UNIVERSITY OF SOUTHAMPTON

School of Medicine

The Effect of Insulin-induced Hypoglycaemia on Myocardial Blood Flow Reserve in Patients with Type 1 Diabetes and Healthy Humans-A Study Using Myocardial Contrast Echocardiography

by

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This work is dedicated to all those who have experienced the ill-effects of hypoglycaemia.

Omar Rana

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Contents

List of Figures

Chapter 1 Figures.

Chapter 2 Figures.

Chapter 3 Figures.

Chapter 4 Figures.

Chapter 5 Figures.

Chapter 6 Figures.

List of Tables

Chapter 1 Tables.

Chapter 2 Tables.

Chapter 3 Tables.

Chapter 4 Tables.

Chapter 5 Tables.

List Of Publications

Papers Published

1. **Rana O**, Byrne C D, Kerr D, Coppini DV, Zouwail S, Senior R, Begley J, Walker J, Greaves K. Acute Hypoglycemia Decreases Myocardial Blood Flow Reserve in Type 1 Diabetes and in Healthy Humans. *Circulation*. 2011 (**In Press**).

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2. **Rana O**, Zouwail S, Coppini D V, Senior R, J Begley, Kerr D, Byrne C D and Greaves K. The Effect of Insulin-induced Hypoglycemia on Myocardial Blood Flow in Patients with Type 1 Diabetes Mellitus. *Circulation*. 2009; 120:S368

3. **Rana O**, Zouwail S, Begley J, Kerr D, Coppini D V, Senior R, and Greaves K. The Effect of Insulin and Insulin-induced Hypoglycaemia on Myocardial Blood Flow and Endothelin-1 Levels Using Myocardial Contrast Echocardiography. *Eur Heart J*. 2009;S456.

4. **Rana O**, Davies S, Thomas P, Kerr D, Coppini D V and Greaves K. The Effect of Insulin and Hypoglycaemia on Myocardial Blood Flow in Healthy Subjects. *Diabetes.* 2009; Suppl:634-P.

DECLARATION OF AUTHORSHIP

I, Omar Aziz Rana, declare that the thesis entitled **'The Effect of Insulin-induced Hypoglycaemia on Myocardial Blood Flow Reserve in Patients with Type 1 Diabetes and Healthy Humans-A Study Using Myocardial Contrast Echocardiography'** and the work presented in the thesis are both my own, and have been generated by me as the result of my own research. I confirm that:

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• Where any part of this thesis has been previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

• Where I have consulted the published work of others, the source is always clearly attributed;

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• Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

• Parts of this work have been published as listed in the section of publications.

Signed.....

Date.....

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List Of Abbreviations

A	Myocardial Blood Volume
ACCORD	Action in Diabetes and Vascular Disease-Preterax and Diamicron Modified Release Controlled Evaluation Study
ADA	American Diabetes Association
ADVANCE	Action to Control Cardiovascular Risk in Diabetes
AHA	American Heart Association
AMI	Acute Myocardial Infarction
APACHE II	Acute Physiology and Chronic Health Evaluation II Score
β	Myocardial Blood Velocity
CAD	Coronary Artery Disease
CVD	Cerebrovascular Disease
CVM	Cardiovascular Mortality
DBP	Diastolic Blood Pressure
DCCT	Diabetes Control and Complications Trial
DIGAMI	Diabetes Mellitus Insulin-Glucose Infusion in Acute Myocardial Infarction Study
DM	Diabetes Mellitus
ECG	Electrocardiogram
ET-1	Endothelin-1
HbA1c	Glycosylated Haemoglobin
HE	Hyperinsulinemic Euglycaemia
HH	Hyperinsulinemic Hypoglycaemia
hs-CRP	High-sensitivity C-reactive protein

HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Inter-cellular Adhesion Molecule 1
ICU	Intensive Care Unit
IGT	Intensive Glycaemic Therapy
IIT	Intensive Insulin Therapy
IL-1	Interleukin-1
IL-6	Interleukin-6
MBF	Myocardial Blood Flow
MBFR	Myocardial Blood Flow Reserve
MCE	Myocardial Contrast Echocardiography
NICE-SUGAR	Normoglycaemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulation Study
NO	Nitric Oxide
Non-STEMI	Non ST-Elevation Myocardial Infarction
PET	Positron Emission Tomogram
QTc	Corrected QT Interval
SBP	Systolic Blood Pressure
SCD	Sudden Cardiac Death
STEMI	ST-Elevation Myocardial Infarction
SPECT	Single Photon Emission Computed Tomogram
TIMI	Thrombolysis in Myocardial Infarction
UKPDS	United Kingdom Prospective Diabetes Study
VADT	Veterans Affairs Diabetes Trial
VEGF	Vascular Endothelial Derived Growth Factor
VISEP	The Efficacy of Volume Substitution and Insulin Therapy in

	Severe Sepsis Study
VPT	Vibration Perception Threshold

Chapter 1 Introduction

1.1 Background

Several observational studies have shown that the prevalence of diabetes mellitus (DM) is increasing world wide.[1, 2] More worryingly, premature cardiovascular disease is a recognized complication in patients with DM.[2-4] A large study including 3.3 million patients demonstrated that patients with DM have a cardiovascular risk profile similar to that of non-diabetic patients with a history of previous myocardial infarction.[4] The presence of DM increases the risk of future cardiovascular complications by two-fold and infact, younger patients with DM may have up to a 10-fold increased risk of major adverse cardiovascular events (MACE) in comparison to patients without DM.[5, 6] This excess risk occurs not only in patients with type 2 DM but also in patients with type 1 DM.[3, 7] Although the physiological mechanism for this association is multifactorial, hyperglycaemia has emerged as a putative cause.[8]

The Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS) showed that intensive glycaemic therapy (IGT), aiming for a glycosylated haemoglobin (HbA1c) of <7.0%, reduced the risk of microvascular complications associated with DM in a log-linear manner.[9, 10] Although there was a similar trend towards reduction in macrovascular events in the UKPDS, this effect did not reach statistical significance. However, long term follow-up analyses from these trials have confirmed a significant decrease in developing macrovascular complications with IGT (aiming for an HbA1c of less than 7.0%).[11, 12] This led to the development of guidelines by the American Heart Association (AHA) and American Diabetes

Association (ADA) recommending IGT in patients with DM with of a target HbA1c <7.0%.[2]

In contrast to the aforementioned trials, recent studies aiming for a stricter glycaemic control (HbA1c <6.0%) including the Veterans Affairs Diabetes Trial (VADT) and Action in Diabetes and Vascular Disease-Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) studies have failed to confirm this benefit.[13, 14] In fact, the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial demonstrated a significant increase in mortality, particularly cardiovascular mortality (CVM) with the use of IGT which led to an early termination of the study on the recommendation of the study's data safety monitoring board.[15]

Furthermore, in accordance with guidelines from the AHA and ADA, IGT (in the form of insulin) is recommended as discussed below (with a target glucose range of 5.0-7.8 mmol/L) in patients with hyperglycaemia during an acute illness.[16, 17] Initial studies have shown that IGT is associated with a significant reduction in MACE in such patients.[18-20] However, more recent trials have failed to confirm this benefit.[21, 22] In fact, the Normoglycaemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulation (NICE-SUGAR) trial (aiming for a more strict glucose range of 4.5-6.0 mmol/L) demonstrated an increase in CVM in the groups treated with IGT in comparison to the group receiving conventional treatment.[23]

The mechanisms through which IGT may exert negative impact on CVM are unclear however, hypoglycaemia is considered as a strong possibility.[24-26] This is due to the alarming rates of hypoglycaemic episodes observed with the use of IGT. In fact, patients on IGT may have up to a 13-

fold increased rate of severe hypoglycaemic episodes (requiring external help).[23] To date, there is no established direct causal link between hypoglycaemia and increased CVM. Several well-conducted randomised, physiological and observational studies as well as anecdotal case reports suggest hypoglycaemia as the main cause for increased CVM.[15, 23, 25, 27-32] However, to understand the consequences of hypoglycaemia on the human body, we should examine the evidence highlighting hyperglycaemia as a risk factor for increased CVM. This review will encompass the various studies which have used IGT to treat patients with and without DM and in the presence and absence of acute illnesses. Furthermore, it will explore the complex association between hypoglycaemia and MACE and provide a physiological link.

Finally, several studies were performed out in the community setting on patients with DM over many years such as the DCCT, UKPDS, VADT, ADVANCE and ACCORD.[9, 13-15, 33] In these studies apart from DCCT (which exclusively utilised insulin treatment for patients with type 1 DM), intensive glycaemic therapy (IGT) was achieved with oral hypoglycaemic drugs as well as insulin. However, in studies that aimed to counter hyperglycaemia in the more acute setting, for example in the intensive care unit (ICU) or in patients with acute coronary syndromes (ACS), intensive insulin therapy (IIT) was used as the preferred IGT.[18, 23] I have purposefully used the term IGT to avoid any confusion for the reader and have explained where necessary the dominant treatment option utilised to achieve IGT.

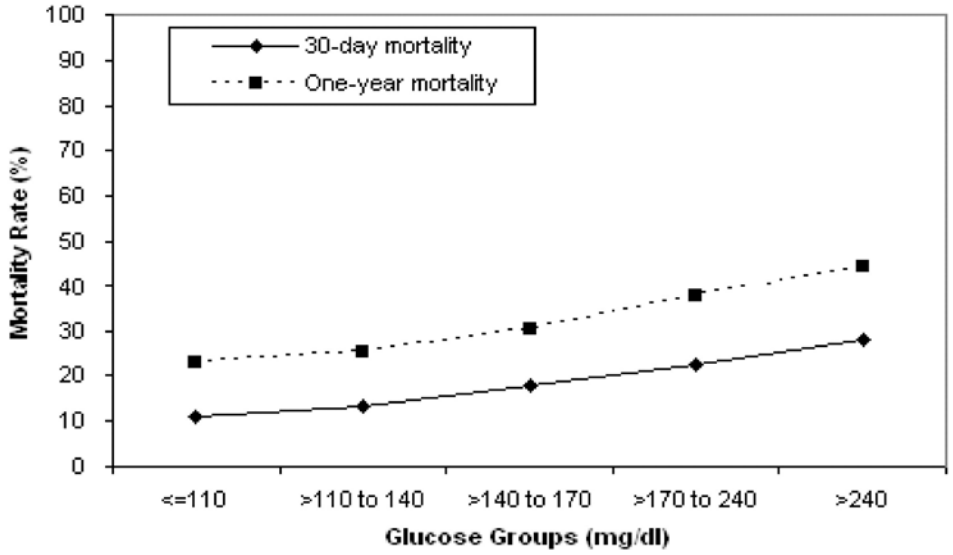
1.2 Hyperglycaemia and Increased Cardiovascular Mortality

Several studies have demonstrated that in-patient hyperglycemia is associated with a significant increase in short- and long-term mortality.[34] Kosiborod and co-workers examined the relation between elevated glucose concentrations on admission and all-cause mortality in 141,680 elderly (mean age 77 years) patients following an acute myocardial infarction (AMI). They found a step-wise increase in total mortality with increasing levels of hyperglycemia. For example, admission glucose levels between 9.4-13.3 mmol/L, (170-240 mg/dL) led to a 52% increase in 30-day mortality after multivariable adjustment in comparison to 77% in patients with levels greater than 13.3 mmol/L (>240 mg/dL). Interestingly, admission hyperglycemia was a relatively weak predictor of increased mortality in patients with diabetes mellitus (DM) in comparison to patients with no antecedent history of DM. There was a 32% relative increase in 30-day mortality in patients with DM and admission glucose concentrations higher than 13.3 mmol/L (>240 mg/dL). Furthermore, one-year mortality rates remained significantly elevated in patients presenting with hyperglycemia on admission (Figure 1.1).

Similarly, another study examined the association between admission, mean and maximum glucose values in 1826 patients admitted to an intensive care unit (ICU) and all-cause mortality.[35] The total in-hospital mortality rate was 9.6% (lowest) and 42.5% (highest) in patients with mean glucose values between 4.4-5.5 mmol/L (80-99 mg/dL) and greater than 16.7 mmol/L (>300 mg/dL) respectively. Furthermore, within each of the three groups of Acute Physiology and Chronic Health Evaluation II (APACHE II) scores, which

represent increasing severity of illness, higher mean and admission glucose values were observed in non-survivors in comparison to patients who survived. Interestingly, the 2.0% of patients with a mean glucose of <3.9 mmol/L had a 22.9% in-hospital mortality rate (2.2-fold increase).

