutes for both groups in the euglycaemic and hypoglycaemic arms.

7.3.5 Statistical Analysis

Data was checked for normality and data are shown as mean± SE except for age and duration of diabetes which was shown as medians (interquartile range). Comparisons between diabetic and control groups in baseline data were made using an independent t-test for parametric data and a Mann Whitney U test for nonparametric data. Blood glucose and hormone concentrations were compared between equivalent euglycaemic and hypoglycaemic time points using a paired t-test or Wilcoxon signed-

rank test, and between groups at equivalent time points using an independent t-test.

Platelet parameters were analysed using a linear mixed model with repeated measures. The mixed model allows for internally correlated measures from repeated measures (with respect to both the glycaemic arm and time) within an individual and also allows for missing values (Galbraith et al., 2010). To investigate whether there was a significant change in platelet parameters within the euglycaemic or hypoglycaemic arms, time point was specified as a fixed effect in the linear mixed model. Changes in platelet parameters between glycaemic arms were also compared using a linear mixed model, where glycaemic arm, time and interaction between the glycaemic arm and time were specified as fixed effects. In both analyses the glycaemic arm and time point were specified as repeated measures and fitted with an unstructured, compound symmetry or autoregressive 1 covariance structure. The model with the best fit (lowest Akaike's information criterion) was selected. p values were obtained by restricted maximum likelihood estimation. Changes in P-selection expression in response to ADP and 5HT stimulation in the presence and absence of P2Y12 blockade were compared using independent t-tests. A p value of ≤ 0.05 was considered significant and results were analysed using SPSS (version 20.0, IBM, Chicago,Ilinois).

7.4 Results

7.4.1 Baseline Characteristics

Participant characteristics are as previously described in Section 4.4.1 and baseline platelet parameters are shown in Table 7-1. There were no significant differences between euglycaemia and hypoglycaemia at baseline. All platelet reactivity measures were generally higher in the diabetic subjects compared to the nondiabetic group.

Table 7-1: Baseline platelet reactivity in T2DM subjects and controls
Data mean ±SE. p values for diabetic versus nondiabetic groups as determined by an independent t-test

	Diabetic (n=12)	**Nondiabetic** (n=11)	**p-value**
Platelet aggregation by impedance aggregometry			
AUC ADP (U)	75±11	41±8	0.03
AUC collagen (U)	87±8	74±6	0.21
Platelet aggregation by WBSPC			
1 µM ADP	16.0±5.7	13.0±3.9	0.67
3 µM ADP	40.3±10.9	54.4±6.6	0.29
P selectin expression (CD62P MFI)			
Unstimulated	4016±1040	3236±1681	0.19
5HT	3984±1111	2866±1488	0.07
ADP	5629±2206	4512±2203	0.11
P selectin expression (% CD62P positive cells)			
Unstimulated	4.45±1.42	3.16±0.81	0.43
5HT	5.38±1.90	4.14±0.80	0.57
ADP	28.7±4.2	36.2±4.4	0.23

7.4.2 Hyperinsulinaemic Clamp

At the end of the morning euglycaemic clamp, glucose was 5.81±0.09 and 5.96±0.05 mmol/L in diabetic and control groups, respectively, and following the morning hypoglycaemia, blood glucose concentrations were

2.55±0.06 mmol/L in the diabetic group and 2.56±0.03 mmol/L in the control group, which was not significantly different between the two groups (Table 7-2). Blood glucose targets were also achieved during the afternoon clamp within each arm.

Median free insulin levels at 120 minutes were 576 (IQR: 468-627) pmol/L during euglycaemia and 689 (IQR: 477-1076) pmol/L during hypoglycaemia in the diabetic group. In the nondiabetic group, they were 865 (IQR: 509-952) pmol/L in the euglycaemic arm and 665 (IQR: 468-967) pmol/L in the hypoglycaemic arm. A comparison of the insulin levels between the groups under both euglycaemic and hypoglycaemic conditions were not statistically different ($p = 0.23$).

Counter-regulatory hormones did not change during the euglycaemic clamp (Table 7-2). During acute hypoglycaemia, adrenaline, noradrenaline and cortisol increased significantly at the end of the clamp in both groups (all $p<0.05$ versus baseline), and the peak levels were not significantly different between the diabetic and nondiabetic groups.

7.4.3 Blood Glucose and Counter-regulatory Hormones at day 1 and 7

Blood glucose at day 1 and day 7 are shown in Table 7-2. Blood glucose was not significantly different between the glycaemic arms except at day 1, where it was higher following hypoglycaemia in the diabetic group. Plasma

adrenaline and cortisol were not significantly different from baseline at day 1 and day 7 following euglycaemic and hypoglycaemic clamps in either group. However, in the nondiabetic group, plasma noradrenaline levels increased significantly at day 1 after the euglycaemic clamp.

Table 7-2: Blood glucose and counter-regulatory hormones in the euglycaemic and hypoglycaemic arms

Data Mean ±SE *p<0.05, **p<0.01 Euglycaemia versus hypoglycaemia within each group was determined by a paired t-test †† p<0.01 significant difference between diabetic and nondiabetic groups at equivalent euglycaemic or hypoglycaemic time points was determined by an independent t-test.

	Diabetics		**Nondiabetics**	
	Euglycaemia	**Hypoglycaemia**	**Euglycaemia**	**Hypoglycaemia**
Blood glucose (mmol/L)				
Baseline	6.24±0.33	6.44±0.37	5.10±0.15†	4.87±0.09†
End of clamp	5.81±0.09	2.55±0.06**	5.96±0.06	2.56±0.03**
Recovery	6.98±0.33	6.63±0.55	6.37±0.19	8.40±0.59**
Day 1	7.88±0.84	11.4±1.09*	5.08±0.28†	5.00±0.29†
Day 7	9.30±0.78	10.3±1.04	5.09±0.13†	4.96±0.21†
Adrenaline (nmol/L)				
Baseline	0.15±0.04	0.12±0.03	0.17±0.03	0.15±0.02
End of clamp	0.16±0.04	3.05±0.71**	0.14±0.02	3.83±0.86**
Day 1	0.23±0.12	0.17±0.06	0.17±0.03	0.20±0.08
Day 7	0.10±0.02	0.16±0.08	0.22±0.06	0.11±0.02
Noradrenaline (nmol/L)				
Baseline	1.23±0.14	1.25±0.15	1.56±0.15	1.46±0.16
End of clamp	1.27±0.09	2.37±0.24**	1.56±0.12	2.69±0.43**
Day 1	1.97±0.15	1.93±0.10	3.08±0.44†	2.32±0.21
Day 7	2.07±0.17	2.52±0.34	2.56±0.24	2.78±0.34
Cortisol				
Baseline	277±25	329±37	338±43	300±39
End of clamp	253±25	726±36**	256±33	656±45**
Day 1	243±35	178±28	312±35	230±29
Day 7	243±16	198±30	285±28	233±22

7.4.4 Platelet Count

There were no changes in platelet count during the euglycaemic clamp (Table 7-3). In the diabetic group, the platelet count tended to increase during hypoglycaemia and returned to baseline at recovery. In the nondiabetic group the platelet count increased during hypoglycaemia from 211±12 to 236±27 x10^9/L at the end of the clamp but fell to 188±16 x10^9/L at recovery (p=0.01 for time). There were no significant changes in platelet count downstream at day 1 or day 7 following hypoglycaemia or euglycaemia in either group.

Table 7-3 Platelet count following euglycaemic or hypoglycaemic clamps in diabetic and nondiabetic groups
Data Mean±SE x 10^9/L

	Diabetes (n=12)		**Nondiabetic** (n=11)	
	Euglycaemia	**Hypoglycaemia**	**Euglycaemia**	**Hypoglycaemia**
Baseline	198±11	219±23	206±18	211±12
End of clamp	204±15	256±21	190±15	236±27
Recovery	215±12	214±25	185±11	188±16
Day 1	221±16	224±25	207±19	215±14
Day 7	219±18	234±28	219±20	232±24

7.4.5 Platelet Activation

In diabetic subjects, platelet activation as measured by unstimulated P-selectin expression (CD62P median fluorescence intensity (MFI)) decreased following euglycaemia maximally at day 1 compared to an increase following hypoglycaemia (p=0.04 for the glycaemic arm) (Figure 7-1). In nondiabetic subjects, no significant changes in platelet activation occurred

during euglycaemia. However, platelet activation tended to increase at the end of the hypoglycaemic clamp (as shown by increases in CD62P MFI and % CD62P positive cells) and at day 1 but there was no overall difference between the euglycaemic and hypoglycaemic arms (Figure 7-1).

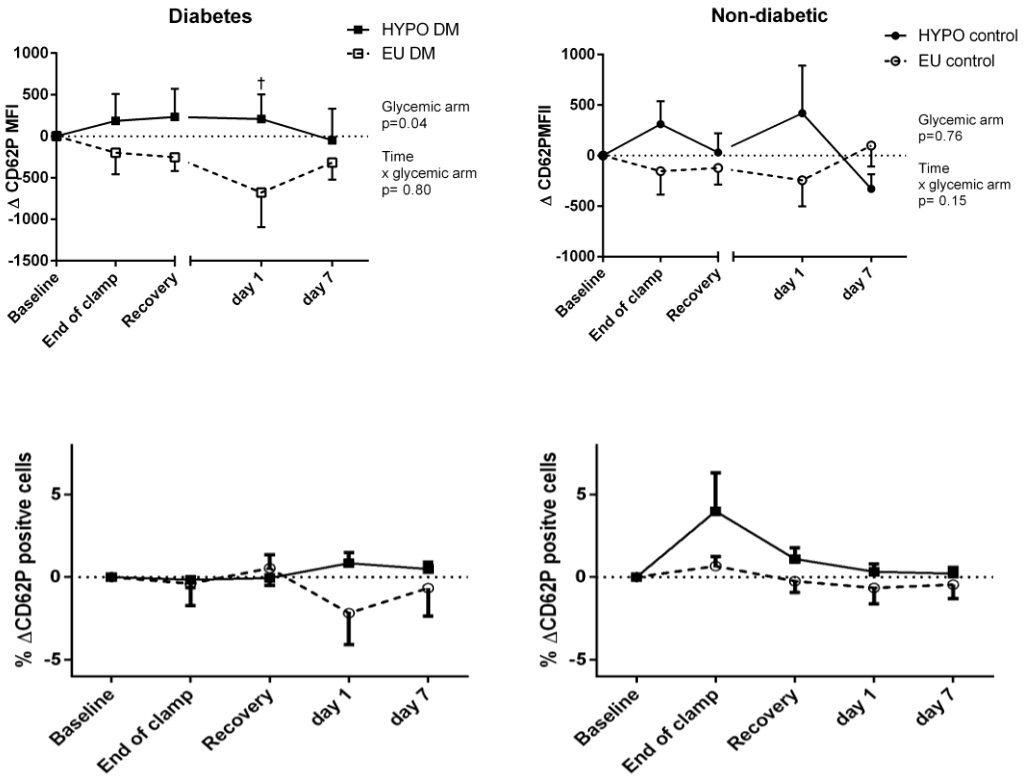

Figure 7-1: Changes in platelet activation following euglycaemia and hypoglycaemia in diabetic and nondiabetic groups

Data mean (SE). † Change in CD62P median fluorescence intensity (MFI) top and change in percentage CD62P positive cells (bottom) $p<0.05$. Euglycaemia (open circle) vs. hypoglycaemia (square) at equivalent time points. p values determined by a linear mixed model for differences between glycaemic arms and the interaction between time and glycaemic arm.

7.4.6 Platelet Reactivity

7.4.6.1 Impedance Aggregometry

In diabetic subjects, platelet aggregation to collagen decreased during the euglycaemic clamp but had returned to baseline at day 1. Platelet macroaggregation to collagen by impedance aggregometry increased

acutely during hypoglycaemia in diabetic (Δ 9±6 U) and nondiabetic subjects (Δ 14±4 U) compared with decreases during euglycaemia (Δ -15±7 and Δ -9±6 U respectively, p=0.05 for the glycaemic arm). Platelet aggregation to collagen returned to baseline at day 1 and 7 after hypoglycaemia (Figure 7-2).

Platelet aggregation to ADP also decreased during euglycaemia compared with an increase at the end of the hypoglycaemic clamp (p =0.03 for the difference between glycaemic arm). Platelet aggregation to ADP remained higher at day 1 and day 7 after hypoglycaemia (Figure 7-2).

In nondiabetic subjects, there was a non-significant decrease in platelet aggregation to collagen during euglycaemia, whilst platelet aggregation to collagen increased during the hypoglycaemic clamp but had resolved at recovery. There were further increases in platelet aggregation to collagen and ADP at day 1 following both euglycaemia and hypoglycaemia but these fell again at day 7 (Figure 7-2).

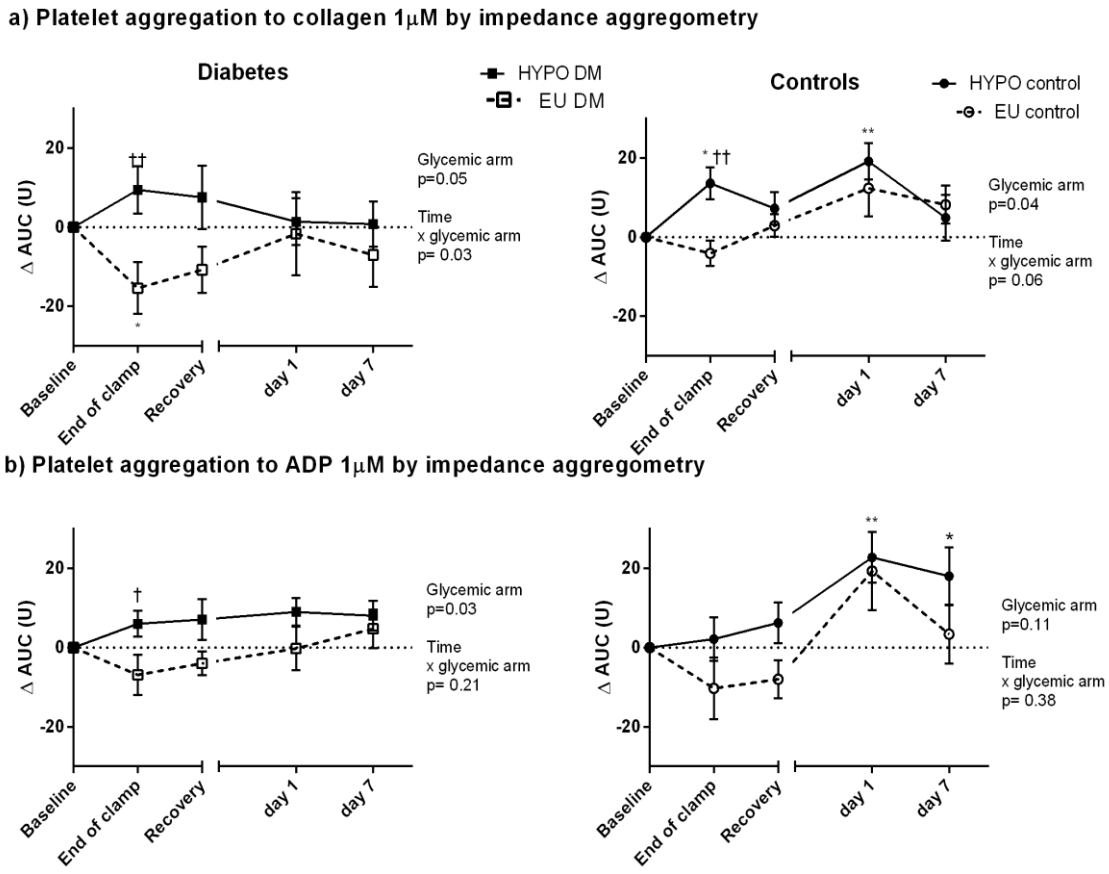

Figure 7-2: Platelet aggregation by impedance aggregometry to a) collagen 1 μM and b) ADP 1 μM

Data mean (SE), area under the curve (AUC). p values are determined by a linear mixed model for the fixed effects of glycaemic arm and the interaction between time and the glycaemic arm * p<0.05 versus baseline †p<0.05 †† p <0.01 euglycaemia (EU) versus hypoglycaemia (HYPO)

7.4.6.2 WBSPC

In the diabetic group, the percentage platelet aggregation to 3 μM ADP decreased at the end of the euglycaemic clamp, Δ- 26±40% (Figure 7-3), before returning towards baseline by day 1 and day 7. % platelet aggregation to ADP 3 μM did not change significantly during the hypoglycaemic clamp. In the nondiabetic group, the percentage platelet aggregation decreased during euglycaemia and hypoglycaemia up to

recovery to a similar extent but returned towards baseline levels at day 1 and day 7 (Figure 7-3) (p=0.001 for time, p=0.26 for glycaemic arm).