Figure 1.1. Relationship between admission glucose values and crude 30-day and 1-year mortality in all patients (30-day mortality for glucose ≤110 mg/dL, 11.0%; glucose >110 to 140 mg/dL, 13.4%; glucose >140 to 170 mg/dL, 17.7%; glucose >170 to 240 mg/dL, 22.4%; glucose >240 mg/dL, 27.8%; 1-year mortality for glucose ≤110 mg/dL, 22.8%; glucose >110 to 140 mg/dL, 25.4%; glucose >140 to 170 mg/dL, 30.4%; glucose >170 to 240 mg/dL, 37.5%; glucose >240 mg/dL, 44.6%). Reproduced with permission.[34]



More recently, Kosiborod explored the association between persistent hyperglycemia and all-cause mortality in 16,781 patients following an AMI.[36] Blood glucose levels were determined on admission, a mean of all blood glucose readings during admission, time averaged glucose (TAG, derived from the area under the curve of all glucose values divided by the length of the observation period) and hyperglycemic index (HGI, area under the curve for hyperglycemic values divided by the in-hospital stay). Mean glucose levels, TAG and HGI were markers of persistent hyperglycemia. Following multivariable adjustment, patients with mean glucose concentrations of greater than 13.8 mmol/L (>250 mg/dL) had a 10-fold increase in in-hospital mortality. There was an increase in hospital mortality rate with each 0.6 mmol/L (10 mg/dL) incremental rise in mean glucose concentrations above the threshold of 6.7 mmol/L (120 mg/dL). Importantly, persistent hyperglycemia was a stronger predictor of in-hospital death in patients without DM in comparison to patients with DM. For example, the odds ratio for in-hospital mortality in patients with mean glucose concentrations of more than 11.1 mmol/L (>200 mg/dL) in the presence and absence of DM were 4.1 and 15.4 respectively.

It is important to note that elevated glucose concentrations on admission may not be associated with increased CVM in all groups of patients. In the largest study to date, Kosiborod and co-workers did not find an association between admission-hyperglycemia and increased 30-day or 1-year mortality in 50,532 patients admitted with acute heart failure.[37] However, the study had important differences to earlier studies. It was a retrospective study including a more elderly population (mean age 80 years). The sample group was selected from discharge diagnosis on patient files. A significant proportion of patients

did not have underlying coronary disease (≈ 40%). Furthermore, markers of myocardial injury (troponin, creatine kinase) were not included in the final analyses. Therefore, it is still not clear whether hyperglycemia on admission predicts mortality in the setting of acute heart failure secondary to AMI. Finally, the group limited their analyses to admission glucose levels and did not measure mean glucose, TAG or HGI which have been shown to be more reliable predictors of all-cause mortality than admission glucose levels.

Subsequently, Timmer and co-workers investigated the prognostic value of admission glycosylated haemoglobin (HbA1c) and admission blood glucose on short- and long-term mortality in 4176 patients presenting with ST-elevation myocardial infarction requiring primary percutaneous intervention.[38] A separate comparator group consisting of 598 patients had pre-existing diabetes. One-year mortality rates were 11.0% in the patients with diabetes in comparison to 4.6% in the patients without. Higher admission blood glucose levels were associated with both 30-day mortality and long-term mortality. For example, an admission blood glucose of more than 9.6 mmol/L was associated with a five- and four-fold increase in 30-day and one-year mortality rates respectively. Interestingly, an admission blood glucose of <6.9 mmol/L was associated with a 30% and 65% increase in 30-day and one-year mortality rates respectively. There was a positive correlation between admission blood glucose and infarct size. HbA1c levels on admission were also associated with mortality rates regardless of admission blood glucose levels (although this difference was not significant in patients with an admission glucose of >9.6 mmol/L). An HbA1c level of >5.8% was associated with a 66% increase in one-year mortality rates. When the cases who had died within 30 days were excluded, only HbA1c

levels were associated with long-term mortality rates (Figure 1.2). These results suggested that both admission glucose and HbA1c levels reflect different patient populations. High admission glucose levels were associated with more unstable patients with larger infarct size and higher 30-day mortality rates. In contrast, elevated HbA1c levels were associated with more gradual mortality rates over time.

1.3 Guidelines for in-patient Glycaemic Control

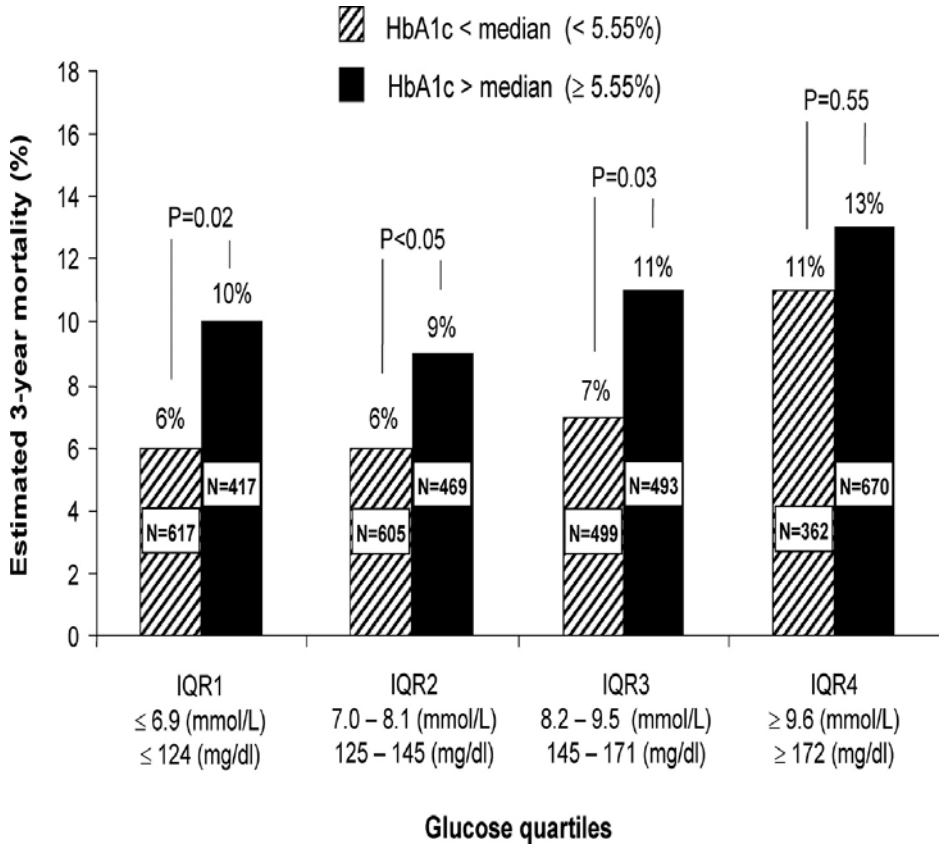
In light of these studies, various national guidelines have been issued and are in common practice. The American Heart Association (AHA) issued guidelines in 2008 for the management of glycemic control during acute coronary syndrome.[16] These guidelines recommend intensive glucose control aimed to maintain normoglycemia at around (5.0-7.8 mmol/L, 90-140 mg/dL). Special emphasis has been laid on the need "to avoid hypoglycaemia." Intravenous insulin has been suggested as the most effective method to control glucose levels in patients admitted to the ICU in contrast to subcutaneous insulin regimens in the non-ICU patients. However, subsequently the NICE-SUGAR trial demonstrated a 2.6% increase in absolute 90-day mortality (mostly driven by an increase in cardiovascular mortality) in patients with a mean blood glucose of 115 mg/dL (6.4 mmol/L) in comparison to patients receiving standard therapy and having a mean glucose of 144 mg/dL (8.0 mmol/L).

Table 1.1 Clinical Outcome of Non-diabetic patients based on Admission glucose and Glycosylated Haemoglobin Levels Requiring Primary Percutaneous Corornary Intervention. Reproduced with permission.[38]

	Admission HbA_{1c} Levels					Admission Glucose Levels				
	IQR 1 (≤5.35%)	IQR 2 (5.36%–5.54%)	IQR 3 (5.55%–5.80%)	IQR 4 (≥5.81%)	*P*	IQR 1 (≤6.9 mmol/L)	IQR 2 (7.0–8.1 mmol/L)	IQR 3 (8.2–9.5 mmol/L)	IQR 4 (≥9.6 mmol/L)	*P*
Infarct size										
Peak CK in the first 24 h, U/L	1469 (558–3272)	1540 (572–3185)	1500 (647–3000)	1486 (509–3105)	NS	903 (327–2050)	1367 (564–2923)	1912 (827–3540)	2046 (898–4195)	<0.001
Clinical outcome, %										
30-d mortality	2.0	2.3	2.3	3.1	NS	1.3	1.0	2.6	4.9	<0.001
1-y mortality	3.1	4.1	4.9	6.8	<0.001	3.8	2.3	4.9	8.0	<0.001

IQR=Interquartile range, CK=creatine kinase. Values are represented as median(IQR) or group percentage.

Figure 1.2 Bar Graph showing unadjusted Kaplan Meier-estimated 3-year mortality stratified on admission blood glucose quartile and according to Glycosylated Haemoglobin (HbA1c) level (median value) in patients without diabetes mellitus. P value was calculated with log-rank analysis. IQR indicates inter-quartile range. Reproduced with permission.[38]



The cause of this increased CVM was not clear, although a 14-fold increase in severe hypoglycaemic events (<40 mg/dL, <2.2 mmol/L) was observed. Despite the lack of a causal link between hypoglycaemia and increased CVM, it was considered prudent to relax the target glycaemic control range between 140-180 mg/dL (7.8-10 mmol/L) in patients with acute myocardial infarction in a 2009 update.[39]

The American Diabetes Association (ADA) and the American Association of Clinical Endocrinologists (AACE) have also issued comprehensive guidelines for the management of in-patient glucose control.[39] Intravenous insulin infusion is again preferred to maintain glucose levels between 7.8-10.0 mmol/L (140-180 mg/dL) in critically ill patients. Similar to the guidelines issued by the AHA, subcutaneous insulin regimens are preferred to maintain pre-meal glucose levels below 7.8 mmol/L (<140 mg/dL) in conjunction with random blood glucose values of less than 10 mmol/L (<180 mg/dL). Frequent glucose monitoring is encouraged especially with intravenous insulin therapy "to minimize the occurrence of hypoglycaemia." The prolonged use of a standard sliding scale insulin was discouraged as it was suggested that this increased the rates of hyperglycemia and hypoglycemia.

1.4 Prevalence of Hypoglycaemia

Premature coronary artery disease (CAD) is a major cause of morbidity and mortality in patients with type 1 DM with an up to 4- to 8-fold increased risk of cardiovascular disease in males and females with type 1 DM respectively.[3, 7, 40] Although this association appears to be multifactorial,

hyperglycaemia has emerged as a putative cause.[8] For example, in a study recruiting 292 patients with type 1 DM (aged <21 years) and followed up for 20-40 years, it was shown that CAD accounted for 35% of all deaths as evidenced on post-mortem examination.[6] More importantly, 11% of deaths were classified as sudden. Post mortem examination on a significant proportion of these patients did not reveal any cause of death. Mortality rates were not influenced by the age of onset of DM. Furthermore, the cumulative prevalence of CAD in patients aged 45-59 years was 33% (as evidenced by past history, changes on resting an electrocardiogram, or a positive stress test). Intensive glycaemic control, aiming at treating hyperglycaemia and thereby reducing CVM risk, comes at some cost with a several-fold increase in severe hypoglycaemic episodes (defined as the inability of the patient to self administer glucose).[41] For example, Pramming and co-workers showed that out of 411 randomly selected type 1 DM subjects, 36 % had experienced hypoglycaemic coma in their life time.[42] The frequency of hypoglycaemia was positively associated with the increased insulin dose, more frequent insulin injections or low HbA_{1c} levels. Macleod and colleagues demonstrated that nearly 30% of 600 type 1 DM patients had experienced at least one episode of severe hypoglycaemia in the preceding year with an estimated frequency of 1.7 episodes per patient per year.[43] These findings were independently confirmed in another study which showed that 37% of patients with type 1 DM suffered one or more episodes of severe hypoglycaemia (requiring assistance to restore normoglycaemia) during a one year period.[44] The rate of self-treated hypoglycaemic episodes was 8 per person per annum and of those over 10% were severe hypoglycaemic events. A separate study conducted in the UK

included 23,752 insulin-treated diabetic patients with a mean follow-up of 13.4 years.[40] These patients had been diagnosed with DM at an early age (<30 years) of whom 94% had type 1 diabetes. A total of 949 deaths (4%) were recorded during the follow-up period. Of concern, 18 % of male deaths and 6% of female deaths were attributed to hypoglycaemia.

1.5 Trials showing benefit of Intensive Glycaemic Therapy in the out-patient setting in patients with Diabetes Mellitus

Worldwide nearly 246 million people suffer from diabetes mellitus (DM) and these numbers are predicted to increase to 380 million over the next twenty years.[2] This projected rise represents a 20-fold increase in the actual number of people suffering from DM within forty years. The complications of DM are well recognised and represent a disease spectrum, ranging from a micro- to macro-vascular level. Cardiovascular disease (CVD) is one of the major complications of DM with a high morbidity and mortality. The biological mechanisms by which DM lead to the development of CVD are yet poorly understood but multiple factors are likely to be involved such as, endothelial dysfunction, oxidative stress, inflammation, hypercoagulability, hypertension, hyperglycaemia (possibly with the formation of advanced glycosylated end products, AGE) and dyslipidaemia.[8] These factors interplay leading to atherosclerosis, plaque formation and rupture with thrombosis causing myocardial ischemia and death.

The cardiovascular (CV) complications of DM are particularly important as up to 80% of patients with DM will suffer a CV event.[45] Furthermore, the

risk of developing CVD is 2-4 times higher than that of the general population.[4] Young patients suffering from DM (males <45 years and females <55 years) have a 10-fold increased risk of death related to CVD.[5] The importance of targeting risk factors for CVD in DM such as optimal glycaemic and blood pressure control, in addition to aggressive cholesterol management is now well recognised and has been incorporated into national guidelines.[2]

The Diabetes Control and Complications Trial (DCCT) was a landmark multi-centre trial in the US and Canada which examined the effect of IGT (in the form of subcutaneous insulin) on patients with Type 1 DM aged 13-39 years with no history of coronary artery disease (Table 1.2).[9] A total of 1441 patients receiving either IGT (insulin) or conventional treatment were followed up for a median duartion of 6.5 years. The goals for intensive therapy included a pre-prandial glucose of 3.9-6.7 mmol/L, post-prandial glucose of <10mmol/L, weekly 3 a.m glucose of >3.6mmol/L and monthly glycosylated haemoglobin (HbA1c) of <6.05%. HbA1c levels in the IGT and conventional treatment group were 7.4% and 9.1% respectively with a 98% compliance with intensive therapy in the IGT group. This study demonstrated for the first time that strict glycemic control lowered the risk of developing microvascular complications in Type 1 DM significantly (severe non-proliferative retinopathy or worse by 54%, clinical neuropathy by 60% and nephropathy as defined by urinary albumin excretion of $\geq$ 300 mg over 24 hours by 39%.)[11] Furthermore, there was a 41% reduction in macrovascular complications albeit not significantly, with the use of IGT (0.5 event per 100 patient-years in the IGT group vs. 0.8 event per 100-patient years in the conventional group). This

could perhaps be explained by the fact that the patients were young at randomisation and the follow-up was relatively short (6.5 years).

A subsequent analysis of the DCCT examined the effect of IGT on the prevalence of CV complications in patients with Type 1 DM from the original cohort after an extended follow-up.[11] Ninety three percent of the original DCCT cohort of 1441 patients participated with a mean follow-up of 17 years. Following the initial study duration of 6.5 years, both the conventional and intensive treatment groups received IGT. Forty six cardiovascular events (nonfatal myocardial infarction, stroke or death due to cardiovascular disease) occurred in 31 patients belonging to the intensively treated group in comparison to 98 events in 52 patients who had originally received conventional treatment. There was a 3.6% absolute risk reduction in major cardiovascular events with IGT which translated into a 42% relative risk reduction in the development of cardiovascular disease (p=0.02), defined as death due to cardiovascular disease, non-fatal myocardial infarction (MI) or stroke, angina (induced by exercise testing with ECG changes or on coronary angiography) and finally revascularisation (percutaneous coronary angioplasty, PCA or coronary artery bypass grafting, CABG). In addition, there was a 57% relative risk reduction in non-fatal myocardial infarction, stroke and CVM. Therefore, this study showed for the first time that IGT in young patients with type 1 DM (albeit for a limited period of 6.5 years in accordance with the original trial duration) not only significantly decreased microvascular complications but also led to less cardiovascular events. The authors termed this phenomenon as the ‘metabolic memory’. Importantly, there was a three-fold decrease in CVM. Finally, the duration of DM, presence of microvascular

complications and elevated HbA1c levels emerged as independent predictors for the development of macrovascular complications. For example, a 10% incremental increase in HbA1c was associated with a hazard ratio of 1.25 of developing cardiovascular disease.

The United Kingdom Prospective Diabetes Study (UKPDS) was a randomized, prospective, multi-centre trial which examined the effects of intensive glucose treatment on patients with a new diagnosis of Type 2 DM (Table 1.2).[10] Three thousand eight hundred and sixty seven patients with a median age of 54 years participated in the study. Participants were either assigned to conventional treatment with a target fasting plasma glucose (FPG) of <15 mmol/L (with diet alone, failing which drug treatment was prescribed) or IGT receiving sulfonylureas (chlorpropamide, glibenclamide, glipizide) or insulin therapy with a target FPG of <6mmol/L. A 11% reduction in the median HbA1c levels were achieved in the IGT group (7.0%) as compared to the conventional group (7.9%) after a median follow-up of 10 years. This translated into a 25% relative risk reduction in the development of microvascular complications with IGT. There was a 16% reduction in the rate of myocardial infarction (fatal and non-fatal) and sudden death but this did not reach statistical significance (p=0.052). Subsequently, a further analysis of UKPDS was published which examined the effect of IGT on long-term MACE in the original cohort after an extended 10-year follow-up following the cessation of the randomized interventions.[12] It recorded a 15% reduction in the rate of myocardial infarction (p=0.01) and a 13% reduction in mortality rate (p=0.007) in the group receiving IGT (sulfonylureas or insulin) in comparison to the conventional group. These results were in agreement to that of the

DCCT investigators. The authors termed this protective effect of IGT as the 'legacy effect' which appeared to be sustained despite the trial stopping 10 years earlier.

In summary, these data demonstrated that IGT was responsible in decreasing micro- and possible macro-vascular complications in patients with type 1 and type 2 DM alike. However, more importantly, both studies demonstrated a three-fold increase in severe hypoglycaemic episodes which raised serious concerns in the scientific community.

The DCCT conducted a full epidemiological investigation of the original cohort of 1441 patients that had participated in the trial (Table 1.2).[46] In fact, this was performed due to the observation that episodes of severe hypoglycaemia were 3-fold higher in patients on IGT in comparison to the conventional group. Severe hypoglycaemia was defined as an episode during which the patient required assistance and blood glucose level of <2.8 mmol/L or prompt recovery with oral carbohydrate, glucagon or intravenous glucose. Of note several patients were excluded from the trial for example those with a history of hypoglycaemic unawareness with more than episode of neurological impairment or history of >2 seizures or coma-like episodes due to any cause. The IGT group (711 patients) experienced a cumulative 2896 episodes of severe hypoglycaemia with an event rate of 62 episodes per 100 patient-years of follow-up (relative risk 3.28, $p<0.001$). This included a total of 770 episodes of coma and or seizure due to hypoglycaemia (relative risk of 3.02 $p<0.001$). In contrast, the conventional therapy group experienced a total of 892 episodes of severe hypoglycaemia at a rate of 19 episodes per 100 patient-years of follow-up including 257 episodes of coma and or seizures.

Table 1.2 Studies that showed benefit of IGT in patients with hyperglycemia in the outpatient setting.

Study	Design	Glycaemic Targets	Hypoglycaemia (definition and prevalence)	Outcome
Diabetes Control and Complications Trial (DCCT[9,11]	1441 patients with type 1 DM with a median follow-up of 6.5 years. Age between 13-39 years. 726 patients had no retinopathy (primary-prevention group). 715 patients had early retinopathy (secondary-prevention group).	IGT group: 1. HbA1c < 6.05% 2. Pre-meal glucose 3.9-6.7 mmol/L. 3. Post-meal glucose <10.0 mmol/L. Conventional group: Avoidance of hyperosmolar symptoms, ketonuria, hypoglycaemia and maintenance of normal body weight and development.	Defined as plasma glucose levels <2.8 mmol/L. IGT group: 62 episodes of severe hypoglycaemia per 100 patient-years. Conventional group: 19 episodes per 100 patient-years. Relative risk of severe hypoglycaemia in IGT group of 3.28 (p<0.001).	**1.** IGT decreased the development of retinopathy by 76% (primary-prevention), progression of retinopathy by 54% (secondary-prevention), development of nephropathy by 39% and neuropathy by 60%. **2.** Subsequently, a 17-year follow-up of DCCT demonstrated that IGT in type 1 DM was associated with 42% decrease in any cardiovascular event and a 57% reduction in non-fatal myocardial infarction, stroke and cardiovascular mortality by 57% (p=0.02).
United Kingdom Prospective Diabetes Study (UKPDS)[10,12]	3867 patients with a new diagnosis of type 2 DM. Median age 54 years (inter-quartile range 48-60 years).	IGT group: Fasting plasma glucose (FPG) levels <6.0 mmol/L. Conventional group: FPG levels <15.0 mmol/L through diet. Medications added if hyperglycaemic symptoms or target of FPG <15.0 mmol/L not achieved.	Severe hypoglycaemia defined as a hypoglycaemic episode requiring external assistance. IGT group: 1.8% with insulin and 1.4% with glibenclamide. Conventional group: 0.7% (p<0.0001).	**1.** IGT decreased the composite end-point of development of microvascular complications by 25% (p=0.0099). **2.** Subsequently, a 10-year follow-up of UKPDS demonstrated a 15% decrease in myocardial infarction (p=0.01) and all-cause mortality by 13% (p=0.007).

IGT was independently associated with an increased risk of subsequent episodes of severe hypoglycaemia. It was estimated that during one year 27% of IGT group would experience at least one episode of severe hypoglycaemia in comparison to 10% of patients receiving conventional therapy. Furthermore, for the same level of HbA1c as compared to the conventional group, a patient receiving IGT had a 45% and 28% greater risk of severe hypoglycaemia and coma (and or seizure) respectively Other risk factors for severe hypoglycaemia included male sex with a 4.4-fold increased risk of severe hypoglycaemia in comparison to a 2.5-fold risk in females, previous episodes of severe hypoglycaemia, adolescents, higher dose of insulin and higher HbA1c at screening. Interestingly, the relationship between log(HbA1c) levels and log(relative risk of hypoglycaemia) was linear in patients receiving IGT with a 22% increased risk of severe hypoglycaemia for every 10% decrease in HbA1c levels. However, this association in patients on conventional treatment was not linear. For example, a reduction of 10% from an HbA1c of 11% would increase the risk of severe hypoglycaemia by 74% while a 10% decrease from 7% would translate into a 47% increase.

Similarly, there was a significant increase in the number of severe hypoglycaemic episodes associated with IGT in the UKPDS study.[10] The risk of severe hypoglycaemic episodes (defined as patient requiring assistance) were 0.7% per year with conventional treatment. In the IGT group this risk was 1.4% with sulfonylureas and around 2.0% with insulin. The annual risk of severe hypoglycaemic episodes was 3% in the patients on IGT. Indeed at the time of randomisation the mean HbA1c levels were 6.2% (conventional), 6.3%

(sulfonylureas) and 6.1% (insulin). This could have resulted in the relatively low rates of severe hypoglycaemic events as described above.

In summary, the aforementioned randomised control trials have demonstrated that IGT not only reduces the incidence of microvascular complications but perhaps more importantly, has a protective role against the development of macrovascular complications such as cardiovascular disease in patients with DM. Importantly, there is also strong evidence that IGT increases the risk of severe hypoglycaemic episodes by an estimated three-fold in patients in the out-patient setting.