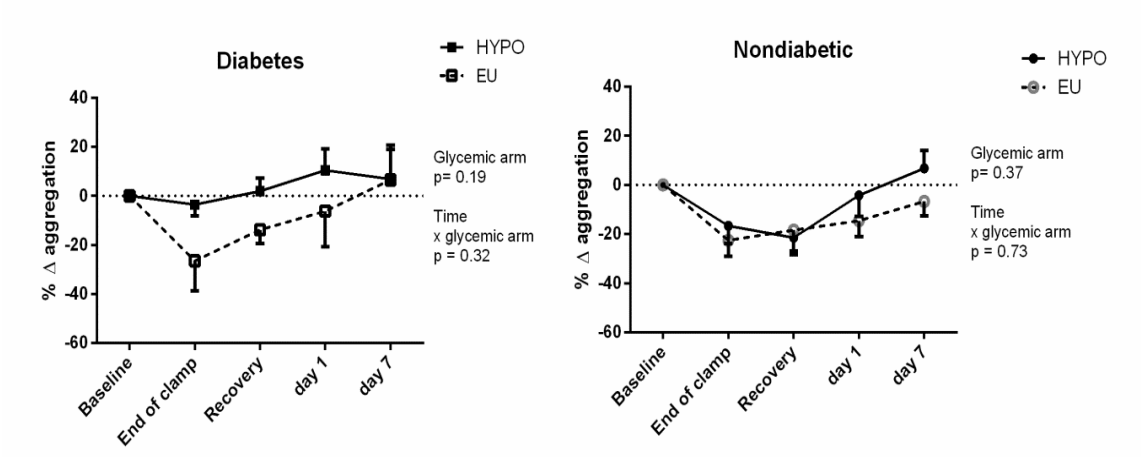

Figure 7-3: Percentage platelet aggregation to 3 µM ADP by whole blood single platelet counting in the euglycaemic and hypoglycaemic arms
Data mean (SE) Change in percentage platelet aggregation, EU-euglycaemia, HYPO-hypoglycaemia

7.4.6.3 P-selectin Expression

5HT

In the diabetic group there was a general trend towards a decrease in P-selectin expression to 5HT (3 µM) in the euglycaemic arm which was maximal at day 1. Platelet reactivity to 5HT as measured by CD62P MFI increased during the hypoglycaemic clamp but returned to baseline at recovery (Figure 7-4).

In the nondiabetic group there were no changes during euglycaemia. P-selectin expression measured by the percentage of CD62P positive cells and CD62P MFI increased acutely during hypoglycaemia and returned to baseline at recovery. There were significant differences in the percentage

of CD 62P positive cells following euglycaemia versus hypoglycaemia (p = 0.04 for glycaemic arm).

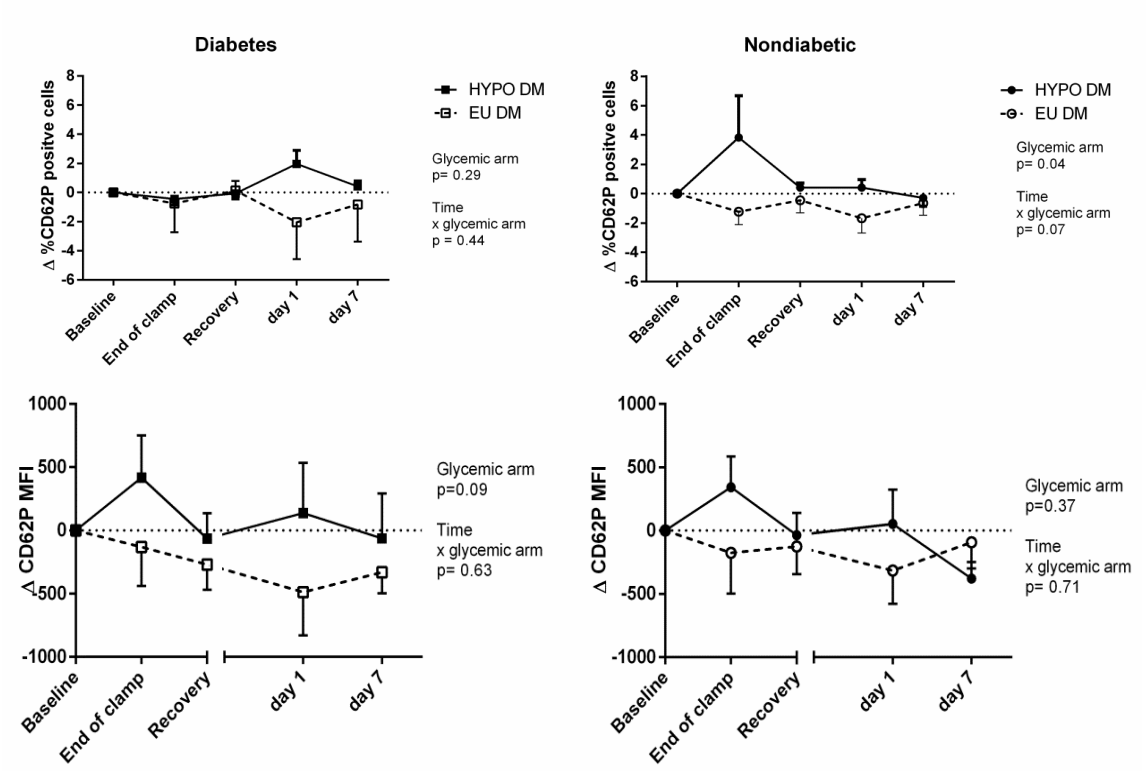

Figure 7-4: P-selectin expression to 5HT (3 μM) by flow cytometry following euglycaemia and hypoglycaemia in diabetic and nondiabetic groups

Data mean (SE) Change in percentage CD62P positive cells (top) and CD62P median fluorescence intensity (bottom). p values as determined by a linear mixed model for the glycaemic arm and interaction between time and glycaemic arm as fixed factors

ADP

In the diabetic group, P-selectin expression to ADP tended to decrease at day 1 after euglycaemia (CD62P MFI Δ-1179±634) compared to no change in the hypoglycaemic arm (Figure 7-5), but the differences between the glycaemic arms did not reach statistical significance. In the nondiabetic group, P-selectin expression to 1 μM ADP similarly decreased at day 1 following the euglycaemic clamp but returned towards baseline at day 7. In the hypoglycaemic arm there was an increase at day 1 but this returned to

baseline at day 7 (Figure 7-5). There was a significant interaction between the time point and glycaemic arm (p=0.04).

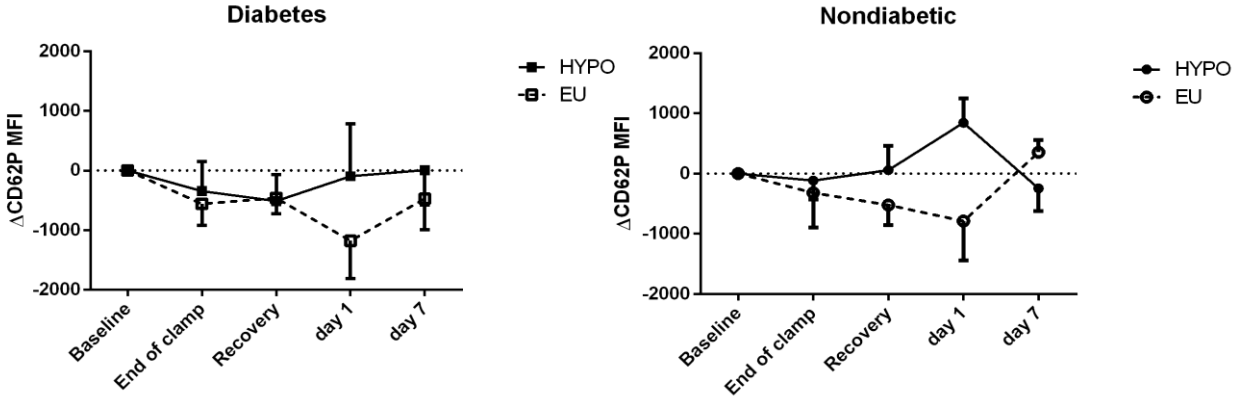

Figure 7-5: P-selectin expression to 1 µM ADP following euglycaemia and hypoglycaemia in diabetic and nondiabetic groups

Data Mean (SE) change in CD62P median fluorescence intensity (MFI). Closed square- Diabetes HYPO, open square – Diabetes EU; closed circle – nondiabetic HYPO; open circle- nondiabetic EU

Effect of P2Y12 blockade on P-selectin expression to ADP and 5HT

During the euglycaemic clamp in diabetic and nondiabetic subjects, the decrement in P-selectin expression tended to be attenuated in the presence of cangrelor to both ADP and to a lesser extent to 5HT (Figure 7-6a).

During the hypoglycaemic clamp, the increment in 5HT-induced P-selectin expression as measured by CD62P MFI was non-significantly attenuated in the presence of cangrelor in the nondiabetic group but did not change in the diabetic group (Figure 7-6b). In contrast, there was a decrease in P-selectin in response to 1 µM ADP at the end of the hypoglycaemic clamp in both diabetic and nondiabetic subjects. This decrement was reversed in the presence of cangrelor, as shown by increases in CD62P MFI (Figure 7-6b).

Similarly, the percentage of CD62P positive cells at the end of hypoglycaemia decreased with ADP alone (-Δ5.46±2.72 versus Δ0.10±0.51) in the presence of cangrelor (p = 0.05) among diabetic subjects, and in nondiabetic subjects the percentage of CD62P positive cells decreased with ADP alone (-Δ4.50±3.09% versus Δ 0.31±0.95%) in the presence of cangrelor (p = 0.15).

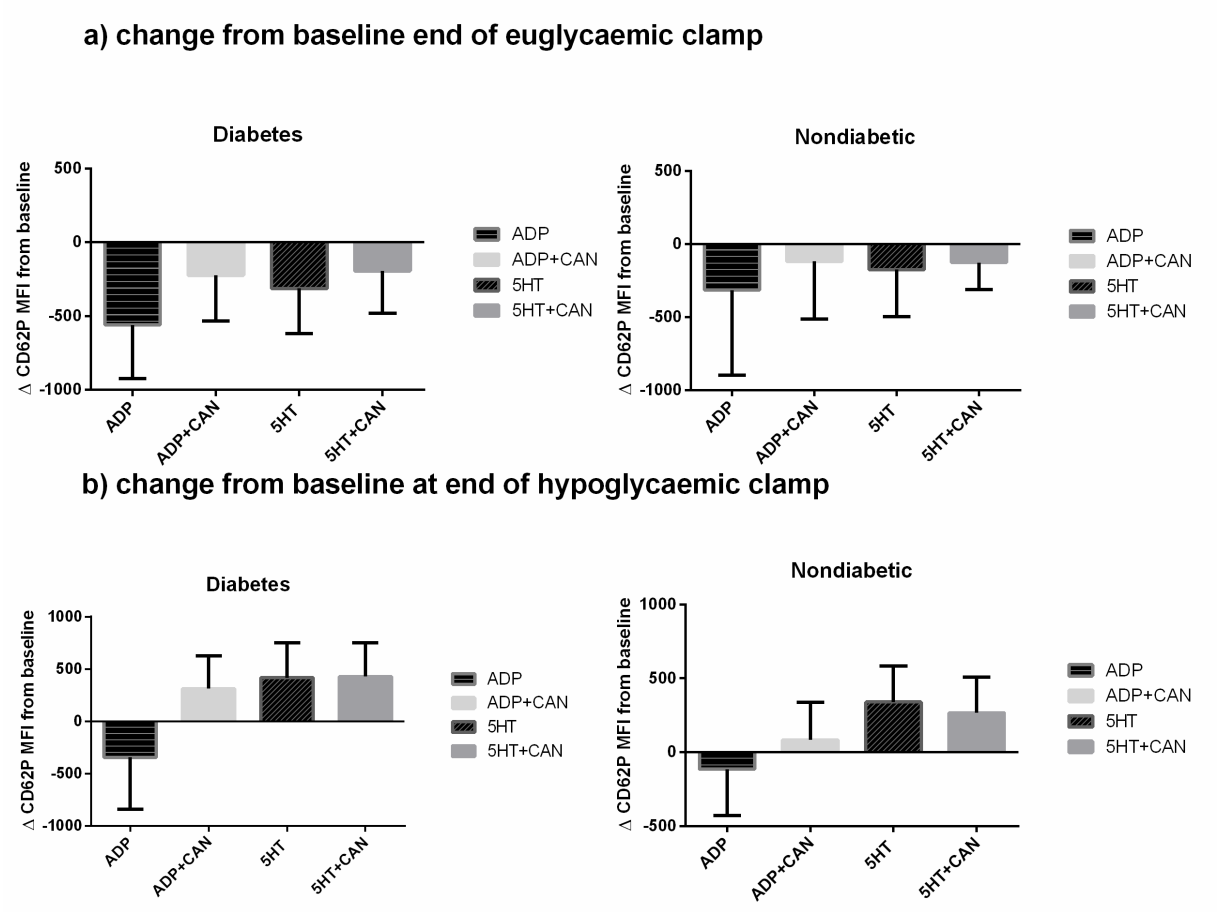

Figure 7-6: Effect of P2Y12 inhibition with cangrelor on P-selectin expression to 1 µM ADP and 3 µM 5HT in the euglycaemic and hypoglycaemic arms

(a) Change in CD62P median fluorescence intensity (MFI) at the end of the euglycaemic clamp compared to the baseline in response to 1 µM ADP, ADP and cangrelor (ADP+CAN), 3 µM 5HT, 5HT and cangrelor (5HT+CAN). (b) Change in CD62P median fluorescence intensity (MFI) at the end of the hypoglycaemic clamp compared to baseline in response to 1 µM ADP, ADP and cangrelor (ADP+CAN), 3 µM 5HT, 5HT and cangrelor (5HT+CAN). Data mean (SE).

7.5 Discussion

The main findings of this study are as follows: 1) platelet activation as shown by P-selectin expression increased during and after hypoglycaemia in diabetic and nondiabetic groups; 2) there were increases in platelet macroaggregation to collagen during hypoglycaemia which decreased following euglycaemia in both groups; 3) There were no increases in platelet microaggregation during hypoglycaemia in either group; and 4) P2Y12 inhibition influenced 5HT and ADP induced P-selectin expression during both euglycaemia and hypoglycaemia.

During euglycaemia which reflects the effect of insulin alone, acute decreases in platelet macroaggregation to collagen and ADP and platelet microaggregation to ADP in both groups were observed. *In vitro* studies have shown contradictory effects of insulin on platelet function, as insulin may inhibit platelet aggregation at physiological concentrations but increases aggregation at supraphysiological levels (Anfossi et al., 1996). *In vivo* studies have generally shown inhibitory effects of insulin on platelet aggregation (Trovati et al., 1988), where insulin may act directly via the insulin receptor substrate-1 (IRS-1) through tyrosine phosphorylation, which results in calcium mobilisation and inhibition of Gi activity and P2Y12 inhibition (Ferreira et al., 2003). Alternatively, insulin may rapidly activate a constitutive NO NOS_3 via the PI3K/Akt pathway to increase NO release in platelets (Trovati et al., 1988). Insulin can also reduce platelet aggregation via indirect mechanisms, such as increases in prostaglandin I2 production

from endothelial cells (Kahn et al., 1991). In particular, platelet activation as shown by unstimulated P-selectin expression remained decreased at day 1 after euglycaemia, suggesting persistent anti-inflammatory mechanisms may be pertinent. Greater decreases in platelet aggregation in diabetic compared to nondiabetic subjects were observed, and these findings differ from previous work which reported diminished anti-aggregatory effects of insulin among obese versus non-obese subjects (Westerbacka et al., 2002), although the reasons for these differences are unclear.

During the hypoglycaemic clamp, there were trends towards an increase in platelet count in diabetic and nondiabetic groups. Hutton et al. (1979) reported increases in platelet counts in 3 out of 10 nondiabetic subjects during insulin-induced hypoglycaemia but no overall change in 7 subjects. The increase in platelet count could relate to the increase in adrenaline (Kjeldsen et al., 1995), which mobilises splenic reserves of platelets (Bakovic et al., 2012).

Platelet activation increased following hypoglycaemia as shown by P-selectin expression via flow cytometry. There was an acute increase in overall P-selectin expression in the total platelet population in both groups but increases in the percentage of P-selectin positive cells were seen in nondiabetic subjects only. Similar to a study by Trovati et al. (1986), platelet factor 4 and beta thromboglobulin were significantly higher at the hypoglycaemic nadir following an insulin bolus in nondiabetic individuals, suggesting platelet activation. Takeda et al. (1988) induced hypoglycaemia

using an insulin bolus in T2DM individuals and found increases in beta thromboglobulin were prevented by midaglizole, suggesting hypoglycaemia-related platelet activation is mediated via alpha 2 – adrenoreceptors.

This study demonstrated increased platelet aggregation to collagen and ADP, as measured by impedance aggregometry in both diabetic and control groups during acute hypoglycaemia. 5HT-induced P-selectin expression, as measured by flow cytometry, also increased acutely during hypoglycaemia. Earlier studies have demonstrated increases in platelet reactivity as defined by platelet aggregation to Born aggregometry in platelet rich plasma (Trovati et al., 1986). Dalsgaard-Nielsen et al. (1982) also induced transient hypoglycaemia in seven nondiabetic and 8 T1DM individuals. The study found that platelet sensitivity to ADP was significantly increased as measured by turbidimetric assays. The increase in platelet sensitivity to agonists, including ADP and thrombin, during hypoglycaemia was abolished by an alpha blockade, implicating alpha-2 adrenoreceptors in mediating this effect (Trovati et al., 1986). Adrenaline on its own is a weak agonist but it has been shown to potentiate platelet aggregation to other agonists, such as ADP, collagen and thrombin, both *in vitro* and *in vivo* (Trovati et al., 1986). Furthermore, *in vitro* studies have shown that adrenaline can neutralise the inhibitory effects of insulin by blocking the tyrosine phosphorylation of both $G_i\alpha_2$ and the insulin receptor β subunit (Ferreira et al., 2003).

In contrast to the changes demonstrate by impedance aggregometry, platelet microaggregation to ADP as measured by single platelet counting did not change during acute hypoglycaemia in diabetic individuals. In the nondiabetic group, the percentage aggregation decreased during acute hypoglycaemia to a similar extent to euglycaemia. Impedance aggregometry is influenced by platelet counts and correlates linearly with platelet counts even in the normal range (Femia et al., 2013). Thus an increase in platelet macroaggregation by impedance aggregometry may in part be explained by trends in increased platelet counts during acute hypoglycaemia. The net effect on platelet microaggregation may reflect a balance between pro-aggregatory effects of adrenaline versus anti-aggregatory effects of insulin. However, other groups have also reported a lack of potentiation by epinephrine on ADP/5HT platelet aggregation *in vitro* when assessed by the disappearance of single platelets (Kerry and Scrutton, 1985).