1.6 Trials that failed to show benefit with Intensive Glycaemic Therapy in the out-patient setting in patients with Diabetes Mellitus

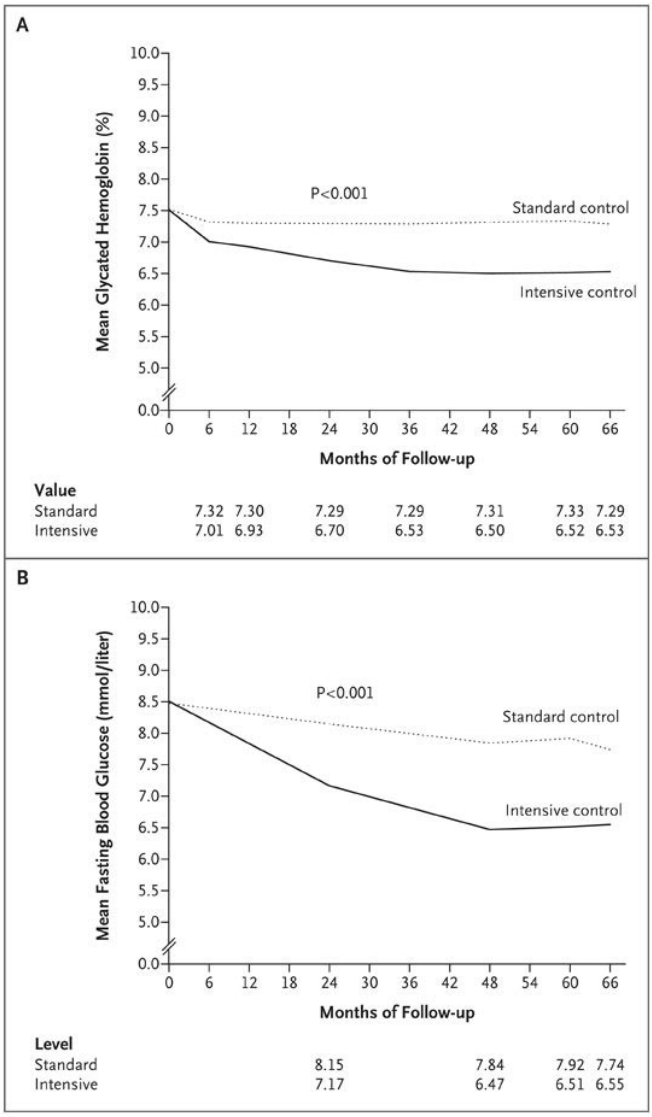
As discussed above, the DCCT and UKPDS trials showed that IGT in patients with DM led to a significant reduction in macrovascular complications.[9, 10, 33] In the DCCT trial, over a period of 6.5 years, HbA1c levels decreased from a mean of 9.1% to 7.4% in the IGT group. Furthermore, in the UKPDS study the median HbA1c concentrations fell to 7.0% from 7.9% in the IGT group. Therefore, current guidelines recommend a target HbA1c levels of <7.0% as part of primary prevention for the development of macrovascular disease in patients with DM.

Several recent trials have however, attempted more strict glycaemic control with a target HbA1c of ≤6.5%.[13-15] These include the Veterans Affairs Diabetes Trial (VADT), Action in Diabetes and Vascular Disease-Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) studies which

failed to confirm any benefit. In fact perhaps surprisingly, the Action to Control Cardiovascular Risk in Diabetes (ACCORD) showed an increased harm with IGT in patients with type 2 DM. These studies are summarised below (Table 1.4).

The first of these was the ADVANCE study which was a multi-centre randomized controlled trial.[14] A total of 11,140 patients with type 2 DM aged ≥55years and either with a history of cardiovascular disease or one other risk factor (1 in 6 patients had established macrovascular complications) were either randomised to an intensive glycaemic strategy (as defined by a HbA1c of <6.5%) or received standard care. Patients were followed up for a median duration of 5 years and the effects of IGT on macrovascular outcomes was examined. IGT consisted of the sulfonylurea agent gliclazide (with the discontinuation of any other sulfonylurea), and if required the step-wise addition of metformin, thaizolidinediones, acarbose or insulin. Particular care was taken to gradually decrease the HbA1c levels of the patients in the IGT and standard therapy group. In fact, following six months of therapy, there was a mean 0.5% difference in the HbA1c levels which increased to a mean of 0.67% point separation between the two groups. Forty one percent of patients in the IGT group were taking concomitant insulin at the end of the trial. Macrovascular events were defined as death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke. The mean HbA1c was 7.5% in the IGT group and control group at randomisation which decreased to 6.5% and 7.3% respectively.

Figure 1.3. Overall glucose control during the ADVANCE study. The average difference between the intensive-control group and the standard-control group for the follow-up period was 0.67% point for HbA1c (Panel A) and 1.22 mmol/L (21.9 mg/dL) for fasting blood glucose (Panel B). Reproduced with permission.[14]



There was a significant reduction in the incidence of microvascular complications 18.1% in the IGT group vs. 20.0% in the control group (p=0.01)

which was driven primarily by a reduction in the incidence of nephropathy. More importantly, there was no difference in the incidence of macrovascular complications for example myocardial infarction or death due to cardiovascular disease between the groups. The rate of severe hypoglycaemia (<2.8 mmol/L) was increased by two-fold in the IGT group ($p<0.001$) with a 50% increase in the frequency of hypoglycaemia-led hospitalisations in comparison to the control group ($p=0.04$).

In a subsequent sub-analysis, the ADVANCE group collaborators examined the effect of severe hypoglycaemia on micro- and macrovascular complications.[47] A total of 1031 (9.3%) deaths were reported in the entire study cohort during a median follow-up of five years. It was noted that IGT led to a two-fold increase in the rate of hypoglycaemic events per patient per year. For example, 2.7% (150/5571 patients) experienced severe hypoglycaemia in the IGT group in comparison to 1.5% (81/5569 patients) in the standard therapy group. In addition, there was a significant increase in the annual rate of severe hypoglycaemia (suggesting an increase in the frequency of hypoglycaemic episodes) in the IGT group ($p<0.001$ for trend) as the study progressed. In contrast, this remained relatively constant for the group receiving standard treatment ($p=0.38$ for trend) as depicted in the figure below. Multiple regression analysis revealed that the risk factors for developing severe hypoglycaemia were advanced age, renal impairment, cognitive impairment, longer duration of diabetes, multiple glucose lowering agents and intensive glycaemic therapy. Among patients who reported severe hypoglycaemia, 16.8% had a subsequent macrovascular event, 11.5% had a subsequent major microvascular event and 19.5% patients died. The respective rates for patients

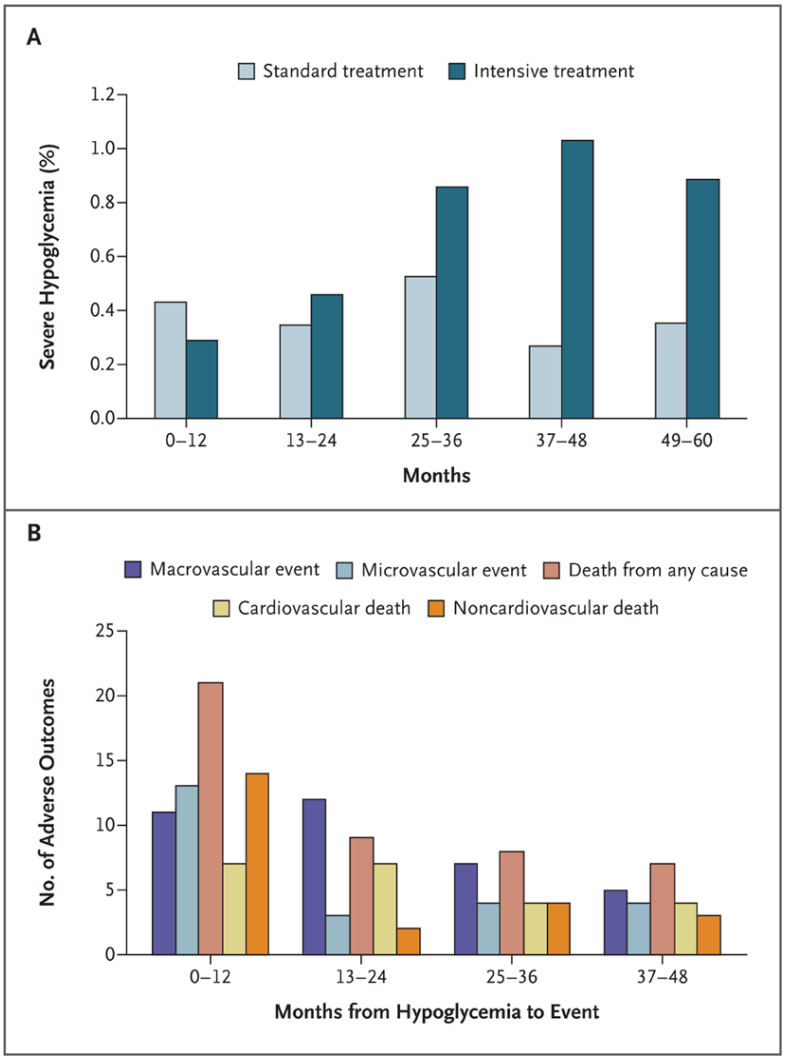
who did not experience severe hypoglycaemia were 10.2%, 10.1% and 9.0%. Importantly, most of the micro- and macrovascular complications occurred within one year of the index episode of severe hypoglycaemia as shown in Table 1.3.

Table 1.3 Median Times from an Episode of Severe Hypoglycaemia to a Subsequent Major Micro- or Macrovascular Event in the ADVANCE Study.

Event	Time in years (interquartile range)
Macrovascular event	1.56 (0.84-2.41)
Microvascular event	0.99 (0.40-2.17)
All-cause mortality	1.05 (0.34-2.41)
Death due to a cardiovascular cause	1.31 (0.80-2.41)
Death due to non-cardiovascular cause	0.74 (0.13-2.60)

A total of 45 patients died out of the 231 patients who had had experienced severe hypoglycaemia. This comprised 26/150 and 19/81 deaths in the IGT and standard therapy groups respectively. Although, this equated to 3.6% and 5.1% annual mortality rates in the IGT and standard therapy groups respectively, suggesting decreased mortality in the IGT group despite higher episodes of severe hypoglycaemia, this observation is most likely due to chance. There was no relationship between repeated episodes of severe hypoglycaemia and adverse events although only 35 out of the total of 11,140 patients experienced two or more episodes of severe hypoglycaemia. Therefore, it is difficult to draw any firm conclusions from such a small number. Finally, the adjusted 6-month hazard ratio following severe hypoglycaemia was 2.75 for

Figure 1.4. Annual Rates of Severe Hypoglycaemia and Adverse Clinical Outcomes among Patients with Severe Hypoglycaemia as seen in the ADVANCE Study. Reproduced with permission.[47]



Panel A shows the rates of severe hypoglycaemia in the two groups, ($p<0.001$ for trend in intensive treatment group) and ($p=0.38$ for trend in standard treatment group). Panel B shows the annual frequency of major complications following an index episode of severe hypoglycaemia.

macrovascular events (p=0.01), 2.41 for microvascular events (p=0.03), 4.28 for all-cause mortality (p<0.001) and 3.57 for cardiovascular mortality (p=0.01). In addition, there was a significant increase in death due to non-cardiovascular mortality with a hazard ratio of 4.95 (p<0.001).

Therefore, hypoglycaemia was associated with an increase in cardiovascular- and non-cardiovascular mortality rates in patients with type 2 DM receiving IGT. The authors were unable to explain a direct causal link between hypoglycaemia and increased CVM and concluded that although severe hypoglycaemia was associated with adverse outcomes, the mechanistic basis could not be determined.

The VADT was a multi-centre trial conducted in the USA and consisted of 1791 military veterans either randomised to IGT or standard therapy.[13] The mean age of the participants was 60 years and 40% of the participants had a history of a cardiovascular event with mean HbA1c values of 9.4%. Patients with a body mass index (BMI) of >27 mg/kg^2 received metformin with rosiglitazone while patients with a BMI <27 mg/kg^2 received glimepiride and rosiglitazone. Patients in the IGT group received maximal doses while the control group were commenced on half the maximal doses. Insulin was added to the regime of the patients in the IGT group if an HbA1c of <6.0% was not achieved (<9.0% for the control group). The goal for HbA1c was an absolute reduction of 1.5% in the IGT group vs. the control group. Cardiovascular events were defined as myocardial infarction, stroke, death from cardiovascular disease, new or worsening heart failure or the need for surgical revascularisation. There was no difference in the rate of cardiovascular events in both the groups (p=0.14). The rate of severe hypoglycaemia (<2.8 mmol/L)

was increased by four-fold in the IGT group in comparison to the control group (203 vs. 53 episodes, p<0.001). Twenty one percent of patients in the IGT group experienced severe hypoglycaemia in comparison to 10% of the group receiving standard therapy. Furthermore, there was a trend towards an increase in the number of sudden deaths in the IGT group as compared to the control group (11 vs. 4, p=0.07). It is plausible that with a larger cohort of patients, this difference would have achieved statistical significance. Finally, serious adverse events (defined as life-threatening events or death and events leading to hospitalisation, disability or incapacity) were more common in patients on IGT (severe hypoglycaemic events, 9% vs. 3%, p<0.0001 and dyspnoea, 11% vs. 7%, p=0.006).