During euglycaemic clamps there was a decrease in P-selectin expression as expressed by the percentage of CD62P positive platelets to ADP and 5HT in both groups. This decrement was attenuated in the presence of cangrelor, suggesting the anti-aggregatory effects of insulin are partially mediated via a P2Y12 dependent pathway. Insulin has previously been shown to inhibit ADP-induced calcium mobilisation via Gi pathways (Ferreira et al., 2003). During hypoglycaemic clamps 5HT-induced P-selectin expression increased but was attenuated in the presence of cangrelor in

nondiabetic subjects. Inhibition of P2Y12 may reduce the ability of secreted ADP to amplify aggregatory responses in response to 5HT analogous to other platelet agonists. It has been shown, for example, that adrenaline potentiates platelet responses to thrombin that were dependent on secreted ADP (Selheim et al., 2000).

Conversely, there was a decrease in P-selectin expression in response to 1 μM ADP at the end of the hypoglycaemic clamp in both diabetic and nondiabetic subjects. This decrement was reversed in the presence of cangrelor and reasons for this are uncertain. It is possible that exposure to adrenaline and stimulation via alpha2-adrenoreceptors *in vivo* cross-desensitises ADP-mediated aggregation pathways. It is known that desensitisation of platelet responses to adrenaline occurs following an insulin-induced hypoglycaemia after an insulin infusion for 60 minutes (Trovati et al., 1990). It has also been shown that there is cross-desensitisation between thrombin and ADP pathways, such that pre-activation of one receptor may heterologously desensitise aggregation responses to the other mediated via protein kinases (Barton et al., 2007). One potential explanation is that desensitisation of alpha 2 adrenoreceptors (Gi subfamily) after hypoglycaemia can lead to cross-desensitisation of P2Y1 mediated P-selectin expression (Gq) but not P2Y12. The fact that P2Y12 antagonism can reverse the inhibition suggests that the mediator of this desensitisation may be a downstream effector that is common to all three pathways. There is accumulating evidence that graded

activation of the Gi subfamily, including P2Y12 and alpha 2-adrenoreceptor, regulates platelet aggregation response and controls the release of platelets from a state of tonic inhibition, whereas signalling via Gq coupled receptors, including P2Y1 and 5HT2A, are necessary for initiation of aggregation responses (Maayani et al., 2001).

One of the strengths of this study is the inclusion of a euglycaemic arm to control for the effects of hyperinsulinaemia. All the platelet function assays were processed as soon as possible within two hours of collection in order to minimise the chances of observing a catecholamine effect. Platelet reactivity was analysed using different assays that measured both platelet macroaggregation and microaggregation effects at a single platelet level. However, the study also has a number of limitations. The number of participants are relatively small. Another potential limitation is the slightly higher number of males in the diabetic group. Most of the subjects were not on any antiplatelet treatment apart from two diabetic subjects who were on aspirin. This can affect collagen-induced platelet aggregation via inhibition of the formation and release of thromboxane A_2. However, these subjects remained on aspirin throughout the study in both arms and displayed similar patterns of collagen-induced platelet aggregation to the rest of the group (data not shown). For ethical reasons, hypoglycaemia was not induced in individuals with known CVD. Intensive glucose lowering exerts greater cardiovascular benefits in patients with low cardiovascular risk compared to those with established CVD (Holman et al., 2008; Reaven

et al., 2009). What is not known is whether the deleterious effects of hypoglycaemia may be exaggerated and the protective effects of insulin attenuated in patients at high cardiovascular risk.

The findings of this study are relevant to intensive glycaemic control, particularly in the setting of ACS, where platelets play an important role in early disease. Vivas et al. (2011) compared the effect of intensive glycaemic control (4.4-6.6 mol/L) versus conventional control (<10 mmol/L) with intravenous glucose/insulin/potassium infusion for 24 hours in ACS patients. Hypoglycaemia (blood glucose <3.8 mmol/L) occurred significantly more often in intensively treated patients (37 vs 2%). Nevertheless, the study demonstrated significant reductions in platelet activation (P-selectin and GPIIb/IIIa expression) and reduction in platelet aggregation to ADP, collagen, adrenaline and thrombin at 24 hours. Worthley et al. (2007) compared the effects of intravenous versus subcutaneous insulin administration on platelet responsiveness in 76 ACS patients. They demonstrated that intravenous insulin infusion improved platelet responsiveness to NO and reduced superoxide production. However, in both studies it was not possible to demonstrate whether better glucose control or higher insulin doses *per se* were responsible for improvements in platelet function. Intensive glycaemic control had short term effects on platelet reactivity which did not persist at long term follow up (Vivas et al., 2014). This echoes findings in this study that reductions in platelet macroaggregation is limited to the period of acute

hyperinsulinaemic euglycaemia and do not persist beyond day 1. Taken together, this would support the benefits of intensive glycaemic control in ACS, however, hypoglycaemia may mitigate antiaggregatory effects of insulin.

In summary, increases in platelet activation and platelet reactivity were observed in response to hypoglycaemia in T2DM and controls. This contrasts with the anti-aggregatory effects of euglycaemic hyperinsulinaemia. Acute hypoglycaemia, via increases in platelet activation and reactivity, may predispose to acute coronary thrombosis and cardiovascular mortality.

Chapter 8 - Effects of Hypoglycaemia on Fibrin clot properties and Inflammation in Type 2 Diabetes

8.1 Summary

Altered fibrin clot characteristics, impaired fibrinolysis, and subclinical inflammation can predispose to acute coronary events in individuals with T2DM. This has previously been attributed to increased glucose levels but recent work has shown that hypoglycaemia can increase pro-coagulant factors, proinflammatory cytokines and inhibit fibrinolysis in nondiabetic and T1DM subjects. The aim of this current study is to investigate the effect of hypoglycaemia on fibrin-related thrombosis risk acutely and up to 7 days after an experimental hypoglycaemic episode in individuals with T2DM.

A total of 12 T2DM and 11 nondiabetic subjects underwent paired hyperinsulinaemia euglycaemic (blood glucose 6 mmol/L for two 60 minute periods) and hypoglycaemic clamps (2.5 mmol/L for two 60 minute periods) on separate occasions. *Ex vivo* fibrin clot properties and fibrinolysis was investigated using a validated turbidimetric assay. Fibrin clot ultrastructure was further studied using laser scanning electron microscopy. Fibrinogen, PAI-1, complement component C3, hsCRP and inflammatory cytokines were measured at the end of the clamp to elucidate mechanisms for altered fibrin clot properties.

In diabetic subjects, hypoglycaemia increased fibrin clot density (mean ±SE), peaking at day 7 (clot maximum absorbance Δ from baseline 0·030±0.016 AU), but decreased after euglycaemia (Δ –0·039±0·025 AU, p=0·007). Clot lysis times were prolonged after hypoglycaemia for up to day 7 but decreased after euglycaemia (Δ 66 ±32 *vs* Δ –52±29 sec; p=0·006). There were no changes in clot density or lysis times following hypoglycaemia in nondiabetic subjects. The fibre network density decreased during euglycaemia and increased following hypoglycaemia for up to 7 days in both groups, whereas an increase in fibre diameter was only evident following hypoglycaemia in diabetic subjects. Fibrinogen, C3 and hsCRP, but not PAI-1, increased after hypoglycaemia up to day 7 in diabetic subjects.

Hypoglycaemia had sustained prothrombotic, hypofibrinolytic, proinflammatory effects for at least one week in individuals with T2DM. This may provide a potential mechanism for downstream increases in cardiovascular mortality associated with hypoglycaemia observed in interventional trials.

8.2 Introduction

Hypoglycaemia has been associated with an increase in cardiovascular deaths (Duckworth et al., 2009; Zoungas et al., 2010). In the previous chapters, it has been suggested that hypoglycaemia may enhance thrombotic risk via an increase in platelet reactivity acutely. However, in interventional trials, hypoglycaemia was associated with increased

mortality for up to 90 days after the event (Duckworth et al., 2009). Mechanisms by which hypoglycaemia is linked to increased cardiovascular mortality downstream of the event are unclear.

Hypoglycaemia has been associated with an increase in coagulation activity, as shown by a rise in coagulation factor VIII activity during insulin-induced hypoglycaemia in nondiabetic individuals (Grant et al., 1987). Receptor blockade with propranolol prevents the rise in factor VIII associated with hypoglycaemia, which suggests that this is also mediated by adrenergic control (Grant, 1990). vWF, an endothelial-derived factor that promotes thrombosis, is increased in response to hypoglycaemia (Fisher et al., 1991). In addition, a reduction in APTT has been reported following insulin-induced hypoglycaemia (Dalsgaard-Nielsen et al., 1982).

In contrast, some studies have suggested that the fibrinolytic system is activated in response to hypoglycaemia induced by an insulin bolus. An increase in tPA and plasminogen activator activity and decrease in inhibitory factors such as PAI-1 have been reported during hypoglycaemia (Fisher et al., 1991; Wieczorek et al., 1993). However, these earlier studies lacked a euglycaemic control arm and it is unclear if the observations were due to the effects of insulin *per se* or due to low glucose. Impaired fibrinolysis has been observed during more sustained hypoglycaemia and recent studies have found increases in PAI-1 and clot lysis times during hyperinsulinaemic hypoglycaemia maintained for more than an hour (Ajjan

et al., 2009). PAI-1 was also found to be increased during hypoglycaemia in nondiabetic but not T1DM subjects (Gogitidze Joy et al., 2010).

Thrombosis and inflammation are interlinked; for example, IL-6 is known to increase fibrinogen as part of an acute phase response (Ray, 2000). Hypoglycaemia induces acute increases in inflammatory cytokines in nondiabetic and T1DM subjects. A study by Galloway et al. (2000) has shown that CRP increases for 24 hours after insulin induced hypoglycaemia in T1DM and healthy volunteers. Acute hypoglycaemia has also been associated with an acute rise in markers of inflammation, including soluble CD40 ligand, VCAM and ICAM (Gogitidze Joy et al., 2010; Wright et al., 2010). No study to date appears to have examined the effect of hypoglycaemia on markers of thrombotic risk in T2DM individuals who are known to have an elevated risk of atherothrombotic events secondary to insulin resistance and other metabolic factors. Furthermore, no previous studies have examined markers for thrombosis risk, either acutely or in the days after an event when an elevated cardiovascular risk may persist. Therefore it was hypothesised that hypoglycaemia might oppose the cardiovascular benefits of intensive glycaemic control by modulating fibrin-related thrombosis risk.

The aim of this study was to delineate the effect of acute and downstream effects of hypoglycaemia on fibrin clot properties and inflammation in patients with T2DM versus nondiabetic controls.

8.3 Research Design and Methods

These are described in detail in Section 2.3 and are outlined in brief below.

8.3.1 Participants

Twelve individuals with T2DM and no known CVD were recruited from Sheffield Teaching Hospitals diabetes outpatient clinics. Patients were on one or more oral hypoglycaemic agents and/or GLP-1 analogue and insulin <2 yrs. Eleven nondiabetic age and BMI matched controls were recruited from staff at the University of Sheffield and Sheffield Teaching Hospitals. Patients taking beta-blocking agents were excluded. Written informed consent was obtained from all participants and the study received local ethics approval.

8.3.2 Hyperinsulinaemic Clamp Protocol

All diabetic and nondiabetic subjects participated in paired hyperinsulinaemic euglycaemia and hypoglycaemia studies separated by 4-8 weeks. In the hypoglycaemic arm, glucose was maintained at 2.5 mmol/L for 60 minutes in the morning and 60 minutes in the afternoon. In the euglycaemic arm, arterialised blood glucose was maintained at the target level of 6 mmol/L for 60 minutes in the morning and 60 minutes in the afternoon. Details of the hyperinsulinaemic clamp protocol are described in section 2.3.5. To investigate the effect of acute hypoglycaemia versus euglycaemia, prothrombotic and inflammatory markers were measured at the end of the morning clamp and at recovery (30 minutes after end of

clamp). To investigate downstream effects, thrombotic and inflammatory markers were also measured at 1 and 7 days after the euglycaemic and hypoglycaemic clamp visits. Study visits were conducted in the morning to control for diurnal variation.

8.3.3 Turbidimetric and Lysis Assay

Ex vivo fibrin polymerisation characteristics of plasma samples were investigated using a turbidimetric clotting assay. The turbidimetric assay was performed as previously described (Carter et al., 2007). Blood was collected into tubes containing 3.2% sodium citrate on ice. Briefly, one volume of plasma was treated with 3 volumes of activation mix containing 0.03 U/mL of thrombin and 7.5 mmol/L calcium. Turbidity was monitored in a 96 well plate every 12 seconds using a microplate reader to measure optical density. A turbidimetric lysis assay was conducted as above in the presence of tPA at a final concentration of 83 ng/mL. Clot maximum absorbance and lysis time (time for the initiation of clot formation to fall 50% in maximum absorbance) were calculated. Plasma clot maximum absorbance (MA) reflects the fibrin clot density, and lysis time the fibrinolytic potential (Alzahrani et al., 2012).

8.3.4 Markers of Fibrin Dynamics and Inflammation

Fibrinogen was measured using a validated Clauss method. PAI-1 was assayed using an ELISA (PAI1 (SERPINE 1) Human SimpleStep ELISA Kit, Abcam), and hsCRP using an immunoturbidimetric assay (Cardiac CRP (Latex) high sensitivity, Roche Diagnostics, Indianopolis, USA). Levels of IL-

6, MCP-1 and IL-10 were determined using a CBA (Human Il6, MCP-1, IL-10 Flex sets, BD Biosciences, Oxford, UK). Complement C3 plasma levels, a protein that is incorporated into the fibrin clot and modulates fibrinolysis (Hess et al., 2012a), were determined by ELISA (Biosources, San Diego, USA).

8.3.5 Scanning Electron Microscopy

Pooled samples of plasma were analysed from 10 diabetic and 10 nondiabetic subjects. Fibrin clots were made as previously described (Hooper et al., 2012) and fixed overnight in 2% glutaraldehyde with clots recovered and further processed via stepwise dehydration with an acetone gradient and sputter coated with platinum palladium. Samples were viewed and photographed using a field-emission scanning electron microscope (LEO1530 FEGSEM, Leo Electron Microscopy, Cambridge, United Kingdom) across four different areas of each clot. Images were captured using Leo 32 version 03.0210 software (Leo Electron Microscopy) and cropped using Paintshop Pro version 8.0 (Corel, Minneapolis, MN). Fibre diameters of all the clots were measured using the image analysis software package ImageJ 1,23y (National Institutes of Health, Bethesda, MD). In all, 160 fibrin fibres per sample (40 fibres in four clot areas) were measured with the operator blinded to the sampling time point. The fibre network density was estimated by counting the number of fibres crossing an arbitrary line of fixed length drawn through a single optical section. Three lines were drawn per image and this was analysed on four different

micrographs from different areas of a clot for each sample (Hethershaw et al., 2014).

8.3.6 **Biochemical Analysis**

Catecholamines were assayed by HPLC. Whole blood (6 mL) was collected in chilled lithium heparin tubes containing 50 µL EGTA/glutathione preservative, subjected to centrifugation at 1000 ×*g* for 10 minutes, and the supernatant plasma removed and stored at -80^{o}C prior to assaying. Free insulin was analysed by an immunometric assay (Invitron Insulin ELISA, Invitron Ltd, Monmouth, UK). Whole blood (3 mL) was drawn into 6 mL lithium heparin tubes, subjected immediately to centrifugation at 4^{o}C, 2400 rpm for 2 minutes. A 0.5 mL aliquot of the resulting plasma was added to a chilled plastic tube containing 0.5 mL PEG for precipitation of immune complexes and mixed and the centrifugation step was repeated for 30 minutes. The product was then stored at -80^{o}C until analysed by immunometric assay, where the inter-assay coefficient variation was 7.1%. The assay has 100% cross reactivity with Actrapid insulin. Serum cortisol was measured using a commercial electrochemilluminescence assay (Cobas, Roche Diagnostics, UK). Biochemical parameters were measured at baseline and at the end of the euglycaemic and hypoglycaemic arms in both groups.

8.3.7 Statistical Analysis

Baseline demographic data and prothrombotic and inflammatory markers are summarised as mean (SE) for parametric data unless otherwise stated,

or median (IQR) for nonparametric data. hsCRP and IL-6 were logarithmically transformed and expressed as geometric means (95% CI) due to a skewed distribution. Comparisons between diabetic and control groups in baseline data were made using an independent t-test for parametric data and Mann Whitney U test for nonparametric data. Blood glucose and hormone concentrations were compared between equivalent euglycaemic and hypoglycaemic time points using a paired t-test or Wilcoxon signed-rank test.

Fibrin thickness and fibrin network density from pooled samples were analysed using a two way repeated measures ANOVA for each subject group. Clot lysis, clot maximum absorbance, inflammatory and coagulation protein levels were analysed using a linear mixed model with repeated measures. The mixed model allows for internally correlated measures from repeated measures (with respect to both the glycaemic arm and time) within an individual and also allows for missing values (Galbraith et al., 2010). To investigate whether there was a significant change in prothrombotic markers over time within the glycaemic arms, time point was specified as a fixed effect in the linear mixed model. To analyse differences between glycaemic arms within each subject group, the glycaemic arm, time and interaction between glycaemic arm and time were specified as fixed effects. In both analyses, the glycaemic arm and time point were specified as repeated measures. Repeated measures were fitted with an unstructured, compound symmetry or autoregressive 1

covariance structure and the model with the best fit (lowest Akaike's information criterion) was selected. P values were obtained using a restricted maximum likelihood estimation and a p value of ≤ 0.05 was considered significant. Results were analysed using SPSS (version 20.0, IBM, Chicago, Illinois).

The sample size calculation took into account the repeated measure design, assuming a correlation of 0.5 between repeated measures (Frison and Pocock, 1992). A sample size of 12 had 80% power to detect a clinically important difference of 25% in overall clot lysis times between glycaemic arms with a type 1 error rate of 0.05. This assumes a SD of 200 ms, based on a previous study of T2DM patients (Alzahrani et al., 2012) .

8.4 Results

8.4.1 Baseline Characteristics

The demographics of the participants are as previously described. Twelve patients with T2DM (9 male, 3 female) and 11 nondiabetic controls (5 male, 6 female) participated in the study. Participants with diabetes were similar in age, 54 (50-58) years compared to the nondiabetic group, 52 (47-59) (p= 0.90), and the mean BMI was also comparable, 34±1 kg/m^2 in the diabetic group versus 31±2 kg/m^2 in the nondiabetic group (p=0.18). The median duration of diabetes was 10 (8-12) years and mean HbA1c was 7.8±0.4% (62±4 mmol/mol).

Baseline prothrombotic and inflammatory markers are shown in Table 8-1. Clot lysis time tended to be shorter, whilst fibrinogen, hsCRP and IL-6 were higher in diabetic subjects. There was no statistically significant difference between euglycaemic and hypoglycaemic baselines for the prothrombotic markers (data not shown).

Table 8-1: Baseline fibrin clot properties and inflammatory markers in diabetic and nondiabetic subjects

Data presented are mean ±SEM. hsCRP expressed as geometric mean (95% CI) p values shown are the comparison between group as determined by an independent t-test

	Diabetic (n=12)	Nondiabetic (n=11)	p value
Fibrin clot properties			
Clot MA (AU)	0.30±0.03	0.32±0.04	0.84
Clot lysis time (s)	617±48	744±66	0.13
Fibrin diameter (mm)	54±1	65±4	
Fibre network density fibres/µm^2	5.52±.54	4.44±0.2	
Acute phase protein and inflammatory biomarkers			
Fibrinogen (mg/mL)	3.01±0.32	2.60±0.21	0.31
PAI-1 (pg/mL)	3289±400	2435±378	0.14
C3 (mg/mL)	1.04±0.04	1.02±0.07	0.79
hsCRP (mg/L)	1.57 (0.66-3.73)	1.01 (0.37-2.77)	0.44

8.4.2 Hyperinsulinaemic Clamp

At the end of the morning euglycaemic clamp, glucose was 5.81±0.09 and 5.96±0.05 mmol/L in the diabetic and nondiabetic groups, respectively, and following morning hypoglycaemia, blood glucose concentrations were 2.55±0.06 mmol/L in the diabetic group and 2.56±0.03 mmol/L in the nondiabetic group. These were not significantly different between the

groups (Table 8-2). Blood glucose targets were also achieved during afternoon clamps within each arm.

Median free insulin levels at 120 minutes were 576 (IQR: 468-627) pmol/L during euglycaemia and 689 (IQR: 477-1076) pmol/L during hypoglycaemia in the diabetic group. In the nondiabetic group it was 865 (IQR: 509-952) pmol/L in the euglycaemic arm and 665 (IQR: 468-967) pmol/L in the hypoglycaemic arm. A comparison of insulin levels between the groups under both euglycaemic and hypoglycaemic conditions was found not to be statistically different ($p = 0.23$).

Counter-regulatory hormones did not change during the euglycaemic clamp (Table 8-2). During acute hypoglycaemia, adrenaline, adrenaline and cortisol increased significantly at the end of clamp in both groups (all $p<0.05$ versus baseline), although the peak levels were not significantly different between the diabetic and nondiabetic groups.

8.4.3 Blood Glucose and Counter-regulatory Hormones at day 1 and 7

Blood glucose was not significantly different between the glycaemic arms except at day 1, where it was higher following hypoglycaemia in the diabetic group (Table 8-2). Plasma adrenaline and cortisol were not significantly different from baseline at day 1 and day 7 following the euglycaemic and hypoglycaemic clamps in either group (Table 8-2). However, in the nondiabetic group, plasma noradrenaline levels increased

significantly at day 1 after the euglycaemic clamp with a smaller non-significant rise similarly following hypoglycaemia.

Table 8-2: Blood glucose and counter-regulatory hormones during euglycaemia and hypoglycaemia in diabetic and nondiabetic subjects

Data Mean ±SE *p<0.05, **p<0.01 euglycaemia versus hypoglycaemia within each subject group at the equivalent time point † † p<0.01 denotes significant difference between diabetic and control groups at equivalent euglycaemic or hypoglycaemic time point by independent t-test

	Diabetic		Nondiabetic	
	Euglycaemia	**Hypoglycaemia**	**Euglycaemia**	**Hypoglycaemia**
Blood glucose (mmol/L)				
Baseline	6.24±0.33	6.44±0.37	5.10±0.15†	4.87±0.09†
End of clamp	5.81±0.09	2.55±0.06**	5.96±0.06	2.56±0.03**
Recovery	6.98±0.33	6.63±0.55	6.37±0.19	8.40±0.59**
Day 1	7.88±0.84	11.4±1.09*	5.08±0.28†	5.00±0.29†
Day 7	9.30±0.78	10.3±1.04	5.09±0.13†	4.96±0.21†
Adrenaline (nmol/L)				
Baseline	0.15±0.04	0.12±0.03	0.17±0.03	0.15±0.02
End of clamp	0.16±0.04	3.05±0.71**	0.14±0.02	3.83±0.86**
Day 1	0.23±0.12	0.17±0.06	0.17±0.03	0.20±0.08
Day 7	0.10±0.02	0.16±0.08	0.22±0.06	0.11±0.02
Noradrenaline (nmol/L)				
Baseline	1.23±0.14	1.25±0.15	1.56±0.15	1.46±0.16
End of clamp	1.27±0.09	2.37±0.24**	1.56±0.12	2.69±0.43**
Day 1	1.97±0.15	1.93±0.10	3.08±0.44†	2.32±0.21
Day 7	2.07±0.17	2.52±0.34	2.56±0.24	2.78±0.34
Cortisol				
Baseline	277±25	329±37	338±43	300±39
End of clamp	253±25	726±36**	256±33	656±45**
Day 1	243±35	178±28	312±35	230±29
Day 7	243±16	198±30	285±28	233±22

8.4.4 Fibrin Clot Properties

8.4.4.1 Clot Lysis Time

In diabetic subjects, clot lysis times decreased during euglycaemia versus an increase following hypoglycaemia up to day 7 (p=0.001 for glycaemic arm). Clot lysis times decreased Δ-81±25 s at the end of the euglycaemic clamp. Conversely, clot lysis times were prolonged at the end of the hypoglycaemic clamp Δ42±24 s returning to baseline at recovery. Clot lysis further increased at day 1 (Δ71±44 s) and day 7 (Δ67±30 s) (Figure 8-1).

In nondiabetic subjects, clot lysis times decreased at the end of the euglycaemic clamp from Δ146±33 (p=0.02 versus baseline). Unlike diabetic subjects, there were no changes in clot lysis times in the hypoglycaemic arm in nondiabetic subjects.

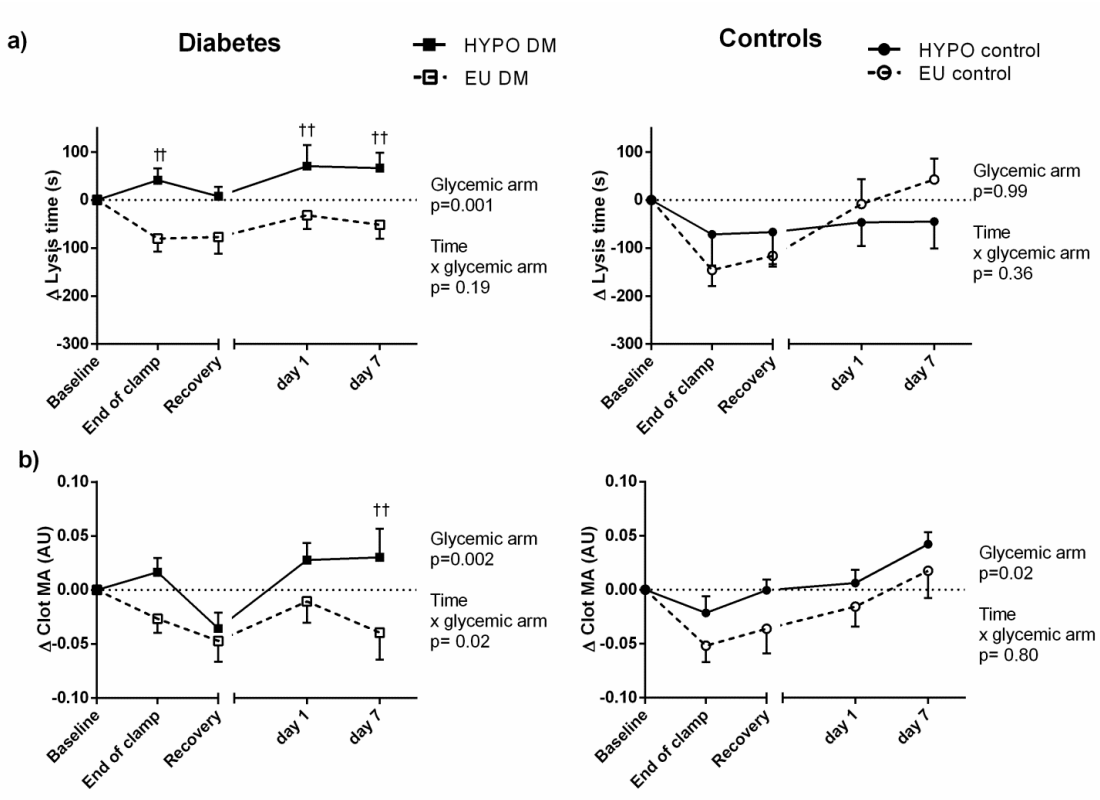

Figure 8-1: Effect of euglycaemia and hypoglycaemia on clot density and clot lysis times by turbidimetric and lysis assay

Diabetic euglycaemia (EU)- open square, diabetic hypoglycaemia (HYPO)- closed square, nondiabetic euglycaemia- open circle, nondiabetic hypoglycaemia - closed circle.† p<0.05 †† p<0.01 euglycaemia versus hypoglycaemia at equivalent time points * p<0.05 versus baseline, ** p<0.01 versus baseline. Differences between glycaemic arm and the interaction between glycaemic arm and time by a mixed model with repeated measures are shown. Data mean (SE). Abbreviations: AU arbitrary units, MA maximum absorbance.

8.4.4.2 Clot Maximum Absorbance

In diabetic subjects, hypoglycaemia increased clot maximum absorbance, peaking at day 7 (Δ from baseline 0·030±0.016 AU), but decreased after euglycaemia (Δ –0·039±0·025 AU, p=0·007). In nondiabetic subjects, clot maximum absorbance decreased at the end of the euglycaemic clamp (Δ-0.05±0.01 AU) but did not change significantly at the end of hypoglycaemia (Δ-0.02 ±0.05 AU) (Figure 8-1). There was a significant difference in the responses between the glycaemic arms (p=0.02).

8.4.5 Scanning Electron Microscopy

8.4.5.1 Fibrin Diameter

Fibrin diameter did not change significantly during euglycaemia or hypoglycaemia in the nondiabetic group (Figure 8-2). In the diabetic subjects, there were no changes in fibrin fibre diameter during euglycaemia, but this increased from Δ from baseline 28±2mm at day 1 and 30±3mm at day 7 following hypoglycaemia ($p<0.0001$ for time). There was a significant difference between glycaemic arms ($p<0.0001$) and the interaction between time and glycaemic arm ($p<0.0001$ by two way repeated measures ANOVA).

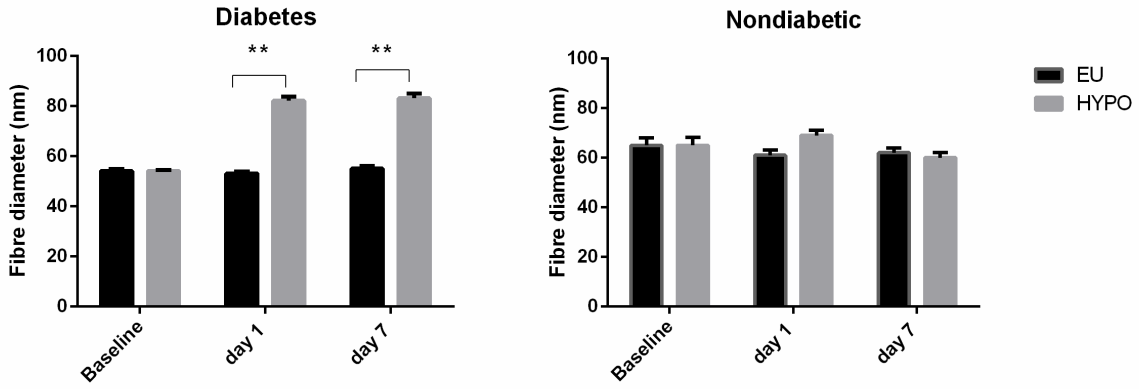

Figure 8-2: Fibre thickness following euglycaemia versus hypoglycaemia at baseline, day 1 and day 7 in diabetic and nondiabetic subjects

Fibrin thickness following euglycaemia (EU) (black) versus hypoglycaemia (HYPO) grey. Fibre thickness was measured for a total number of 160 fibres (40 fibres measured from 4 different clot areas at each time point) ** $p<0.01$ by paired t-test between euglycaemia versus hypoglycaemia Data mean (SE)

8.4.5.2 Fibrin Network Density

In the nondiabetic group, fibre network density decreased Δ 1.82±0.27 fibres/μm^2 at day 1 after euglycaemia ($p<0.0001$), and the fibrin network density increased from Δ 1.15±0.28 fibres/μm^2 at day 7 following the hypoglycaemic clamp ($p<0.01$) (Figure 8-4).

In the diabetes group, the fibre network density decreased Δ-1.68±0.24 fibres/μm^2 at day 7 following euglycaemia ($p<0.001$), whilst following hypoglycaemia, there was an increase in the fibre network density Δ 0.53±0.36 at day 1 fibres/μm^2 and Δ1.43±0.24 fibres/μm^2 at day 7 ($p<0.01$) (Figure 8-4). There were significant differences between the glycaemic arms and the interaction between glycaemic arm and time in both the diabetic and nondiabetic groups (both $p<0.0001$). Examples of scanning electron micrographs of pooled fibrin clots are shown in Figure 8-3.

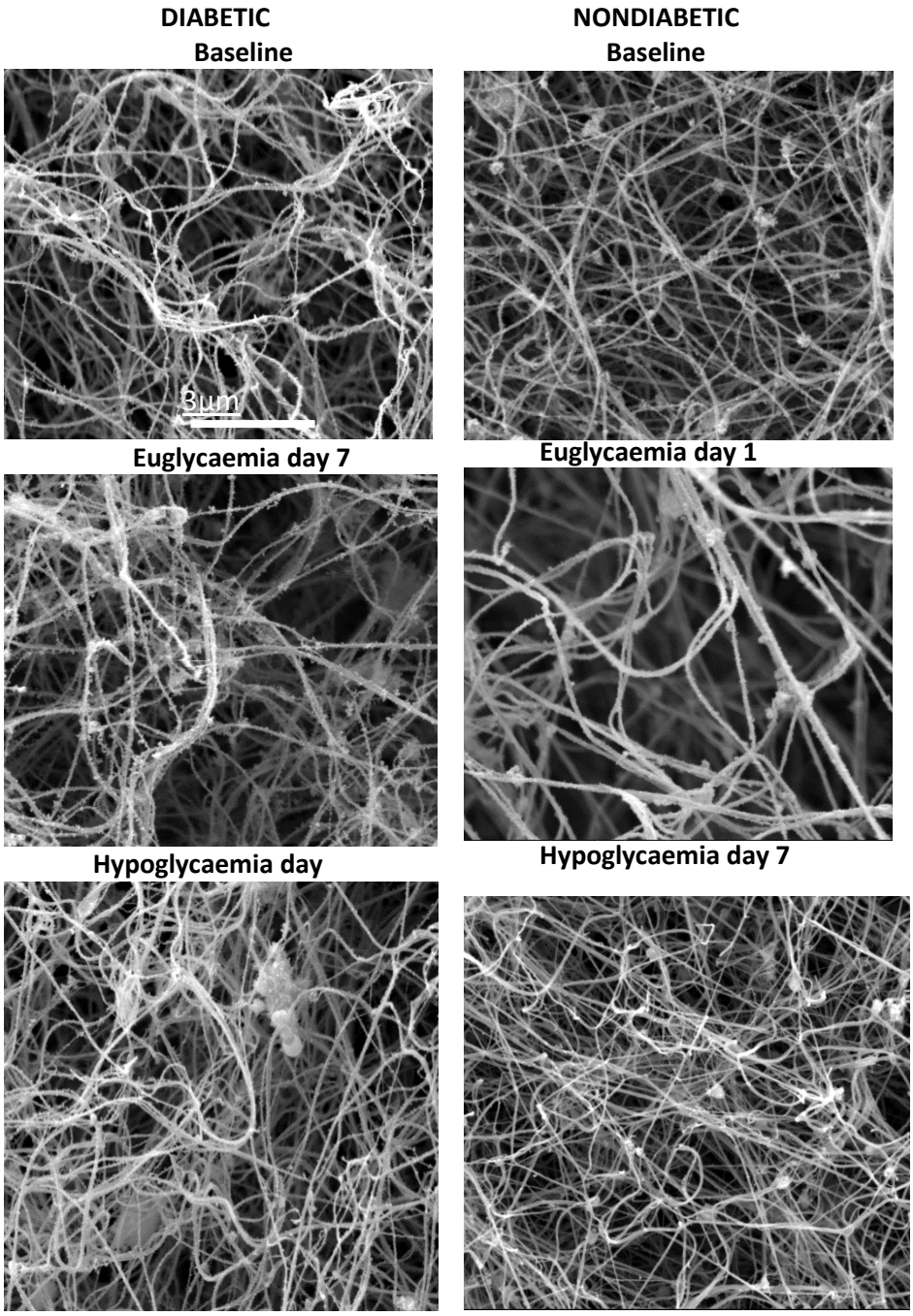

Figure 8-3: Scanning electron micrographs of fibrin clots following euglycaemia versus hypoglycaemia in diabetic and nondiabetic subjects

Visualisation of *ex vivo* fibrin clots from pooled plasma samples in diabetic (n=10) and nondiabetic (n=10) subjects. There is a decrease in the fibre network following euglycaemia in both groups as opposed to an increase in the network density following hypoglycaemia at day 7.