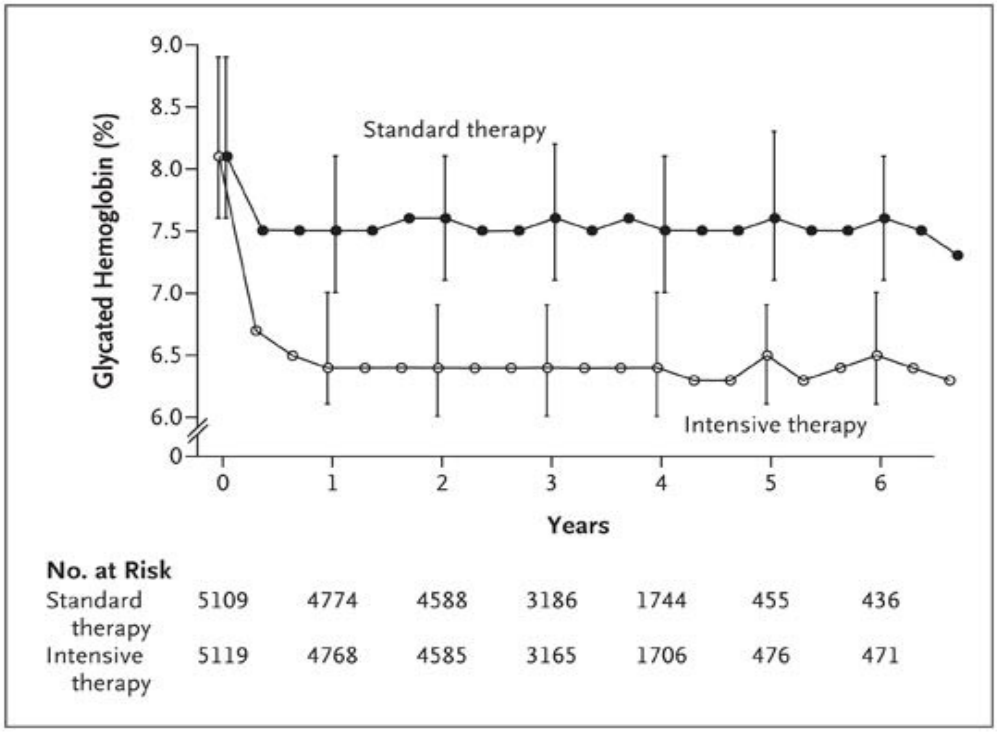
Similar to the above trials, ACCORD was also a multi-centre randomised controlled trial examining the effects of IGT on cardiovascular outcomes in 10251 patients with type 2 DM.[15] In contrast to earlier studies it aimed for a more strict glycaemic control with a target HbA1c level of <6.0% in patients receiving IGT vs. 7.0-7.9% in the group receiving standard care. In comparison to the UKPDS study, the patients were older with a mean age of 62 years and 35% of the participants had a history of a previous cardiovascular event. Importantly, patients with a history of frequent severe hypoglycaemic events and/or hypoglycaemic coma (or seizure) in the preceding 12 months were excluded from the study. The rate of decrease in HbA1c levels in the IGT group was very steep with a separation of 0.8 of a percentage point within 2 months of randomisation. Within 12 months, mean HbA1c levels were 6.4% in comparison to 8.1% in the IGT group. The trial

Table 1.4 Studies that showed no benefit of IGT in patients in the out-patient setting.

Study	Design	Glycemic Targets	Hypoglycemia (definition and rates)	Outcome
VADT[13]	1791 patients with type 2 DM. 40% patients had history of cardiovascular disease. These received IGT in a 1:1 randomised order. Median follow-up of 5.6 years.	IGT group: Absolute decrease in HbA1c levels of 1.5%. Patients received rosiglitazone and glimepiride (BMI <27 kg/m^2) or metformin (BMI >27 kg/m^2). IGT group received maximal doses while the conventional group received half the maximal doses. Insulin prescribed if HbA1c did not decrease below 6.0% in IGT group or 9.0% in the conventional group.	<2.8 mmol/L. 203/100 patient-years in IGT group. 52/100 patient years in the conventional group.	No difference in the rate of progression of microvascular complications, development of macrovascular complications or death.
ADVANCE[14]	11140 patients with type 2 DM and aged >55 years. These received IGT in a 1:1 randomised order. Median follow-up of 5 years.	IGT group: 4.4-6.1 mmol/L (80-110 mg/dL). Conventional group: 10-11.1 mmol/L (180-200 mg/dL).	<2.8 mmol/L. 2.7% in the IGT group vs. 1.5% in the conventional group.	No benefit of IGT on all-cause or cardiovascular mortality or the development of macrovascular complications. ARR in the rate of development of microvascular complications by 1.5%. Severe hypoglycaemia was associated with a 2- to 3-fold increase in cardiovascular mortality and macrovascular complications.
ACCORD[15]	10251 patients with type 2 DM and a mean age of 62 years. These received IGT in a 1:1 randomised order. Mean follow-up of 3.5 years.	IGT group: Target HbA1c levels of <6.0%. Conventional: Target HbA1c of 7.0-7.9%.	<2.8 mmol/L requiring medical or any assistance. 16.2% (10.5%) in the IGT group and 5.1% (3.5%) in the conventional group requiring any (or medical) assistance.	Trial halted due to increased mortality in the IGT group (257 deaths) vs. conventional group (203 deaths), p=0.04.

had to be terminated early after a mean duration of 3.7 years due to concerns with the safety of the study. In fact, it was noted that the rate of severe hypoglycaemia was extremely high in the IGT group, 16.2% vs. 5.1% in the standard therapy group.

Figure 1.5. Median Glycosylated Haemoglobin (HbA1c) Levels During the ACCORD Study. (Note the Acute Decrease Following Trial Initialisation)



Bars denote interquartile ranges (IQR). Reproduced with permission.[15]

A 22% increase in the relative risk of all-cause mortality in the IGT group was observed which equated to an absolute increase of 1%. A total of 257 deaths (5% in total and equivalent to 1.42% per year) occurred in the IGT group (all-cause) in comparison to 203 deaths (4% in total and equivalent to 1.14% per year) in the standard therapy group (p=0.04). Similarly, there was a 1% increase in the absolute risk of CVM (p=0.02) in the group receiving intensive therapy. The authors recognised this association of increased harm between IGT and patients with type 2 DM however, the mechanistic basis of this finding remained unexplained.

Table 1.5 Mortality Rates and Episodes of Hypoglycaemia Among All Participants of the ACCORD Study. (The Mortality Rates are Increased as Hypoglycaemic Episodes Become More Frequent).[48]

Groups	Hypoglycaemic Episodes Requiring Assistance (Medical and Non-medical)		Hypoglycaemic Episodes Requiring Assistance (Medical)	
	No Hypoglycaemic Events	One or More Hypoglycaemic Events	No Hypoglycaemic Events	One or More Hypoglycaemic Events
All Participants	377/9122 (4.13%)	74/1072 (6.90%)	400/9491 (4.21%)	51/703 (7.25%)
Standard Therapy	176/4832 (3.64%)	21/256 (8.20%)	180/4913 (3.66%)	17/175 (9.71%)
Intensive Glycaemic Therapy	201/4090 (4.69%)	53/816 (6.49%)	220/4578 (4.81%)	34/528 (6.43%)

A post-hoc analysis of the ACCORD study was published two years after the initial trial was halted.[48] A total of 451 deaths (4.4%, excluding 9 deaths with incomplete follow-up data) had occurred during the 3.5 years. Higher mortality rates were observed in the group receiving IGT in comparison to the group on standard therapy (4.97% vs. 3.87%). As shown in the Table 1.5, mortality rates were higher in the group receiving IGT in the sub-groups requiring assistance for hypoglycaemic events. In addition, mortality rates were also higher in the sub-groups with one or more hypoglycaemic episodes in comparison to the sub-groups with no previous episodes of hypoglycaemia. Furthermore, there was 28% relative risk increase in the IGT sub-group with a history of hypoglycaemic events that required medical assistance. Finally, there was significant increase in the mortality rates in the IGT and standard therapy patients with more frequent episodes of severe hypoglycaemia. For example, in the IGT group the mortality rate in patients with a history of one episode of hypoglycaemia vs. three or more episodes were 6% and 12% respectively.

More recently, a 5-year follow-up of the ACCORD was published. IGT was associated with a 29% and 19% increase in cardiovascular and all-cause mortality rates respectively, an effect that persisted 5-years after cessation of the intervention.[49] The authors concluded that an IGT strategy to aim for an HbA1c of <6.0% was not safe and was associated with more harm in comparison to a target HbA1c level of 7.0%.

It is difficult to understand at first the reason behind such conflicting results produced by the ADVANCE, VADT and ACCORD trials in comparison to the earlier trials such as the DCCT and UKPDS. However, on careful

examination several dissimilarities begin to emerge in the study designs and methodologies of the above trials which could help explain these differences.

1. Both DCCT and UKPDS studies included patients with new-onset DM.[9, 10, 33] In contrast, the participants had a mean duration of DM for 8 years (ADVANCE), 10 years (ACCORD) and 11.5 years (VADT).[13-15] Using Cox-regression analysis, recent evidence has shown that the duration of DM independently predicts short-term (5-year) risk of CVD. Therefore, the participants in three latter trials had increased risk of future cardiovascular event-rate at baseline. This association has been recognised by the ACCORD investigators.[49]

2. The study population in the VADT, ACCORD and ADVANCE studies were more elderly (>60 years) in comparison to UKPDS (54 years) and DCCT (27 years).

3. Between 32-40% of the study population taking part in ACCORD, ADVANCE and VADT had established CVD at baseline and therefore had a higher risk of future coronary events in comparison to DCCT (0%) and UKPDS (5%).

4. The target HbA1c for ACCORD, ADVANCE and VADT studies was <6.5% in comparison to ≤7.0% for UKPDS and DCCT trials. Therefore, these patients were at increased risk of having hypoglycaemia. This is supported by

evidence from the DCCT study which showed a 22% greater risk of severe hypoglycaemia with every 10% decrease in HbA1c levels.[46]

5. In comparison to the VADT and ADVANCE studies, the rate of decrease in the HbA1c levels in the group receiving IGT was the steepest in the ACCORD study. Perhaps, unsurprisingly, this translated into the much higher rates of severe hypoglycaemia in the IGT group vs. the other studies (Table 1.4) and thus could have influenced the mortality rates.

6. A significant proportion of patients receiving IGT in the VADT (not specified) and ACCORD (91%) studies received rosiglitazone, a thiazolidinedione. This drug has been associated with increased cardiovascular mortality and has subsequently been withdrawn from Europe while in the USA its prescription is extremely restricted.

1.7 Summary of Existing Evidence Regarding the Usage of Intensive Glycaemic Therapy in the Outpatient Setting

In summary, there is strong evidence from the DCCT and UKPDS studies that IGT is associated with a decrease in micro and macrovascular complications, particularly death due to cardiovascular disease in young patients with type 1 and 2 DM with little or no history of antecedent cardiovascular complications. However, there is to date no study which has demonstrated that this beneficial effect of IGT can be translated to older patients with type 2 DM with or without existing cardiovascular disease. In addition, there is no

benefit from adapting more strict glycaemic regimes (aiming for a HbA1c below 6.0%) on all-cause or CV mortality. In fact such a strategy has been associated with an increased risk of all-cause and CVM as seen in the ACCORD study.

1.8 Trials showing benefit of Intensive Glycaemic Therapy in patients with Hyperglycaemia during Acute Illnesses

The prevention of hyperglycemia during acute illness with IGT has been shown to improve survival in patients with and without coronary artery disease as shown in Table 1.6. In the Diabetes Mellitus Insulin-Glucose Infusion in Acute Myocardial Infarction (DIGAMI) study, 620 patients with DM presenting with acute myocardial infarction were randomly assigned to receive either IGT (in the form of intravenous followed by subcutaneous insulin) or conventional treatment for hyperglycemia (>11.0 mmol/L).[18] Patients were risk stratified into high- and low-risk groups on the basis of age >70 yrs, history of MI, history of heart failure and ongoing treatment with digoxin. After 1-year, a significant 29% relative risk reduction in all-cause mortality was observed with IIT (p=0.027). Furthermore, the greatest benefit on survival was observed in the low-risk group with no previous exposure to insulin (relative risk reduction of 52%, p=0.046). This benefit was confirmed in a further analysis of the DIGAMI trial which recorded an absolute risk reduction of 11% in all-cause mortality with the use of IGT after a 3.5-year follow-up.[19] Fifteen percent of patients on IGT experienced hypoglycaemic events including mild episodes

(none in the conventional group). However, very few patients experienced severe hypoglycaemic episodes. Furthermore, these were judged severe enough in only 5 patients to necessitate the cessation of IGT.

In a subsequent study of 1548 patients, Van den Berghe examined the potential benefit of IGT in patients admitted to a surgical intensive care unit (ICU) with hyperglycaemia.[20] More than 95% of participants had had some form of major surgery with the majority of cases being cardiac surgery (63% in IGT group and 62% in conventional group). The primary end-point was all-cause in-hospital mortality. There was a 3.7% absolute risk reduction in the total in-hospital mortality which represented a 34% relative risk with the use of IGT. This benefit was more pronounced in the group with a prolonged ICU stay (>5days, $p<0.01$) demonstrating a 48% relative risk reduction in death during intensive-care and an absolute risk reduction of 9.5% in in-hospital mortality. Therefore, the number needed to prevent one in-hospital mortality was 11 in the IGT group in comparison to the conventional treatment group. The prevalence of DM in both groups was close to 13%. Furthermore, IGT significantly reduced the episodes of septicaemia by 46% ($p=0.003$), requirement of haemodialysis or haemofiltration by 42% ($p=0.007$, number of patients requiring >14 days of ventilatory support 39% ($p=0.003$), patients requiring >14 days of ICU stay by 29% ($p=0.01$) and number of blood transfusions per patient by 50% ($p<0.001$). Thirty nine patients (5%) in the IGT group experienced severe hypoglycaemia (defined as <2.2 mmol/L) in comparison to 6 patients (0.8%) in the conventional therapy group. There were no episodes of seizures or haemodynamic complications.

More recently, Krinsley and co-investigators examined the effect of IGT on patients admitted to their intensive care unit over a 7-year period.[50] Patients were divided into three groups; index cases experiencing severe hypoglycaemia (102 patients, <2.2 mmol/L), case-matched control patients (306 patients at a ratio of 1:3 to the index cases) who did not have severe hypoglycaemia, and patients who did not experience severe hypoglycaemia (5263 patients). There was no set glucose control protocol for the first 4 years following which, the glucose target range was 4.4-7.8 mmol/L except for the last six months, when the control was made more stringent (4.4-6.9 mmol/L). The in-hospital mortality rate was 56% (57 patients) for patients experiencing severe hypoglycaemia in comparison to 40% to the case-matched cases while it was 16.3% in the patients who did not experience severe hypoglycaemia. The index cases and case-matched controls were well-matched for age, Acute Physiology and Chronic Health Evaluation (APACHE II) score, percentage of diabetics, patients requiring mechanical ventilation and patients in septic shock. The total mortality was 19.5% in the group which did not receive IGT and 14.6% in the group that did receive IGT. This represented an absolute reduction of 4.83% in the total mortality equating to a 25% relative risk reduction in in-hospital mortality. More importantly, on multiple regression modelling, severe hypoglycaemia emerged as an independent factor associated with increased mortality (odds ratio 2.28 [1.41-3.70, 95% CI], p=0.0008) along with the need for mechanical ventilation (odds ratio 2.43 [2.00-2.95, 95% CI], p<0.0001). Twenty eight patients died within 24-hours following a severe hypoglycaemic event. In comparison to the 29 patients who died after the initial 24-hours, the 28 patients who had died earlier, had higher APACHE II scores suggesting a

more serious degree of illness (p=0.0043). In addition, further subgroup analyses revealed that non-survivors who had had experienced severe hypoglycaemia had a 10% and 400% increase in in-hospital mortality if they had a high APACHE II score (p=0.002), or were requiring mechanical ventilation (p=0.0127), at the time of severe hypoglycaemia respectively. The investigators noted that out of the total index cases, 62 had had severe hypoglycaemia during the IGT period (comprising 2.3% of the entire cohort receiving IGT) out of which 25% died. The investigators noted that by quadrupling the observed severe hypoglycaemic rate to 9.3% (248 patients) and increasing the mortality attributable to severe hypoglycaemia to 75% (assuming that 186 out of the 248 patients died), the benefit of IGT on total cohort mortality rates would be nullified.

In another study, Furnary and co-workers examined the effect of peri-operative IGT (insulin infusion) on in-hospital mortality in patients with DM undergoing coronary artery bypass grafting (CABG) in a single centre non-randomised study.[51] A total of 3554 patients were included in this study with 2612 patients receiving IGT. Plasma glucose target levels were reduced in step-wise fashion over several years to maintain the plasma glucose between 8.3-11.1 mmol/L (1991-1998), 6.9-9.7 mmol/L (1999-2001) and finally 5.5-8.3 mmol/L (2001). The control group consisted of 942 patients receiving subcutaneous insulin as conventional therapy to maintain the plasma glucose below 11.1 mmol/L. IGT resulted in a 57% relative risk reduction in all-cause peri-operative absolute mortality which was 50% after adjusting for other risk factors with a strong predictive model (c-statistic 0.9). IGT added an

independently protective effect against all-cause peri-operative mortality after multivariate analysis (odds ratio 0.5, p=0.005).

As there was no difference in peri-operative non-cardiac mortality (0.9% in IGT group vs. 1.1% in conventional group p=0.5), the benefit on survival was driven entirely by a decrease in cardiac-related deaths. Furthermore, this study also demonstrated that in comparison to patients receiving IGT the rate of immediate post-operative mortality (within 48 hours of surgery) secondary to cardiac causes (defined as arrhythmia or heart failure) was 6- and 15-fold higher in patients with a mean post-operative glucose of >12 and 14 mmol/L respectively. According to the authors extensive resources had been utilised to train the staff in order to familiarise them with the insulin protocol to avoid episodes of severe hypoglycaemia. Furthermore, the target level for plasma glucose in the IGT group was reduced over several years in a step-wise manner as detailed above to avoid episodes of severe hypoglycaemia. Although, the exact rates of severe hypoglycaemia were not published, these were considered to be very low.

The evidence from these studies has led to the development of guidelines from the American Heart Association (AHA) and American Diabetes Association (ADA) which recommend IGT for in-patient control of hyperglycaemia but also add the caveat that “care should be taken to avoid hypoglycaemia.”[17]

Table 1.6 Studies that showed benefit of IGT in patients with hyperglycemia during acute illnesses.

Study	Design	Glycemic Targets	Hypoglycemia (definition and rates)	Outcome
Malmberg et al[18]	620 patients post AMI with glucose > 11.0 mmol/L (200 mg/dL) ± history of DM.	IGT group: 7-10 mmol/L (126-180 mg/dL) Conventional group: Not specified	<4.0 mmol/L (72 mg/dL) 10% symptomatic in IGT group. 0% in conventional group	29% RRR in 1-year mortality IGT group vs. conventional group (p=0.027)
Van den Berghe et al[20]	1548 primarily surgical patients on ICU. N= 765 (IGT) N=783, (conventional)	IGT group: 4.4-6.1 mmol/L (80-110 mg/dL). Conventional group: 10-11.1 mmol/L (180-200 mg/dL).	<2.2 mmol/L (40 mg/dL) 5% in IGT group 0.8% in conventional group	3.7% ARR in in-hospital mortality p<0.01 3.4% ARR in mortality on ICU (p<0.01)
Krinsley et al[50]	5365 patients admitted to ICU. These received IGT in a 1:1 non-randomised order.	IGT group: 4.4-7.8 mmol/L (4.4-6.9 for last 6 months). Conventional: No target range.	<2.2 mmol/L (40 mg/dL). 2.3% in the IGT group and 1.5% in the conventional group.	4.83% ARR in in-hospital mortality. Severe hypoglycaemia was an independent predictor of mortality.
Furnary et al[51]	3554 patients with DM requiring CABG receiving IGT peri-operatively up to day 3 post-operation	IGT group: 1991-1998 8.3-11.1 mmol/L (150-200 mg/dL) 1999-2001 6.9-9.7 mmol/L (124-175 mg/dL) 2001 5.5-8.3 mmol/L (99-150 mg/dL) Conventional group: <11.1 mmol/L (200 mg/dL)	<2.2 mmol/L (40 mg/dL) Absolute rates not published	57% RRR in all-cause mortality in the IGT group (p=0.001)

AMI = acute myocardial infarction, ARR = absolute risk reduction, DM = diabetes mellitus, IIT = intensive insulin therapy, RRR = relative risk reduction.

1.9 Trials that failed to show benefit with Intensive Glycaemic Therapy during Acute Illnesses

More recently, several trials have further examined the effect of IGT on patients with acute illnesses with and without CAD. In contrast to earlier trials, these have failed to confirm this beneficial effect of IGT on survival. Two studies have shown a 'neutral' effect on survival with IGT while 3 other groups have observed an increase in mortality as shown in Table 1.7. The first of these was the multi-centre, prospectively randomized DIGAMI 2 trial.[21] It examined the effect of IGT (insulin) on 2-year mortality rates in patients with type 2 DM presenting with an acute myocardial infarction (AMI). The patients were recruited into three groups with group 1 receiving 24 hours of insulin infusion followed by subcutaneous insulin (IGT) to maintain a fasting glucose of 5-7 mmol/L and random glucose of <10 mmol/L. Group 2 received intravenous insulin for 24 hours followed by 'standard' therapy and group 3 only received 'standard' therapy. It did not show that IGT was more advantageous in comparison to conventional therapy for the prevention of hyperglycaemia in patients with type 2 DM presenting with AMI. However, there were several inherent flaws in the study design. DIGAMI 2 trial was statistically underpowered as it managed to recruit 1253 patients in comparison to an intended sample size of 3000 patients. Also, the target fasting glucose of 5-7 mmol/L for group 1 patients receiving IGT was never achieved. Furthermore, there was no standard protocol or target level of glucose for the patients receiving standard therapy as this was left to the treating physician's discretion. There was no statistical difference in the glucose levels and HbA1c levels

amongst the three groups. Therefore, the study never answered the question that it set out to address and in fact demonstrated that for a similar glycaemic control there was no difference in the use of insulin (intravenous or subcutaneous) or oral hypoglycaemic therapy in patients with type 2 DM and presenting with AMI. Finally, the rate of symptomatic hypoglycaemia (<3.0 mmol/L) was 13- and 10-fold higher in groups 1 and 2 respectively in comparison to group 3 and it is likely that this could have contributed to the overall 'negative' result.

Table 1.7 Studies that did not show benefit of IIT in patients with hyperglycemia.

Study	Design	Glycemic Targets	Hypoglycemia (definition and rates)	Outcome
Malmberg et al[21]	1253 patients post AMI with established type 2 DM or admission glucose >11.0 mmol/L (200 mg/dL)	IIT group: 7-10 mmol/L on intravenous insulin, fasting 5-7 mmol/L (90-126 mg/dL) and pre-meal <10 mmol/L (180 mg/dL) on subcutaneous insulin. Conventional group: Not specified	<3.0 mmol/L (54 mg/dL) 12.7% in IIT group. 1% in Conventional group	No difference in mortality or morbidity between groups
Van den Berghe et al[22]	1200 patients admitted to medical ICU. N=595 (IIT) N=605 (Conventional)	IIT group: 4.4-6.1 mmol/L (80-110 mg/dL). Conventional group: 10-11 mmol/L (180-200 mg/dL)	<2.2 mmol/L (40 mg/dL) 18.7% in IIT group 3% in Conventional group	No benefit of IIT on total mortality rate.
Brunkhorst et al[53]	488 patients N=247(IIT) N=241 (Conventional)	IIT group: 4.4-6.1 mmol/L (80-110 mg/dL) Conventional group: 10-11 mmol/L (180-200 mg/dL)	<2.2 mmol/L (40 mg/dL) 17% in IIT group 4.1 % in Conventional group	Non significant increase in 90-day mortality with IIT (39.7% vs. 35.4%, p=0.31) Significant increase in life-threatening hypoglycemic events with IIT (5.3% vs. 2.1%, p=0.05)
NICE-SUGAR[23]	6104 patients admitted to medical ICU. N=3054 (IIT) N=3050 (Conventional)	IIT group: 4.5-6.0 mmol/L (81-108 mg/dL) Conventional group: <10 mmol/L (180 mg/dL)	<2.2 mmol/L (40 mg/dL) 6.8% in IIT group 0.5% in Conventional group	2.6% increase in absolute risk of 90-day mortality, p=0.02 CVM 41.6% in IIT group vs. 35.8% in Conventional group, p=0.02

AMI = acute myocardial infarction, DM = diabetes mellitus, IIT = intensive insulin therapy,
NICE-SUGAR = The Normoglycemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulation Study.