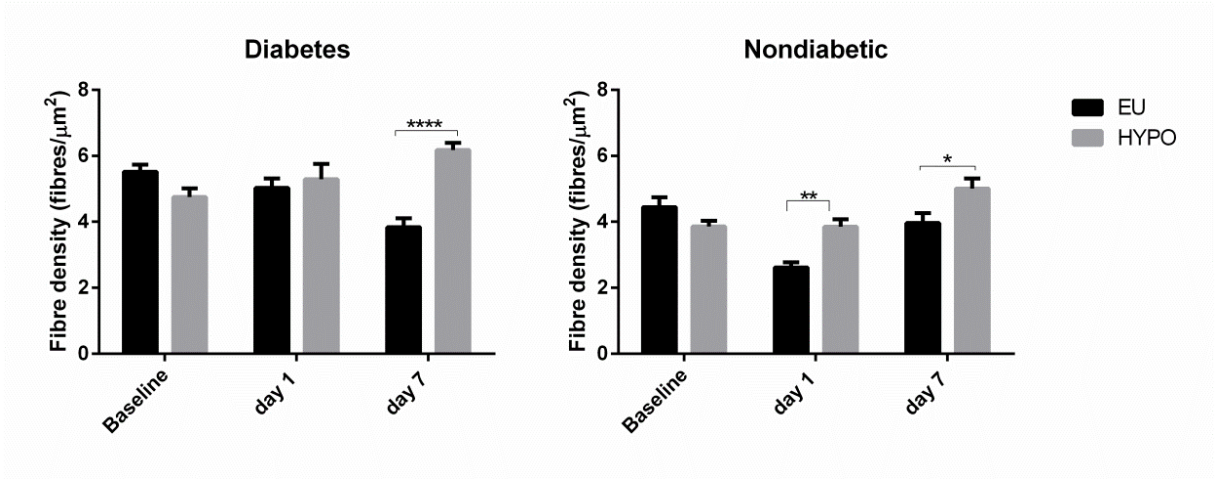

Figure 8-4: Fibrin network density following hypoglycaemia versus euglycaemia in diabetic and nondiabetic subjects

Fibrin fibre density following euglycaemia (EU) (black) versus hypoglycaemia (HYPO) grey. * p<0.01, ** p<0.01, ****p<0.0001 between euglycaemia versus hypoglycaemia. Data mean (SE)

8.4.6 Coagulation Proteins

8.4.6.1 Fibrinogen

Euglycaemic and hypoglycaemic clamps had different effects on plasma fibrinogen levels in the diabetic group (p=0.05) (Figure 8-5). Fibrinogen did not change during euglycaemia but increased Δ 0.20±0.10 mg/mL at day 1 and Δ0.85±0.69 mg/mL at day 7 following hypoglycaemia. In the nondiabetic group, fibrinogen tended to decrease at the end of the clamp and recover similarly in both glycaemic arms (Figure 8-5).

8.4.6.2 Plasminogen Acivator-1

In diabetic subjects, PAI-1 decreased significantly from baseline Δ -811 ±204 pg/mL at the end of the euglycaemic clamp (p<0.001 versus baseline) and remained lower at day 7. PAI-1 decreased to a similar extent during the hypoglycaemic clamp (Δ-888±256, p=0.001 versus baseline) with no

significant differences detected between the glycaemic arms (p=0.99). PAI-1 also decreased in the nondiabetic group during euglycaemia and hypoglycaemia (both p<0.01 for time) with again no significant difference between the glycaemic arms (p=0.66). PAI-1 decreased to a similar extent in both diabetic and control groups during euglycaemia and hypoglycaemia (Figure 8-5).

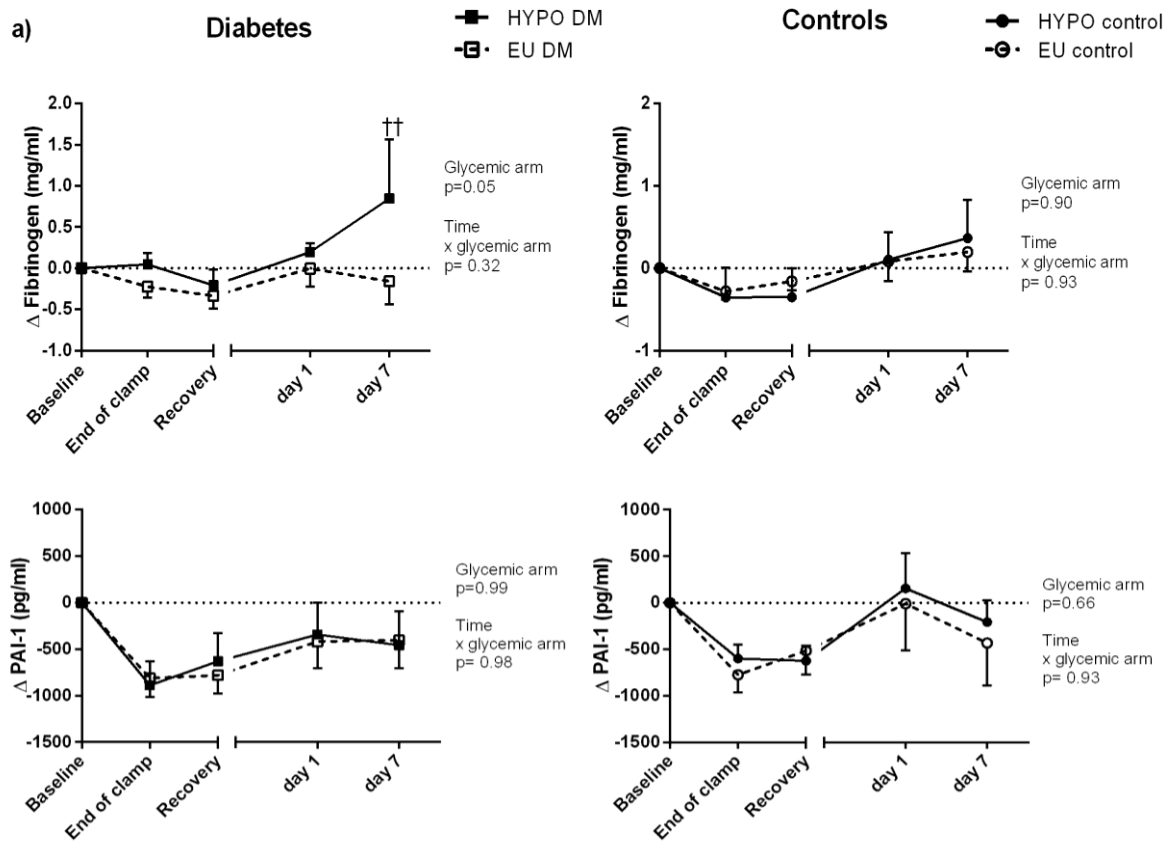

Figure 8-5: Effect of euglycaemia and hypoglycaemia on coagulation proteins in diabetic and nondiabetic subjects

Diabetic euglycaemia (EU)-open square, diabetic hypoglycaemia (HYPO)-closed square, nondiabetic euglycaemia-open circle, nondiabetic hypoglycaemia-closed circle. † p<0.05 †† p<0.01 euglycaemia versus hypoglycaemia at equivalent time points * p<0.05 versus baseline, ** p<0.01 versus baseline. Differences between glycaemic arm and the interaction between glycaemic arm and time by a mixed model with repeated measures are shown. Data mean (SE). Abbreviations: C3, complement component C3, PAI-1,plasminogen activator 1,

8.4.7 Inflammatory Proteins

8.4.7.1 hsCRP

In diabetic subjects, hsCRP decreased following euglycaemia at day 7 (log hsCRP - Δ0.11±0.01 p=0.02 versus baseline) but tended to increase following hypoglycaemia (log hsCRP Δ0.06±0.01 at day 7) and this was significant between the glycaemic arms at day 7 (p=0.002) and there was also a significant interaction between time and glycaemic arm (p=0.04). In nondiabetic subjects, hsCRP increased similarly at day 1 following euglycaemia and hypoglycaemia, with no significant differences between the glycaemic arms (Figure 8-6).

8.4.7.2 Complement Component C3

C3 levels did not change during euglycaemia in the diabetic group but showed a trend towards an increase at day 7 following hypoglycaemia (from 99±5 to 108±4 mg/mL, p=0.10 versus baseline) (Figure 8-6). There were significant differences in the change in C3 at day 7 between the glycaemic arms (p=0.02). In the control group, C3 levels were similar during euglycaemia and hypoglycaemia and did not change significantly.

8.4.7.3 Interleukin 6, interleukin 8 and interleukin 10

In the diabetic group, IL-6 did not change during the euglycaemic arm, although there was a trend towards an increase in IL-6 following hypoglycaemia at day 7. In the nondiabetic group, IL-6 was higher at day 1

following euglycaemia but did not change significantly following hypoglycaemia (Table 8-3).

In the diabetic group, IL-8 demonstrated a trend to increase from 6.10±0.84 pmol/L to 8.64±1.33 pmol/L at day 7 following hypoglycaemia are compared with no change after euglycaemia. There was a significant interaction between the glycaemic arm and time (p= 0.03). IL-10 tended to increase during euglycaemia in the diabetic group (Table 8-3) and also increased following hypoglycaemia at day 7 (p=0.04 for time). IL-8 and IL-10 did not change within the euglycaemic or hypoglycaemic arm in nondiabetic individuals (Table 8-3).

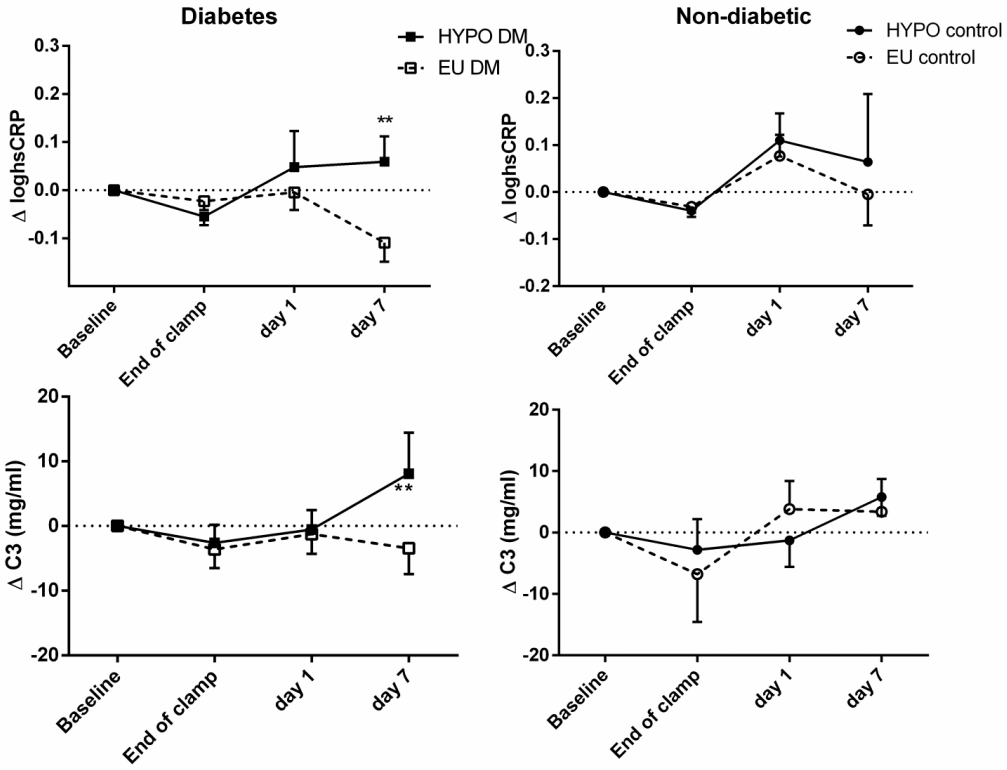

Figure 8-6: Changes in hsCRP and C3 following euglycaemia versus hypoglycaemia in diabetic and nondiabetic subjects

Data mean (SE) ** p<0.01 euglycaemia vs hypoglycaemia at equivalent time points

Table 8-3: Inflammatory cytokines following euglycaemia and hypoglycaemia arms in diabetic and nondiabetic subjects

IL-6 interleukin 6, IL-8 interleukin 8, IL-10 interleukin 10 * p<0.05 versus baseline. Data mean±SE

	Baseline	End of clamp	Day 1	Day 7
IL-6 (pg/mL)				
Diabetic				
Euglycaemia	3.99±1.22	4.08±0.81	4.36±0.58	3.97±0.86
Hypoglycaemia	4.93±0.96	4.97±0.65	5.02±0.79	5.30±2.43
Nondiabetic				
Euglycaemia	3.34±0.72	3,73±0.94	5.07±0.81*	4.75±0.95
Hypoglycaemia	3.62±1.37	3.89±1.22	3.40±0.79	4.03±1.01
IL-8 (pg/mL)				
Diabetic				
Euglycaemia	5.81±1.18	4.87±0.91	7.74±1.25	6.65±1.18
Hypoglycaemia	6.10±0.84	6.77±1.30	5.92±0.75	8.23±1.33
Nondiabetic				
Euglycaemia	4.56±0.85	4.80±0.80	4.97±0.80	4.86±0.86
Hypoglycaemia	3.59±0.59	5.01±1.58	4.19±0.99	4.73±1.03
IL-10 (pg/mL)				
Diabetic				
Euglycaemia	1.37±0.32	1.54±0.22	1.68±0.23	1.92±0.33
Hypoglycaemia	1.65±0.29	1.88±0.21	1.53±0.25	2.11±0.35
Nondiabetic				
Euglycaemia	1.37±0.22	1.37±0.18	1,52±0.27	1.62±0.25
Hypoglycaemia	1.43±0.13	1.71±0.29	1.50±0.22	1.72±0.22

8.5 Discussion

A number of novel findings have emerged from this study : 1) hypoglycaemia was associated with the formation of fibrin clots that are resistant to fibrinolysis, with these effects occurring acutely and persisting

for at least a week; 2) hypoglycaemia-induced changes in the fibrin network structure which were more prominent in diabetic subjects compared to nondiabetic subjects; and 3) increased levels of fibrinogen and C3 are likely to contribute to changes in fibrin clot structure and lysis, whereas PAI-1 does not appear to be a mediator.

The antithrombotic, profibrinolytic and anti-inflammatory response of euglycaemic hyperinsulinaemia is in agreement with previous human studies (Chaudhuri et al., 2004). In this study hyperinsulinaemic euglycaemia acutely reduced clot density, as shown by falls in clot maximum absorbance in diabetic and nondiabetic groups. Such effects may have two mechanistic explanations. The first is related to the dilutional effect of infusing fluid and insulin, whereas the second mechanism is related to the ability of insulin to suppress fibrinogen levels (Barazzoni et al., 2003). The attenuated decrement in fibrinogen and clot density in diabetic individuals may reflect insulin resistance or altered hepatic fibrinogen synthesis in T2DM subjects (Barazzoni et al., 2003). Notably in this study, hyperinsulinaemic euglycaemia was associated with a reduced fibrin network density up to a week later. Previous work has shown that the treatment of T1DM patients with continuous subcutaneous insulin therapy increases clot porosity, regardless of the effect of glycaemic control (Jörneskog et al., 2014).

Hyperinsulinaemic euglycaemia also decreased clot lysis times. This may be mediated by decreases in PAI-1 levels, consistent with earlier studies

conducted in individuals without diabetes (Landin et al., 1991). An attenuated rise in PAI-1 has also been observed in patients with diabetes with myocardial infarction who were infused with intravenous insulin for 48 hours to maintain euglycaemia (Chaudhuri et al., 2004). However, other studies have suggested that insulin induces PAI-1 gene expression and increases PAI-1 plasma levels in insulin resistant and normal subjects (Stegenga et al., 2006). The reasons for these discrepancies in these observations are unclear but may be because higher doses of insulin were infused in the present study. The mean plasma insulin level in the study by Stegenga and colleagues was 400pmol/L which is 1.5 times lower than our clamps.