Van den Berghe and co-workers studied the outcome of IGT on 1200 patients admitted to the medical ICU.[22] They found that there was no benefit of IGT on the in-hospital mortality rate which was in direct contrast to their earlier results in surgical ICU patients. In the group receiving conventional therapy, insulin infusion was only initiated once the plasma glucose levels were >12.0 mmol/L. In contrast, in the intensive therapy group insulin infusion was commenced once the plasma glucose was >6.1 mmol/L with the target range of 4.4-6.1 mmol/L. The number of patients with a history of DM in both groups did not differ (16% in the conventional group vs. 18% in the intensive group, p=0.41). There was a 6-fold increase in the rate of severe hypoglycaemia (>2.2mmol/L) in patients receiving IIT in comparison to patients on conventional treatment (18.7% vs. 3%, p<0.001). Furthermore, patients on IGT were at a 5-fold greater risk of experiencing more than 1 episode of severe hypoglycaemia. The reason for the failure for IGT to show benefit is not clear. One major factor may be the extremely high rates of severe hypoglycaemia. For example, the medical ICU patients had a much higher rate of hypoglycaemia (19%) in comparison to surgical ICU patients (5%) while receiving IGT. Hypoglycaemia emerged as an independent predictor of mortality.

The Efficacy of Volume Substitution and Insulin Therapy in Severe Sepsis (VISEP) study was designed to look at the effect of IGT on the 28-day mortality of patients with sepsis admitted to ICU with hyperglycaemia.[52] The conventional treatment group received insulin once their plasma glucose exceeded 11.1 mmol/L with a target level of <10.0 mmol/L. In contrast, the IGT group received insulin once the glucose levels exceeded 6.1 mmol/L (with

a target range of 4.4-6.1 mmol/L). The IGT arm was terminated early following the recruitment of 488 patients due to a 6-fold increase in severe hypoglycaemic events (<2.2mmol/L). Furthermore, IGT did not improve 90-day survival and in fact, insulin-induced hypoglycaemia was shown to independently increase the 28-day mortality-rate by 3-fold ($p<0.001$) in a regression analysis. The 90-day mortality rate was greater in the IGT group 40% vs. 35.4%, with a relative risk increase of 13%, although this did not reach statistical significance ($p=0.3$). It is possible that with a larger cohort of patients this difference would have been significant. Serious hypoglycaemic episodes were 3-fold higher in the IGT group vs. the conventional group ($p=0.005$) while life threatening hypoglycaemic events were significantly greater in the IGT group in comparison to the conventional group (5.3% vs. 2%). Finally, IGT-induced hypoglycaemia was associated with a prolonged stay on ICU (2.4% vs. 0.3%, $p=0.05$).

The Normoglycemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulation Study (NICE-SUGAR) is the largest study to-date which examined the effect of IGT on the 90-day mortality in 6104 patients admitted to ICU.[23] The group receiving IGT had glucose target range of 4.5-6.0 mmol/L while the conventional therapy group had a target range of 8.0-10.0 mol/L. They demonstrated a significant increase in 90-day all-cause mortality (27.5% in IGT vs. 24.9% in the conventional group). This represented an increase of 2.6% in the absolute mortality rates. Furthermore, the investigators reported that deaths from cardiovascular causes were significantly higher in the group receiving IGT in comparison to the control group (41.6% vs 35.8% respectively, number needed to harm=17). More importantly, the rates of severe

hypoglycaemia (<2.2 mmol/L) were extremely high with the use of IGT (up to 14-fold increased risk) in comparison to patients receiving conventional therapy. The authors recognised this association between IGT and increased all-cause and cardiovascular mortality suggesting that more strict glycaemic control had adverse effects on the cardiovascular system. They also highlighted the need to understand the physiological basis of these results in future research.

1.10 Summary of Existing Evidence Regarding the Usage of Intensive Glycaemic Therapy in the Acute Setting

In summary, earlier studies exploring the effects of IGT in acutely unwell patients provided encouraging results with a decrease in in-hospital mortality. However, subsequent studies aiming for a more strict glycaemic control (4.4-6.0 mmol/L) have been disappointing. In fact, the NICE-SUGAR trial suggested that a more strict glycaemic control was associated with an increase in CVM and was halted early. Importantly, patients were well-matched for baseline characteristics and severity of illness. This association has been duly recognised and such a strategy is not advised by the AHA or ADA.[39]

1.11 Acute Hypoglycaemia and Cardiovascular Mortality

We have thus far examined the association between IGT-induced hypoglycaemia and CVM. However, there is accumulating evidence suggesting a strong association between spontaneous hypoglycaemia and adverse cardiac events (defined by unstable angina, acute myocardial infarction (AMI) and cardiovascular death). For example, Gandevia observed in an unselected group of 55 patients with DM at autopsy that 14 had died of AMI and nearly half of these deaths had coincided with an antecedent episode of hypoglycaemia.[27] Furthermore, Desouza and co-workers examined the relationship between hypoglycaemia and myocardial ischemia in type 2 DM using continuous electrocardiogram (ECG) monitoring.[32] Patients recorded their blood glucose readings four times a day over a 72-hour period and also any symptoms of chest pain or hypoglycaemia. A total of 54 hypoglycaemic episodes were recorded in 21 patients of which 19% were associated with angina, and half had accompanying ischemic ECG changes. No ECG changes were recorded during periods of normo- or hyperglycaemia. The authors concluded that hypoglycaemia was more likely to be associated with cardiac ischemia than normal or elevated blood glucose levels. However, the mechanism for this observed association was not fully understood.

Wei and co-workers looked at the association between low fasting glucose (<3.9 mmol/L) and CVM in the general population.[53] The prospective study included a total of 40,069 volunteers with a mean age of 43 years and an average follow-up of 8 years. Majority of the participants (93%) had no history of coronary disease and only 4% had established DM. Following

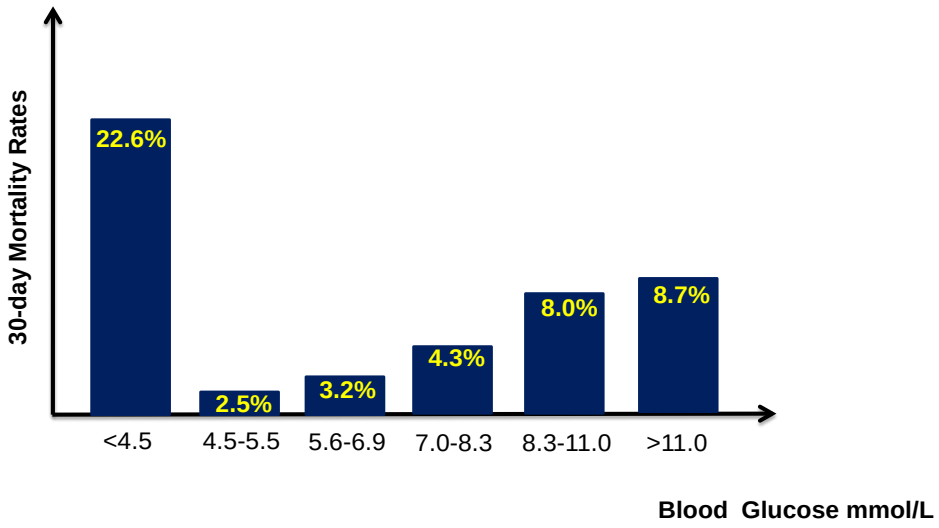
multivariable analysis it was demonstrated that a low fasting plasma glucose (measured after a 12-hour fasting period) was associated with 3.3-fold and 2.3-fold increase in CVM and all-cause mortality respectively ($p<0.0001$ for both associations). Fifty two percent of deaths attributable to CVM were secondary to AMI in patients with low fasting glucose levels.

Subsequently, Fisman recorded the association between fasting hypoglycaemia (<3.8 mmol/L) and all-cause mortality in patients with established CAD.[54] The study included 14,670 patients with a mean age ≈60 years and an average follow-up of 8 years There was 69% increase in the relative risk of all-cause mortality in patients with fasting hypoglycaemia vs. patients with normoglycaemia ($p<0.0001$). Furthermore, consistent with previous studies, fasting hyperglycaemia was associated with an increased risk of all-cause mortality by 2-fold. Finally, following multivariable adjustment there was an increased risk of CVM in patients experiencing fasting hypoglycaemia in comparison to patients with normal glucose levels with a hazard ratio of 1.30 (0.73-2.30 95% CI).

In a recent meta-analysis the association between spontaneous hypoglycaemia on admission and adverse outcomes (defined as 30-day mortality and myocardial infarction) following ST-elevation myocardial infarction (STEMI) was examined.[55] Only 8% of the patients had known DM. Patients with an admission blood glucose <4.5 mmol/L had a 3-fold increased rate of adverse outcomes in comparison to patients with euglycaemia (5.6-7.0 mmol/L). Furthermore, this association was more significant in the cohort of patients with known DM with an 18-fold increased risk of adverse outcomes. More importantly, patients with a Thrombolysis in Myocardial Infarction (TIMI) risk

score >4 and concomitant hypoglycaemia had a >11-fold risk of death within 30 days vs. those with a similar TIMI risk score but normal glucose levels. These data suggest that the detrimental effects of hypoglycaemia may be more pronounced in patients with advanced coronary disease.

Figure 1.6 U-shaped relationship between blood glucose levels and adverse cardiac outcomes in high-risk patients with TIMI risk score >4. Note the high 30-day mortality rates in patients suffering from hypoglycaemia. Reproduced with permission.[55]



Furthermore, Svensson recorded the 2-year all-cause mortality in 713 diabetic patients admitted with unstable angina or Non-ST elevation myocardial infarction (Non-STEMI).[56] All patients had their blood glucose measured

serially following admission. Forty four patients had at least one documented episode of hypoglycaemia (<3.0 mmol/L) during hospitalization. Spontaneous hypoglycaemia and hyperglycaemia were associated with a 2-fold and 48% relative increase in the 2-year all-cause mortality rate respectively.

Finally, Kosiborod examined the association between spontaneous hypoglycaemia and iatrogenic hypoglycaemia (induced by insulin) in 7820 patients admitted with AMI.[57] Hypoglycaemia was defined as blood glucose level below 3.3 mmol/L. Patients with hyperglycaemia on admission and a confirmed diagnosis of AMI were included in this study. The primary outcome defined as all-cause in-hospital mortality. The relative risk of hypoglycaemia was increased by four-fold with the use of insulin (11.4% vs. 2.9% in patients on insulin in comparison to patients not receiving insulin respectively). The study showed that patients experiencing spontaneous hypoglycaemia had a significant increase in in-hospital mortality in comparison to patients who did not, 18.4% vs. 9.2% respectively ($p<0.001$). However, in contrast to earlier studies, patients receiving insulin had no difference in mortality rates whether they experienced hypoglycaemia or not (10.4% vs. 10.2%, $p=0.92$ respectively). However, it is important to note that the study had several limitations which are listed below.

1. It was a retrospective analysis of in 40 hospitals across United States and therefore local practices may have varied. It was not clear whether there was a trend of more hypoglycaemic events in some hospitals in comparison to the others as only 3% of patients experienced spontaneous hypoglycaemia (136 out 4639 patients).

2. It was not a randomised controlled trial and the methodology for patient selection to receive either insulin or oral hypoglycaemic agents following AMI was not defined. This is important as again local practices may have varied and selection bias may have been an important confounder. For example, 80% of the patients receiving insulin had established DM in comparison to only 30% not receiving insulin. Therefore, it possible that any potential harm from insulin-induced hypoglycaemia was off-set by the beneficial effects of improved glycaemic control.

3. The mean glucose levels were significantly different between patients developing hypoglycaemia on insulin in comparison to patients who did not (9.7 mmol/L vs. 11.2 mmol/L, $p<0.001$). This suggests that patients not experiencing hypoglycaemia on insulin never achieved a target glucose as recommended by national guidelines.[16] In fact, the observed in-patient mortality rate of 10.3% in patients receiving insulin is considerably high in comparison to recent trials.[38] Therefore, these data suggest that any potential harm of hypoglycaemia could have been 'neutralised' by controlling hyperglycaemia.