Insulin has anti-inflammatory effects that are independent of its glucose lowering effects. Diabetic myocardial infarction patients who received continuous insulin infusion to maintain euglycaemia for 48 hours showed an attenuated increase in CRP (Chaudhuri et al., 2004), a finding that has been replicated by other groups (Wong et al., 2004). Previous studies have shown decreases in sCD40L, cell adhesion molecules, and IL-6 during hyperinsulinaemic euglycaemia in T1DM and nondiabetic subjects (Gogitidze Joy et al., 2010; Wright et al., 2010). Insulin induces the expression of NO synthetase in the endothelium via the PI3/Akt pathway (Aljada and Dandona, 2000). Insulin also inhibits NFκB and MCP-1 expression in human aortic endothelial cells (Aljada et al., 2001). The onset of action of the anti-inflammatory effect of insulin has been shown to be

rapid, occurring within two hours (Dandona et al., 2009). Relatively few studies have explored the effects of euglycaemic hyperinsulinaemia beyond the period of acute administration. In an endotoxaemic model, rats that were administered with subcutaneous insulin showed reduced inflammatory responses in serum IL-6, TNFα and IL1-β for up to 7 days (Jeschke et al., 2004). Interestingly, in the present study, the anti-inflammatory cytokine IL-10 was increased for up to 7 days following hyperinsulinaemic euglycaemia and hypoglycaemia in the diabetic group, but not in nondiabetic subjects. Insulin has been shown to specifically antagonise proinflammatory signalling at the level of the adipocyte (Andersson et al., 2007) and this may underlie the more pronounced anti-inflammatory effects in the diabetic group.

With regards to the acute effects of hypoglycaemia, the results of this study differ from those of previous studies which have shown that hypoglycaemia induced by insulin resulted in the enhancement of fibrinolysis, with decreases in PAI-1 activity and increases in tPA (Dalsgaard-Nielsen et al., 1982; Fisher et al., 1991). Previous authors have not included a euglycaemic control arm and hence this may reflect profibrinolytic effects of insulin.

A novel and intriguing observation is the ongoing effect of hypoglycaemia on clot density that persisted 7 days after hypoglycaemia. Hypoglycaemia increased fibre density in both diabetic and nondiabetic subjects. However, hypoglycaemia only increased fibre thickness in subjects with T2DM but

the nondiabetic subjects, with no change during hyperinsulinaemic euglycaemia. This may be explained by the increase in fibrinogen which did not occur in the nondiabetic subjects. There is evidence that dysregulated fibrinogen synthesis occurs in patients with T2DM (Tessari et al., 2006).

Hypoglycaemia had a hypofibrinolytic effect for at least 7 days in diabetic subjects. Clot lysis times increased for up to 7 days after hypoglycaemia and were prolonged to a greater extent in diabetic compared to nondiabetic subjects. Denser fibre networks were observed in both groups and an increase in fibre thickness was observed in diabetic subjects but not nondiabetic subjects. These differences cannot be explained by changes in PAI-1 which was similarly depressed in the euglycaemic arm. However, levels of complement component C3 were increased, and this has been associated with increased resistance to clot lysis, particularly in clots composed of diabetic fibrinogen compared to those in healthy subjects (Hess et al., 2012a),

The mechanisms driving sustained changes in fibrin clot density and fibrinolysis following hypoglycaemia are unclear. However, it can be hypothesised that this may be driven by subclinical inflammation, which exhibited similar trends to fibrin clot properties. There were modest rises in markers of subclinical inflammation (hsCRP, IL-6, IL-8, C3 and fibrinogen) following hypoglycaemia up to day 7 in diabetic subjects and this did not occur in nondiabetic individuals. The importance of C3 as a contributor to hypofibrinolysis has recently been confirmed in a T2DM cohort (Hess et al.,

2014). Aggravation of atherothrombosis has also been shown to occur follow AMI in mouse models, where it is thought that sympathetically mediated rise in the mobilisation of haemopoetic stem cells may promote proinflammatory responses in the weeks after the event (Dutta et al., 2012). Furthermore, chronic hyperglycaemia has been shown to prime the innate immune system for an exaggerated inflammatory response to physiological challenges, which may explain why such changes occurred following hypoglycaemia in T2DM subjects but not the nondiabetic subjects (Gyurko et al., 2006).

The present study had some limitations as the number of participants, while adequate to demonstrate changes in clot density, may be short of power to identify subtle differences between diabetic and nondiabetic groups. It was necessary to administer high doses of insulin (120 mU/m2/min in the morning and 240 mU/m2/min afternoon) in order to induce hypoglycaemia in insulin resistant individuals and it was intended as part of the study design to give equivalent amounts of insulin between the glycaemic arms and between groups. Such supraphysiological doses are markedly higher than during routine clinical use and this might artefactually limit prothrombotic and proinflammatory changes due to hypoglycaemia. Another potential limitation is the slightly higher number of males in the diabetic group. For ethical reasons, hypoglycaemia was not induced in individuals with known CVD, although studies have shown that intensive glucose lowering exerts greater cardiovascular benefit in patients

with low cardiovascular risk compared with those with established CVD (Holman et al., 2008; Reaven et al., 2009).

This data supports a causal role for hypoglycaemia in increasing cardiovascular mortality, mitigating the benefits of intensive glycaemic control, and intensive insulin therapy. This is of clinical relevance in several contexts; in the use of intensive insulin therapy in critically care and during intensive glycaemic control in T2DM patients. In the NICE-SUGAR study, critically ill patients randomised to intensive insulin therapy to achieve tight glycaemic targets exhibited excess mortality (Finfer et al., 2009). *Post hoc* analysis showed a strong association between death and hypoglycaemia that persisted despite adjusting for confounding factors. In the study, the median time to death from the occurrence of a hypoglycaemic event was 7 days for moderate hypoglycaemia and 8 days for severe hypoglycaemia, and this time course closely matches the findings from this study of a worsening thrombotic profile one week after hypoglycaemia (Finfer et al., 2012).

An alternative explanation for the lack of improvement in mortality with intensive insulin therapy in some studies is that the insulin dose was not sufficiently high to achieve beneficial anti-inflammatory, antithrombotic effects. In the study by van den Berghe et al. (2001) which demonstrated a reduction in mortality and morbidity in critically care patients receiving intensive insulin therapy, significantly higher doses were used (71 units/day versus 30-50 units/day) compared with other studies that did not

demonstrate any benefit (Brunkhorst et al., 2008; Finfer et al., 2009). The dose and also the duration of insulin infusion may be relevant, as insulin given for three or more days reduced mortality in medical intensive care patients but not in those who received insulin for less than 3 days (Van den Berghe et al., 2006).

The current findings provide a putative mechanism by which hypoglycaemia may increase the risk of cardiovascular death in the weeks and months following an episode of severe hypoglycaemia during intensive glycaemic control in patients with T2DM. The prothrombotic and proinflammatory effects of hypoglycaemia, although modest, may be sufficient to predispose individuals at high risk to an acute coronary event. In the Northwick Park study, fibrinogen was Δ0.35 g/L higher (2.90 g/L versus 3.25 g/L) in men who subsequently died of CVD in the following 5 years (Meade et al., 1986), differences which are of a similar magnitude to those observed in this study.

In conclusion, it has been shown that hypoglycaemia has acute prothrombotic effects in both nondiabetic and T2DM subjects. It has also been demonstrated that the impact of moderate, short-lived hypoglycaemia is maintained for at least 7 days after the event, with adverse effects on fibrin clot properties, fibrinolysis and subclinical inflammation, and these effects were more prominent among individuals with T2DM. These effects oppose the beneficial anti-thrombotic, anti-inflammatory and fibrinolytic properties of intravenous insulin

administered under euglycaemic conditions. Thus potential mechanisms have been identified whereby hypoglycaemia could increase the risk of cardiovascular events during and after an episode, thereby counteracting the benefits of intensive glycaemic control.

Chapter 9 - Final Discussion

9.1 Summary of Findings

The aim of this work was to explore the potential mechanism by which hypoglycaemia can increase the risk of SCD in patients with T2DM in the context of intensive glycaemic therapy. SCD is a multifactorial process involving distal factors promoting atherogenesis, transition to an inflammatory plaque, development of acute coronary thrombosis, and a transient trigger leading to fatal arrhythmia and sudden death. In this work, potential mechanisms have been demonstrated that can increase the risk of SCD using ambulatory and experimental models of hypoglycaemia in T2DM patients. The main findings of this work can be summarised into four main points:

1. Hypoglycaemia has acute proarrhythmic effects, as manifest by altered autonomic balance and abnormal repolarisation. Hypoglycaemia is also prothrombotic, acutely due to enhanced platelet reactivity, and downstream of the event due to altered fibrin clot properties and hypofibrinolysis.

2. There are differential cardiovascular effects of hypoglycaemia dependent on the state of sympathoadrenal activation.

3. Hyperinsulinaemic euglycaemia has beneficial cardiovascular effects that may be opposed by hypoglycaemia

4. The adverse cardiovascular effects of hypoglycaemia on fibrin clot properties and inflammation are enhanced in T2DM patients

9.1.1 Hypoglycaemia Predisposes to Sudden Cardiac Death via Proarrhythmic and Prothrombotic Pathways

Acute hypoglycaemia may have proarrhythmic effects via changes in sympathovagal balance and prolongation of cardiac repolarisation. Here we demonstrate transient vagal withdrawal followed by vagal reactivation during sustained hypoglycaemia in T2DM individuals. QTc is prolonged during acute hypoglycaemia in both spontaneous and experimental settings. The combined electrophysiological effects may serve as a transient trigger for supraventricular and ventricular arrhythmias via abnormal automaticity and triggered activity (Fig 9-1).

The balance between prothrombotic and profibrinolytic factors dictates the propensity for coronary thrombus formation following plaque rupture. Here it has been demonstrated that acute hypoglycaemia causes increases in platelet activation and platelet reactivity. The present study also demonstrated that hypoglycaemia has sustained adverse effects on clot density and depresses fibrinolysis for up to seven days after an episode that are more pronounced in T2DM subjects. Figure 9-1 illustrates a revised hypothetical model which integrates the findings from both studies.

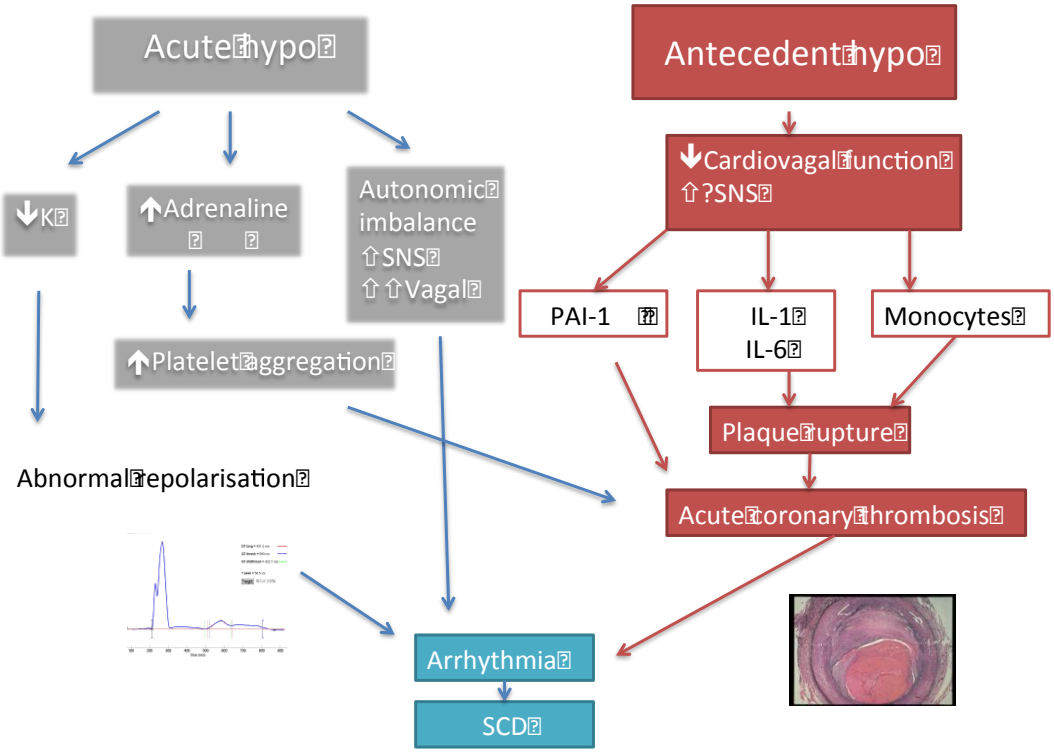

Figure 9-1: Revised model of potential mechanisms of hypoglycaemia-related SCD

Acute effects of hypoglycaemia with associated sympathoadrenal activation and hypokalaemia can cause abnormal repolarisation, increasing the risk of ventricular arrhythmias. Autonomic balance can further enhance this arrhythmic risk. Acute hypoglycaemia is also prothrombotic and enhances platelet aggregation via adrenaline release. However, hypoglycaemia may have longer lasting effects on the cardiovascular system in the days and weeks following an event. A suppression of cardiovagal function leading to unrestrained sympathetic nervous system activity (SNS) can mediate proinflammatory changes and atherothrombotic changes. This might increase the propensity for plaque rupture, acute coronary thrombosis, and SCD.

9.1.2 Differential Cardiovascular Effects of Hypoglycaemia Depend on the state of Sympathoadrenal Activation

In the ambulatory study, daytime hypoglycaemia was associated with a transient cardioacceleration and vagal withdrawal. During nocturnal prolonged hypoglycaemia where sympathoadrenal activation was weak, there was a phasic cardiac response, whereby initial vagal withdrawal and sympathetic activation was followed by excessive vagal counteraction that led to relative bradycardia. Therefore the cardiovascular effects of

hypoglycaemia may significantly differ according to the state of sympathoadrenal activation (Figure 9-2).

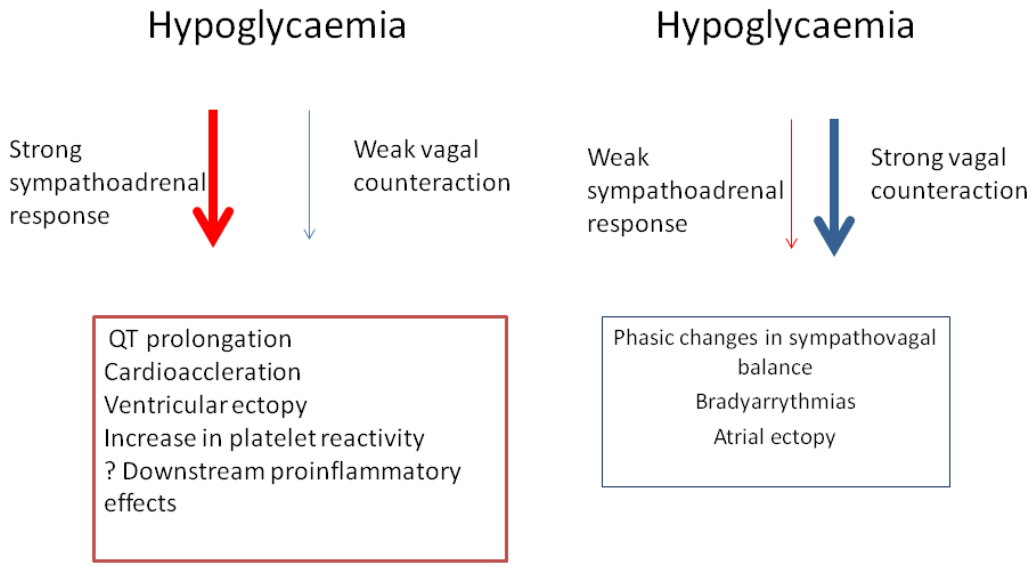

Figure 9-2: Differential cardiovascular effects of hypoglycaemia depending on the strength of sympathoadrenal activation

In situations where there is a strong sympathoadrenal response, as in the case of symptomatic daytime hypoglycaemia or in populations, QT prolongation and increases in platelet reactivity are likely to occur due to catecholamine release. This is in contrast with situations where there is diminished sympathoadrenal activation and high vagal tone, such as in nocturnal hypoglycaemia or in those with hypoglycaemia unawareness, where phasic changes in sympathovagal balance occur.

Furthermore, there is considerable heterogeneity in counter-regulatory responses to hypoglycaemia in T2DM patients. In patients with advanced T2DM and long duration on insulin, sympathoadrenal responses to hypoglycaemia may be diminished. They are more likely to experience frequent hypoglycaemia but these episodes are also more likely to be asymptomatic. These hypoglycaemic episodes are likely to be associated with blunted sympathoadrenal responses and increased vagal counteraction (see Figure 9-2).

In contrast, T2DM patients with early disease and intact counter-regulatory responses are at lower risk of clinical hypoglycaemia. Hypoglycaemic episodes should be infrequent, except under iatrogenic circumstances, such as during rapid intensification of glucose therapy (Gerstein et al., 2008). These episodes are therefore likely to be associated with strong sympathoadrenal responses. Acute sympathoadrenal activation results in cardiac repolarisation prolongation and cardioacceleration. Acute releases of catecholamines may promote increases in platelet aggregation, as shown in experimental models of hyperinsulinaemic hypoglycaemia. What is unknown is whether downstream proinflammatory, prothrombotic changes after an episode, as observed in our data, are related to the strength of sympathoadrenal activation at the time of the event. It was hypothesised that hypoglycaemia, analogous to other stressors, results in a rise in splenic mobilisation proinflammatory cells as a consequence of heightened sympathetic activity (Dutta et al., 2012).

The impact of differential sympathoadrenal responses to hypoglycaemia on cardiovascular outcomes need to be considered, particularly in the interpretation of relationships between reported and asymptomatic hypoglycaemia and cardiovascular events in clinical trials (Seaquist et al., 2012)

9.1.3 Beneficial Cardiovascular Effects of Hyperinsulinaemic Euglycaemia may be mitigated by Hypoglycaemia

The original aim of the euglycaemic arm was to serve as a control for the effects of insulin. However, this has shed light on the actions of insulin in both normal and T2DM subjects. It has been confirmed that hyperinsulinaemic euglycaemia has acute anti-aggregatory, antithrombotic properties when administered intravenously and may have sustained anti-inflammatory effects some days later. These potentially beneficial effects of insulin are opposed by the adverse effects of hypoglycaemia. For example, in the case of platelet aggregation, adrenaline neutralised the anti-aggregatory effects of hyperinsulinaemia blocking the insulin receptor β subunit and affecting G protein coupled pathways (Ferreira et al., 2003). This is of importance when understanding the risk–benefit balance of intensive glycaemic control within clinical settings.

9.1.4 Adverse Cardiovascular Effects of Hypoglycaemia on Fibrin Clot Properties and Inflammation are enhanced in Type 2 Diabetes

This work has highlighted a number of similarities between T2DM and nondiabetic subjects in responses to hypoglycaemia. For example, there was increase in platelet macroaggregation and initial cardioacceleration to acute hypoglycaemia in both groups. However, there were also differences between T2DM and nondiabetic subejcts. In diabetic patients, vagal reactivation occurred during sustained hypoglycaemia leading to cardiac

slowing which did not occur in nondiabetic subjects. Diastolic blood pressure declined to a smaller extent during hypoglycaemia in T2DM compared with nondiabetic subjects. In earlier discussions, it was hypothesised that the differential responses may conceivably relate to differences in baroreceptor and haemodynamic function. It was reasoned that the abrupt increases in pulse pressure during hypoglycaemia in diabetic subjects can increase baroreceptor firing and vagal activation (Bell, 2013; Chapleau et al., 1988), however, these observations are in need of further investigation.

In addition, there were increases in fibrin clot density and hypofibrinolysis up to seven days after hypoglycaemia in T2DM patients compared with no change in nondiabetic controls. This was mirrored by increases in markers of subclinical inflammation for at least seven days after hypoglycaemia in diabetic subjects but not nondiabetic subjects. T2DM subjects in the clamp study did not have overt CVD; however, they did exhibit higher levels of subclinical inflammation, higher levels of prothrombotic markers including higher platelet reactivity, increased fibrinogen and PAI-1, as well as denser clots that were resistant to fibrinolysis. There are suggestions that chronic hyperglycaemia may prime the innate system to exaggerate inflammatory changes in response to physiological challenges, potentially explaining why adverse effects of hypoglycaemia on inflammation and thrombosis may be enhanced in T2DM (Gyurko et al., 2006). However, this remains speculative. The precise mechanisms may be challenging to disentangle in

human studies alone, and might require complementary studies using animal models.

9.2 Future Directions

The current research poses a number of questions and the aim of future work should be to provide deeper mechanistic insight into cardiovascular effects of hypoglycaemia and to translate the current findings into areas of potential therapeutic benefit. Several potential avenues for exploration will be described below.

9.2.1 Mechanistic Studies

9.2.1.1 Effect of Hypoglycaemia on Inflammatory Pathways and the Relationship with Thrombosis

In this study it has been shown that hypoglycaemia is capable of inducing sustained changes in fibrin clot properties, increases in clot density, and reduced clot lysis up to seven days later in T2DM patients. Mechanisms underlying this are uncertain but it is hypothesised that subclinical inflammation, which increased after hypoglycaemia, may underlie the worsening thrombotic profiles. To investigate this hypothesis, collaborations with experts in immune and vascular biology have been established and further preliminary data has been developed demonstrating the effects of hypoglycaemia on monocyte function. This has led to the successful funding of a project currently under way to investigate the effects of hypoglycaemia on inflammatory pathways.

In this experimental study, changes in immune cell function and inflammatory cytokines following an episode of insulin-induced hypoglycaemia will be probed using a low-dose endotoxin challenge model in healthy volunteers. Downstream effects will be investigated at seven and thirty days. In the current study, it was more difficult to demonstrate more modest but clinically important changes with a small sample size, and the priming effects of endotoxin may help to elucidate changes in subclinical inflammation following hypoglycaemia by enhancing the immune response.

9.2.1.2 Effects of Hypoglycaemia on Atherosclerotic Plaques

Results from interventional trials have shown that intensive glycaemic control had neutral or favourable effects on those with a low atherosclerotic burden; however, in patients with a high cardiovascular burden, intensive control was associated with adverse cardiovascular outcomes (Reaven et al., 2009). Although the electrophysiological effects of hypoglycaemia in patients with T2DM and high cardiovascular risk an in ambulatory setting were studied, it was impossible to examine thrombotic mechanisms using experimental hypoglycaemia in this population due to safety and ethical considerations. It would be of value to examine the effect of hypoglycaemia on the atherosclerotic plaque, particularly concerning whether this could lead to the transition to unstable plaques; however, this would be challenging to examine in human models. Therefore, in collaboration with other groups with expertise in murine

models of atherosclerosis and animal models of hypoglycaemia, a proposed study is under way to investigate the effect of hypoglycaemia on changes in atherosclerotic plaques. In the planned study, hypoglycaemia will be induced in ApoE-/- mice and changes in plaque composition will be quantified along with changes in inflammatory cytokines and compared with euglycaemic counterparts.

9.2.1.3 Physiology of Nocturnal Hypoglycaemia and Relationship with Autonomic Control

The frequency of asymptomatic nocturnal hypoglycaemia among insulin-treated T2DM patients was much higher than expected. In the ambulatory study, an undulating pattern of glucose was demonstrated during prolonged nocturnal hypoglycaemia that has previously been reported only in a few studies in T1DM patients (Matyka et al., 1999). The physiological mechanisms underlying this are unclear. Further we reported phasic responses in cardiac autonomic function that parallel changes in glucose during spontaneous nocturnal hypoglycaemia.

To corroborate findings from the ambulatory study, a two-part human experimental study is proposed to investigate glucose, counterregulatory and cardiac autonomic responses to nocturnal hypoglycaemia in a laboratory setting. The advantage is that HRV and blood sampling for determination of catecholamines and electrolytes can be performed at regular intervals. Measurements of blood glucose, as opposed to IG, will

avoid problems of interstitial lag and inaccuracies in the hypoglycaemic range. In the first part of the study, insulin-treated T2DM subjects will be recruited to study counter-regulatory and cardiac autonomic responses during spontaneous nocturnal hypoglycaemia. Given the high frequency of nocturnal hypoglycaemia in the ambulatory cohort of insulin-treated T2DM patients (14 out of 25 subjects), it may be feasible to capture sufficient spontaneous episodes based on two overnight visits per subject. The second part of the study would involve sustained hyperinsulinaemic hypoglycaemia induced overnight with continuous ECG monitoring for assessment of HRV. Pharmacological adrenergic and parasympathetic blockade may be administered at the end of the hypoglycaemic clamp to further probe the interaction between sympathetic and vagal arms. A schematic diagram of the proposed studies is shown in Figure 9-3.

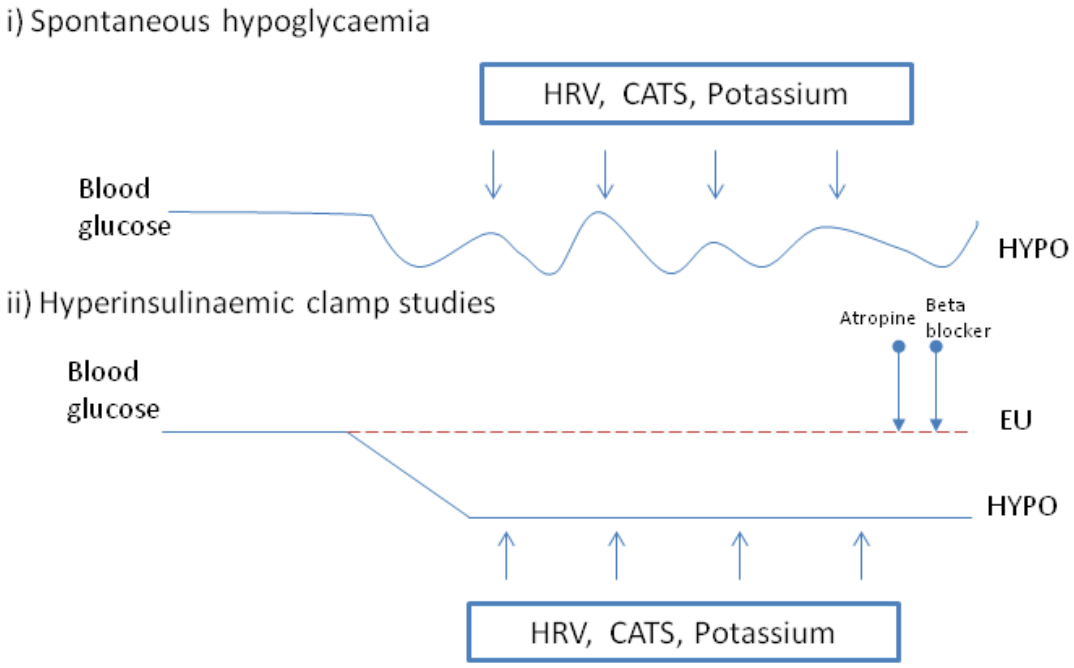

Figure 9-3: Schematic diagram for the proposed studies to investigate autonomic and counter-regulatory responses to nocturnal hypoglycaemia in T2DM subjects

EU-euglycaemia; HYPO-hypoglycaemia, HRV-heart rate variability, CAT- catecholamines (see text for details).

9.2.1.4 Computer Simulation of Cardiac Electrophysiological Changes during Hypoglycaemia in the Presence of Ion Channel Mutations

SCDs are stochastic events, and given that hypoglycaemia is relatively common and arrhythmias and sudden deaths are relatively rare, additional factors must determine the risk of arrhythmias in response to the transient physiological challenge of hypoglycaemia. It has been demonstrated that individuals with a reduced repolarisation reserve displayed highly abnormal repolarisation during spontaneous hypoglycaemia. It is increasingly recognised that genetic or acquired mutations in cardiac ion channels may be responsible for abnormal cardiac repolarisation. For example, it has

been shown that changes to the persistent sodium current in diabetes may be responsible for QT prolongation in diabetes (Lu et al., 2013). Furthermore, low glucose itself has been suggested to affect HERG channel function (Zhang et al., 2003b). The effect of ion channel mutations on ECG waveforms have been simulated using a computerised model based upon human ventricular tissue (K.Q. Wang, 2006). Similar computerised simulations have been applied in other contexts (Xia et al., 2006), such as *in silico* cardiac models which have been applied in risk stratification of LQT1 syndrome patients by predicting complex changes in channel function on the risk of arrhythmic events (Hoefen et al., 2012). One idea currently under development is to simulate physiological effects of hypoglycaemia, with a combination of graded hypokalaemia and sympathoadrenal activation, in the presence of mutated ion channels (SCN5A and HERG) in computerised cardiac models. The impact of hypoglycaemia on accentuating repolarisation abnormalities in the presence of existing ion channel mutations can be explored. Computer models may in future help stratify T2DM patients at high risk of developing arrhythmias during hypoglycaemia, with the possibility of prophylactic measures in the form of medical therapy, ablation or implantable cardiac defibrillators.

9.2.2 Observational Clinical Studies

9.2.2.1 Effect of spontaneous severe hypoglycaemia on thrombotic risk in Type 2 diabetes

In the present work, we have reported increases in platelet reactivity at the time of experimental hypoglycaemia and alteration in fibrin clot properties downstream. However, the transferability of these experimental observations to spontaneous hypoglycaemia in a clinical setting is unknown. Tsujimoto et al. (2014) have examined a retrospective cohort of patients presenting to the emergency room with severe hypoglycaemia and demonstrated similar QT prolongation and hypokalaemia to what we observed experimentally. It would therefore also be of value to investigate thrombotic effects of hypoglycaemia in a multi-centred prospective cohort of T2DM patients presenting with severe hypoglycaemia to the emergency services. The main outcome measures would include indices of platelet reactivity, determined using point-of-care devices at presentation with hypoglycaemia. Furthermore, changes in inflammation, fibrin clot properties and platelet reactivity seven days later may be determined and compared against a matched T2DM ambulatory cohort without a history of hypoglycaemia. However, this study may be logistically challenging to identify and recruit individuals presenting to the emergency services in a timely manner.

9.2.2.2 Changes in cardiac autonomic function following hypoglycaemia in critically ill patients

The second study area of interest is concerned with the long term effects of hypoglycaemia in critically ill patients. During trials of intensive glycaemic control, the increase in mortality was not apparent during the period of intensive care stay but instead rose post ICU/hospital discharge and remained evident at 90 days (Deane and Horowitz, 2013; Finfer et al., 2012). One hypothesis following from the experimental observations in this study is that depressed cardiac autonomic function could mediate long term increases in cardiovascular mortality. It has been established that impaired cardiac autonomic function and HRV predict subsequent mortality up to 60 days in patients who have experienced multiorgan dysfunction (Schmidt et al., 2005; Schmidt et al., 2008). Therefore, it is hypothesised that hypoglycaemia during intensive glycaemic therapy may further impair cardiac autonomic function in these patients leading to an increase in arrhythmic death in the short to medium term. To explore this possibility, collaborations are underway with an international group to study changes in autonomic function and the impact of glycaemic control in a prospective cohort of T2DM patients admitted to critical care. Autonomic function, as measured by HRV and cardiac autonomic function tests, will be performed in the period post ICU discharge and compared against ambulatory T2DM patients. This will be accompanied by Holter monitoring to assess the frequency of arrhythmias in the post-discharge period.

9.2.3 Clinical Implications

9.2.3.1 Intensive Glucose Control with Hyperinsulinaemic Euglycaemia in Critical Care Patients

This present work confirms that hyperinsulinaemic euglycaemia has anti-aggregatory, anti-inflammatory, profibrinolytic properties that may confer cardiovascular protection. However, clinical trials of intensive glucose control in critical care settings and ACS have produced equivocal results, with improvements in mortality in some (Malmberg et al., 1995; van den Berghe et al., 2001) and no benefit or increases in mortality in others (Finfer et al., 2009; Malmberg et al., 2005).

It is believed that the reasons for these are threefold. Firstly, occurrence of hypoglycaemia may oppose beneficial effects of intensive insulin therapy, as it has consistently been shown that a hypoglycaemia is one of the strongest independent predictor of subsequent mortality (Finfer et al., 2012). Secondly, the occurrence of hyperglycaemia and the failure to achieve pre-designated targets may account for the lack of benefit, particularly in trials of glucose-insulin-potassium infusions in ACS. The final reason may be due to the fact that insulin doses were not sufficiently high to confer cardiovascular benefits associated with insulin action. For example, in the Leuven-1 trial subjects received around 70 IU insulin/day (with concomitant intravenous dextrose in the first 24 hours) and survival benefits were demonstrated with intensive control (van den Berghe et al., 2001). This is in contrast with the NICE-SUGAR study, where daily doses of

around 50 units of insulin were used and this was not associated with improvements in clinical outcomes (Finfer et al., 2009). Similarly, other studies of low dose glucose-insulin-potassium infusions have had negative results in ACS patients (Ceremuzynski et al., 1999).

One area where intensive glucose control has been used for many years and been shown to improve mortality is perioperatively during cardiac surgery. Rackley et al. (1981) have developed a regime for a perioperative hyperinsulinaemic euglycaemic clamp for cardiac surgery. In this regimen, following a priming bolus of insulin, intravenous insulin is administered at a rate of 5m U/kg/min with 20% dextrose at a variable rate to maintain arterialised blood glucose at 4-6 mmol/L. Such a regime has been successfully applied in clinical settings with a low risk of hypoglycaemia. It has been shown to improve cardiac enzymes and myocardial function through inhibiting ischaemic-reperfusion injury (Carvalho et al., 2011) and attenuating the systemic inflammatory response (Visser et al., 2005).

Therefore testing a similar hyperinsulinaemic euglycaemic clamp protocols in critical care patients versus conventional glucose control (<10mmol/L) on morbidity and mortality outcomes maybe feasible. In this present work, it has been demonstrated that maintaining glucose at 6.0 mmol/L still had favourable effects on thrombosis and inflammation. Thereofre a glycaemic target of between 4-8 mmol/L, compared to a narrow range as used in previous trials (4.5-6.0 mmol/L) may be safer and still confer benefit (Finfer et al., 2009). The challenges are to design a suitable

hyperinsulinaemic euglycaemic clamp regime in critical care patients. This may be based on hyperinsulinaemic clamp protocols and doses of insulin according to body weight as used in this research study, but will require modification. The feasibility of a lower frequency of blood glucose testing, e.g. every 15 minutes when a steady-state is reached, will require investigation, as this is one of the limiting factors for translating to use in a clinical setting. There are groups that have trialled the use of interstitial CGM in critical care areas (Lee et al., 2011); however, it is unlikely that this can replace blood glucose monitoring due to the limited accuracy of CGMS in the hypoglycaemic range and also the interstitial lag that will influence the accuracy of insulin adjustments. Initial studies could focus on comparing the effects of hyperinsulinaemic euglycaemic regimes versus conventional glucose control on surrogate measures, such as inflammatory indices (hsCRP) and markers of thrombosis (fibrinogen) as a proof-of-concept. This should lead to definitive randomised controlled trials to look at hard outcomes on length of intensive care stay, short and medium term morbidity, and mortality.

9.2.3.2 Intensive Glucose Control in Acute Coronary Syndrome in Patients with Type 2 Diabetes

Glucose-insulin-potassium infusions in ACS without glycaemic control have shown no mortality benefit (Zhao et al., 2010). However the value of insulin-glucose infusions to achieve normo-glycaemic targets in diabetic patients with ACS remains open to debate. In the DIGAMI-1 study, there

were long term survival benefits apparent at one year and even at 20 year follow up as recently repored, where the median survival in the intensive group was 7 years as opposed to 4.7 years in the conventionally treated group (Ritsinger et al., 2014). It remains unresolved as to whether the benefits seen in the DIGAMI-1 study were related to the acute insulin-glucose regimen or subsequent control with subcutaneous insulin. Furthermore, the benefits of insulin-glucose regimens need to be evaluated in the modern era of primary percutaneous coronary intervention.

There is a need for a well-designed, well-conducted trial of intensive glucose control in AMI in diabetic patients. The first step would be to evaluate isolated effects of acute insulin-glucose therapy in the immediate infarct period. Pilot phases should explore safe doses of intravenous insulin and dextrose to be used. In a subset analysis of the Hyperinsulinaemia-Intensive Insulin and Infarction study (HI-5), achieving a mean blood glucose <8 mmol/L in the first 24 hours with insulin-dextrose infusion was associated with a significant reduction of in-hospital, three and six month mortality (Cheung et al., 2006). Therefore the value of a more stringent glucose target of 4-8 mmol/L could be explored. The optimal timing of insulin–glucose therapy in relation to the onset of AMI and reperfusion therapy needs to be investigated. This will hopefully eventually lead to randomised controlled trials of intensive-glucose therapy in diabetic patients in a clinical setting. In addition to mortality and cardiovascular

event rates, secondary outcomes should include effects on inflammation, platelet reactivity, cardiac enzymes and myocardial function.

9.2.3.3 Intensive Glycaemic Control in Type 2 Diabetes Patients and Cardiovascular Outcomes

The final question relates to the original population of interest, the value of intensive glycaemic control in T2DM patients in cardiovascular mortality. In the post ACCORD era, there has been a shift away from a 'glucocentric' view of macrovascular disease prevention, and the risk-benefit of any anti-diabetic regimen has to be weighed against potential microvascular benefits versus the risk of hypoglycaemia. A third important factor to be taken into consideration is the glucose-independent cardiovascular profile of glucose lowering agents. A lesson has been learnt from the withdrawal of rosiglitazone following post-marketing studies demonstrating an increased risk of myocardial infarction and cardiovascular death (Nissen and Wolski, 2007). In contrast, protective effects of metformin have been suggested by the UKPDS study and subsequently been confirmed by meta-analyses which showed lower cardiovascular mortality when used in overweight patients (Selvin et al., 2010). Recent post-marketing studies of DPP-4 inhibitors have shown that they may be 'cardiovascular neutral' with no evidence of an increase in cardiovascular deaths (Scirica et al., 2013; White et al., 2013). However, the safety of GLP-1 analogues are still to be confirmed in ongoing trials. As the present study has highlighted, insulin itself has numerous glucose-independent effects. While it may have anti-

aggregatory, anti-inflammatory, profibrinolytic, vasodilatory effects at high doses with intravenous infusion, it can also have sympathoexcitatory effects that can worsen, for example, platelet function. It is emerging that pleiotropic effects of these glucose lowering agents can have complex influences on overall cardiovascular outcomes.

Nevertheless, the present work has underlined the importance of the minimisation of hypoglycaemia while maintaining good glycaemic control, which remains important in reducing microvascular complications (Ismail-Beigi et al., 2010). There are two aspects of choice for a suitable glycaemic regimen – a suitable 'glycaemic target' and the choice of glucose lowering agent. The current guidelines of the European Association for the Study of Diabetes and the American Diabetes Association recommend an individualised approach to decisions regarding glycaemic targets (Inzucchi et al., 2012). Less stringent targets have been advocated in individuals at higher risk of hypoglycaemia, or those with advanced macrovascular disease, or limited life expectancy. Traditionally, glycaemic targets have equated with 'HbA1c' targets. However, observational studies suggest that there is not a clear linear relationship between HbA1c and the number of hypoglycaemic events; in fact, a U-shaped relationship has been shown, where patients with HbA1c <6% and above 9% were both at higher risk of hypoglycaemia (Lipska et al., 2013). This current study showed that the high prevalence of asymptomatic hypoglycaemia was only detected on CGMS in insulin treated T2DM patients. Part of the problem is that

intensive self-monitoring of blood glucose is often impractical in this group of patients given that they are often elderly with multiple comorbidities and complications. Other studies have confirmed the value of CGMS in detecting asymptomatic hypoglycaemia in T2DM patients (Chico et al., 2003), and there may be a role for CGMS as a clinical tool to aid treatment adjustment which will require confirmation in larger studies of T2DM subjects (Kim et al., 2014).

The choice of glucose lowering agents is also of importance. Metformin and incretin based therapies are associated with lower hypoglycaemic potential among oral hypoglycaemic agents; however, insulin-treated patients are the most vulnerable to hypoglycaemia. Both the regimen and type of insulin has some effect on the risk of hypoglycaemia. The 4T study which compared different regimens of analogue insulins showed that prandial regimes were associated with the highest risk of hypoglycaemia, followed by premixed insulin, whilst basal insulin was associated with the lowest risk of hypoglycaemia (Holman et al., 2009). In the present ambulatory study, the only difference between T2DM patients who experienced hypoglycaemia was in the type of insulin used. Out of 14 patients who experienced hypoglycaemia, thirteen were on human insulin and only one on analogue insulin ($p=0.02$). Systematic reviews of randomised controlled trials have shown that basal analogue insulins are associated with a lower risk of nocturnal hypoglycaemia compared with human isophane insulin (Monami et al., 2008). Similarly, in studies of

premixed human versus premixed analogue insulins, there was a difference in the rate of nocturnal hypoglycaemia as captured on CGMS (McNally et al., 2007). A question which remains unanswered is whether there are any differences between analogues versus human insulins on overall cardiovascular risk when controlled for HbA1c. There is evidence from observational cohorts that patients on analogue insulin as opposed to human regular insulin had a lower incidence of cardiovascular outcomes (Rathmann and Kostev, 2012). However, the type of insulin regime will influence more than one glucose variable, so it is difficult to attribute whether differences are due to reduced hypoglycaemia alone or other aspects of glucose variability.

9.3 Conclusions

It is encouraging that a nationwide study has demonstrated that there has been a dramatic reduction in cardiovascular complications in the past decade (Gregg et al., 2014). The most marked reduction has been in the incidence of AMI, which fell by 67%. This has partly been brought about by improvements in revascularisation techniques, but also early disease detection and multifactorial risk factor modification. However, the rates of CVD remain significantly higher among diabetic patients. For example, the relative risk of AMI remains 2-fold higher than in nondiabetic individuals, although it was nearly 4-fold higher in the previous decade (Gregg et al., 2014). The question for the future is how to further reduce macrovascular

risk with the armoury of glycaemic, lipid lowering, antithrombotic and cardiovascular therapies.

Hypoglycaemia remains a limiting factor for intensive glycaemic therapy in T2DM patients and the magnitude of the problem of hypoglycaemia in T2DM has previously been underestimated. The message from this current work is to alert physicians to the potentially harmful effects of hypoglycaemia, which may not be limited to the acute hypoglycaemic episode but persist in the days and weeks after an event. Such proarrhythmic, prothrombotic effects, may in concert, ultimately lead to an increased propensity for SCD. It is hoped that this current work will stimulate new ideas and new thinking about the role of glucose lowering in CVD prevention in T2DM. This could lead to new paths of research that may change the picture of glycaemic management for the 21st century for the benefit millions of T2DM patients worldwide and in generations to come.

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Appendix 1: Event definitions for arrhythmia analysis in the Pathfinder ECG Analysis system

N: Normal beat

A: Aberrant beat

Atrial ectopic

A normal beat (N) followed by a normal beat, such that the R-R interval between these two beats is less than 66% of the prevailing NN interval.

Ventricular premature beats (VPB)

Any beat followed by an aberrant (A) beat, such that the R-R interval between these two beats is less than 66% of the prevailing NN interval.

Bradycardia

Run of any 4 beats at a rate of less than 45 bpm

Complex VPB

Trigeminy: repeated ANN sequence

Bigeminy: repeated AN sequence

Couplet: NAAN sequence

Salvo: NAAAAN sequence

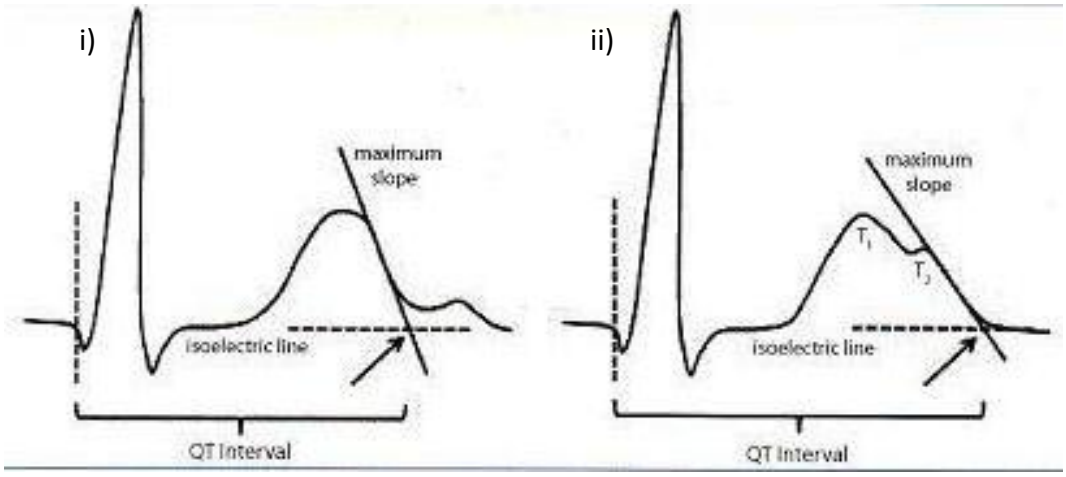
Ventricular tachycardia: AAAAA…N, sequence of ≥ 5 aberrant beats at > 100 bpm, terminated by a normal beat

Appendix 2: Classification of abnormal T wave morphologies

Normal (Figure i)

G1 Notched T (T1 apex >T2 apex) (Figure ii)

G2 Notched T (T1 apex <T2 apex)



QTU Fusion

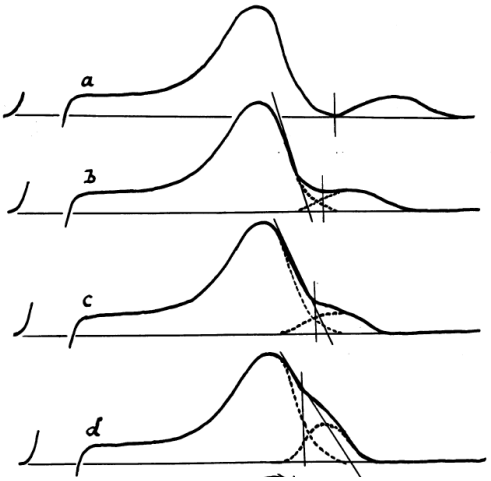


Figure a: F0: No QTU fusion (No fusion between T and U wave)

Figure b: F1: 1st degree fusion (nadir<25% of T1 peak)

Figure c: F2: 2nd degree fusion (nadir 25-50% of T1 peak) -

Figure d: F3: 3rd degree fusion (nadir>50% of T1 peak)

Appendix 3: Ethics approval for Study 1: Cardiac electrophysiological responses to spontaneous hypoglycaemia in type 2 diabetes

Derbyshire Research Ethics Committee
1 Standard Court
Park Row
Nottingham
NG1 6GN

Telephone: 0115 8839481
Facsimile: 0115 9123000

10 August 2010

Mr Scott Williams
Cardiothoracic Dept
Chesterman Unit
Northern General Hospital
Herries Road, Sheffield
S5 7AU

Dear Mr Williams

Study Title: **Exploring the role of glycaemic instability in sudden cardiac death: how does the electrophysiological response to spontaneous hypoglycaemic episodes differ between subjects with type 1 and type 2 diabetes?**

REC reference number: **10/H0401/60**

Thank you for your letter of 06 August 2010, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

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

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. I will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

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

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Appendix 4: Ethics approval for Study 2: Cardiovascular responses to experimental hypoglycaemia in type 2 diabetes

NRES
Health Research Authority

NRES Committee Yorkshire & The Humber - South Yorkshire
Millside
Mill Pond Lane
Meanwood
Leeds
LS6 4RA

Telephone: 0113 30 55128
Facsimile: 0113 85 55134

15 March 2012

Dr Elaine Chow
Sheffield Teaching Hospitals NHS Foundation Trust
Diabetes Centre
Northern General Hospital
Herries Rd
Sheffield
S57AU

Dear Dr Chow

Study title:	**Mechanisms of hypoglycaemia related sudden cardiac death in type 2 diabetes mellitus**
REC reference:	**12/YH/0080**
Protocol number:	v. 1

Thank you for your letter of 12 March 2012 responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

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

Non-NHS sites

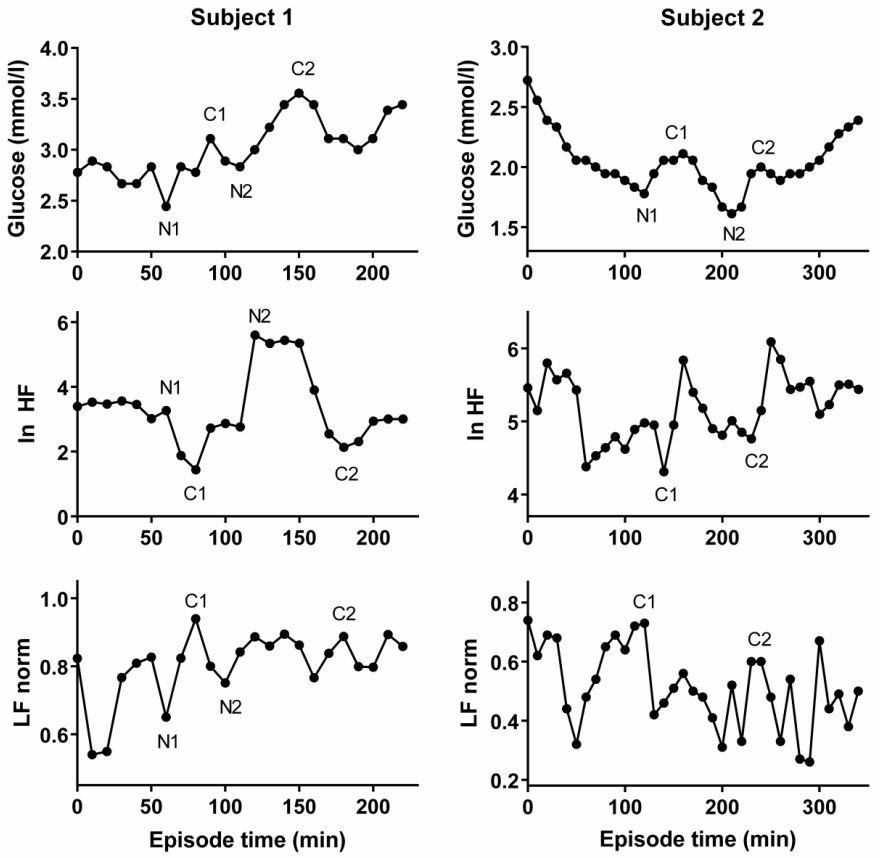
Conditions of the favourable opinion

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

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Appendix 5: Example of phasic changes in HRV during nocturnal hypoglycaemic episodes in two further participants

In both instances, following falls in glucose at N1 or N2, there was glucose counter-regulation (C1) and (C2) (top panels). There was a decrease in ln HF (middle panels) indicating vagal withdrawal at C1 and C2, followed by a subsequent rise in ln HF within 20 minutes, similar to the example in Figure 2. The bottom panels illustrate the reciprocal changes in normalised LF power.



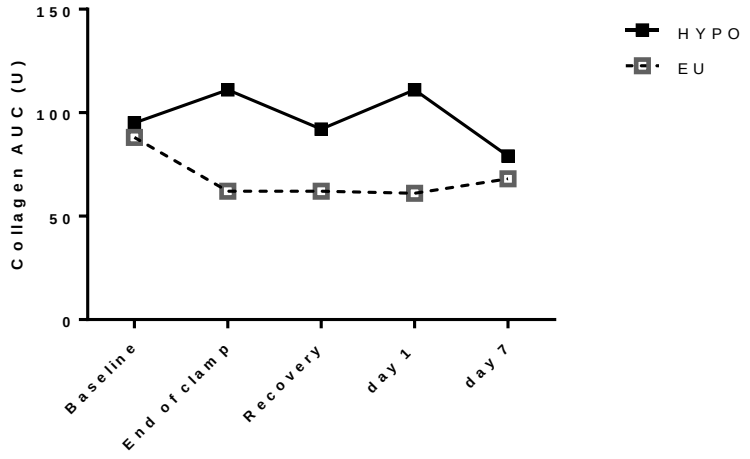
Appendix 6: Respiratory frequencies during heart rate variability recordings

Respiratory frequencies (Hz) mean ±SD during euglyacemic and hypoglycaemic clamp studies in diabetic and control groups

Time		0	30	60	90	120
Diabetes	**EU**	0.25±0.05	0.27±0.07	0.26±0.05	0.29±0.06	0.24±0.05
	HYPO	0.25±0.06	0.25±0.07	0.26±0.08	0.31±0.06	0.29±0.05
Controls	**EU**	0.24±0.03	0.25±0.05	0.24±0.04	0.26±0.05	0.26±0.07
	HYPO	0.24±0.04	0.26±0.04	0.27±0.06	0.30±0.08	0.27±0.08

Appendix 7: Platelet aggregation to 3 μM ADP by whole blood single platelet counting in euglycaemic and hypoglycaemic arms

Data for mean (SE) change in percentage platelet aggregation.



EU – euglycaemia, HYPO - hypoglycaemia