1.12 Physiological Effects of Hypoglycaemia on the Cardiovascular system

Hypoglycaemia provokes a number of physiological responses. These include the release of counter-regulatory hormones to restore normal blood glucose levels and several haemodynamic changes.[58] More than five decades ago, French and Kilpatrick studied the effects of hypoglycaemia on the

cardiovascular system following an injection of 0.15unit/k.g of body weight of insulin.[59] Their study included 12 healthy subjects, 8 subjects with hypertension and 5 subjects who had had sympathectomies for the treatment of hypertension. They observed a significant increase in the heart rate and systolic blood pressure with a drop in the diastolic blood pressure and subsequent widening of the pulse pressure in healthy subjects as well as subjects with hypertension. Interestingly, although the volunteers with bilateral sympathectomies demonstrated a significant increase in their heart rates with a concomitant decrease in diastolic blood pressure, they also exhibited a drop in systolic blood pressure in contrast to the other groups following hypoglycaemia. Urinary adrenaline levels were measured before and 3-hours following the acute hypoglycaemic reaction in 3 patients each with hypertension with and without bilateral sympathectomies. The hypertensive volunteers demonstrated an 8- to 17-fold increase in urinary adrenaline excretion an effect not present in volunteers who had undergone bilateral sympathectomies. The authors concluded that release of adrenaline in response to hypoglycaemia was responsible for a majority but not all of the haemodynamic responses seen. In a subsequent study, Allwood and co-workers measured blood flow though the peripheral circulation using venous plethysmography in 17 normal subjects. They recorded a 5- and 2-fold increase in blood flow through the forearm and calf respectively during acute symptomatic hypoglycaemia.[60]

In non-diabetic individuals an intact glucagon response is essential to restore glucose levels following hypoglycaemia.[58, 61] However, there is direct evidence to suggest that this response is blunted in patients with DM. For example, Gerich and co-workers induced symptomatic hypoglycaemia in 15

healthy subjects and 6 patients with type 1 DM.[62] They recorded a 3-fold increase in the plasma glucagon levels ($p<0.001$) in the non-diabetic subjects following hypoglycaemia. In contrast, the patients with type 1 DM showed no change. Furthermore, the investigators also recorded an up to 14-fold increase in growth hormone levels with a small but significant increase in cortisol levels which were comparable in the diabetic and non-diabetic volunteers. These findings were subsequently confirmed in another study including 12 non-diabetic volunteers and 9 volunteers with type 1 DM.[58] Symptomatic hypoglycaemia was induced following a 0.15 units/k.g of bodyweight injection of short acting insulin. Their data showed that urinary adrenaline excretion in volunteers with type 1 DM was increased by 14-fold in response to symptomatic hypoglycaemia. The baseline and peak adrenaline levels were not different in the non-diabetic or diabetic volunteers. Importantly, the ratio of peak to baseline adrenaline levels were significantly higher in patients with type 1 DM in comparison to the healthy controls 18.8±6.2 vs. 5.6±1.3 ($p<0.01$). However, it is important to note that the baseline glucose levels were significantly higher in the patients with type 1 DM in comparison to healthy volunteers (13.7±4.8 mmol/L vs. 5.0±1.6 mmol/L). Furthermore, these were decreased to a nadir of 1.2±0.1 mmol/L and 1.3±0.1 mmol/L in the diabetic and non-diabetic volunteers respectively suggesting a larger magnitude of change in the blood glucose levels of the patients with DM. Finally, as seen in earlier studies, there was no change in the plasma glucagon levels in patients with type 1 DM in contrast to the non-diabetic volunteers which recorded a 2- to 3-fold increase.

Through a series of elegantly designed studies, Amiel et al showed that strict glycaemic control in type 1 DM was associated with a lower plasma threshold for the release of epinephrine in response to hypoglycaemia.[63] Seven young subjects with type 1 DM were included. These were studied on two separate occasions: the first with a conventional insulin regimen (HbA_{1C} 9.6±1.1%) and the second, following a period of IGT with insulin (HbA_{1C} 7.1±0.7%). The participants were admitted overnight and their insulin was maintained at around 5.0 mmol/L. The following morning a multi-step insulin clamp study was performed and the plasma glucose was decreased by 0.6 mmol/L to a nadir of 2.2 mmol/L. There was a 32% reduction in the glycaemic threshold for adrenaline release, 3.7±0.2 mmol/L during conventional treatment vs. 2.6±0.1 mmol/L during intensive treatment ($p<0.01$). Furthermore, there was 53% decrease in the peak adrenaline levels during hypoglycaemia following intensive treatment, 690±130 pg/ml (on conventional treatment) vs. 320±64 pg/ml (on IGT) ($p<0.01$). Finally, there was a significant decrease in the glycaemic threshold to induce an increase in the heart rate by 10 beats per minute as well as other symptoms such as sweating and 'feeling low'. It was therefore shown that with IGT, the counter-regulatory response to hypoglycaemia was impaired which could potentially increase the risk of future hypoglycaemic attacks. In a subsequent study the same investigators showed that patients with type 1 DM receiving conventional glycaemic control had a higher threshold for adrenaline release (3.3-3.9 mmol/L) in comparison to patients receiving IGT (2.2-2.4 mmol/L). This observation suggested an increased risk of repeated hypoglycaemic attacks following the implementation of IGT thereby potentially increasing the risk of future adverse events.

Hilsted and co-workers examined the effect of insulin-induced hypoglycaemia on the haemodynamic changes in seven healthy volunteers.[64] Hypoglycaemia was induced acutely following the injection of 0.15 units/k.g bodyweight. The mean heart rate increased by 20 beats per minute ($p<0.05$) and returned to pre-hypoglycaemic levels within 10 minutes. As seen in previous studies there was significant 10% increase in the mean SBP ($p<0.05$) during hypoglycaemia which returned to pre-hypoglycaemic levels within 20 minutes and a 20 mmHg decrease ($p<0.05$) in the mean DBP which returned to pre-hypoglycaemic levels within 90 minutes of the hypoglycaemic reaction. Cardiac output was measured using the acetylene wash-in method with a coefficient of variation of 5%. The investigators recorded a 50% increase in the cardiac output, an effect which lasted 1 hour. Furthermore, there was a significant increase in the peripheral blood flow (measured in the legs using venous plethysmography) which lasted 30 minutes. As expected, there was a 7- to 8-fold increase in the plasma adrenaline levels accompanied by a 2-fold increase in plasma noradrenaline levels ($p<0.05$). In a subsequent study Fisher examined the effect of insulin-induced hypoglycaemia on the left ventricular ejection fraction (LVEF) in 6 non-diabetic subjects.[65] Hypoglycaemia was induced following an injection of 0.15 units/k.g of bodyweight. The mean glucose levels fell from 4.6±0.1 mmol/L to 1.0±0.2 mmol/L and were associated with a 55-fold increase in adrenaline levels. The heart rate increased from 63±4 beats/minute to 87±3 beats/minute ($p<0.005$), SBP increased from 104±4 mmHg to 127±5 mmHg ($p<0.01$) and DBP decreased from 64±3 to 52±1 mmHg ($p<0.001$) within 15 minutes of the hypoglycaemic reaction. More importantly, there was a 50-60% increase in the LVEF and stroke volume

resulting in a 2-fold increase in the cardiac output in comparison to baseline (all p values <0.05).

In a separate study, Russell and group investigated the effect of insulin-induced hypoglycaemia on cardiac responses in 9 healthy controls and 9 patients with type 1 DM receiving IGT in the form of insulin (HbA1c 6.2±1%).[66] The subjects were randomised to have a hyperinsulemic euglycaemia (HE) clamp or hyperinsulinemic hypoglycaemia (HH) clamp on two separate occasions. The HE clamp consisted of a 20-minute baseline period (baseline measurements were made) followed by 2 hours of euglycaemia (5.0 mmol/L), at the end of which repeat measurements were made. HH clamp consisted of an initial 20-minute baseline period leading to 30 minutes of euglycaemia followed by a 30-minute stage during which blood glucose levels were decreased and finally a 60-minute hypoglycaemic phase (2.8 mmol/L). LVEF was measured using radionuclide angiography with a coefficient of variation of 1.7%. During hypoglycaemia, plasma adrenaline levels rose by 20-fold in the controls in comparison to a 8-fold increase in the patients with DM ($p<0.05$). Also noradrenaline levels were increased by 3-fold in the healthy volunteers as compared to a 60% increase in patients with type 1 DM ($p<0.05$) Furthermore, the control subjects demonstrated a small but significant 4% increase in LVEF during euglycaemia in comparison to baseline ($p<0.05$) which further increased during hypoglycaemia (13% increase vs. baseline, $p<0.001$ vs. baseline and $p<0.05$ vs euglycaemia). In contrast, in patients with type 1 DM there was a small increase in the LVEF of 7% in comparison to baseline ($p<0.05$ vs. baseline) which did not increase further during hypoglycaemia. The authors concluded that similar to a blunted counter-regulatory response, the cardiac

response to hypoglycaemia was also blunted in patients with type 1 DM receiving IGT which could increase the risk of adverse cardiac events in the clinical setting.

1.13 Myocardial Blood Flow Reserve in Diabetes

The volume of blood in the entire coronary circulation (epicardial arteries, arterioles, capillaries, venules and epicardial veins), is close to 12ml/100grams of heart muscle. At any given time, one-third of this blood is in the myocardium, in vessels smaller than 300 microns in diameter >90% of which are capillaries.[67] Furthermore, 90% of this 'myocardial blood volume' resides within the capillaries during systole while the remaining is in the arterioles and venules.[68]

Importantly, myocardial blood flow reserve (MBFR) is determined by measuring myocardial blood flow (MBF) at rest (basal flow) and under maximal hyperaemia (peak stress).[69-71] The ratio of MBF (peak stress) to MBF (rest) is equal to MBFR. In clinical practice, this can be measured by an intracoronary injection of either adenosine or acetylcholine or an intravenous infusion of dipyridamole or adenosine. In patients with CAD, the extent of the reduction in MBFR is directly related to the severity of stenosis.[72, 73] However, in patients with normal arteries on coronary angiography, a decreased MBFR is a marker of microvascular dysfunction. In such patients an MBFR of less than 2.0 is considered abnormal.[74]

There is sufficient evidence that demonstrates that MBFR is reduced in DM. Nitenberg and co-workers were amongst the very first to measure MBFR

using intracoronary Doppler during coronary angiography in 11 patients with DM.[75] They used acetylcholine as the vasodilating agent in patients with type 1 (n=6) and type 2 DM (n=5) with angiographically normal coronary arteries. They demonstrated that MBFR was 20% less ($p<0.02$) in patients with DM in comparison to healthy controls (n=7) which was attributed to microvascular dysfunction. Furthermore, these results were confirmed in another larger study including 24 patients with DM which showed a 24% decrease in MBFR ($p<0.001$) in comparison to control subjects (n=31).[76] However, there were several confounding factors in these studies such as varying blood glucose levels, hypertension and hyperlipidaemia all of which have been shown to independently reduce MBFR.[77-80] The investigators repeated the analysis after excluding patients with known hypertension (which comprised the majority of the subjects) and found that MBFR was less in patients with DM (2.7±0.2, n=4) vs. control subjects (3.6±0.2, n=12, $p<0.05$). In addition, coronary vascular resistance was measured following an increase in heart rate induced by rapid atrial pacing. The heart rates increased in the patients with DM (increase by 45±1 per minute) and controls equally (increase by 48±2 per minute). A normal haemodynamic response would have been arteriolar vasodilatation with decrease in coronary vascular resistance. However, the decrease in coronary vascular resistance was less in the patients with DM (14±3%) vs. the controls (24±2%, $p<0.05$) suggesting altered myocardial vascular reactivity.

In a subsequent study, Pitkänen and co-workers used positron emission tomography (PET) to show that MBFR was decreased in patients with type 1 DM (n=11) in comparison to control subjects (n=12) during hyperinsulinemic euglycaemic clamps.[81] Resting MBF was not different between the two groups

(0.88±0.25 ml/min/g, DM) vs. (0.84±0.18 ml/min/g, controls). However, peak MBF was less in the patients with type 1 DM as compared to the controls (3.2±1.6 ml/min/g vs. 4.4±1.4 ml/min/g, $p<0.05$). As expected, MBFR was 28% less in the DM group vs. controls (3.8±1.7 vs. 5.3±1.9, $p<0.05$). In addition, coronary vascular resistance was calculated by dividing the mean arterial blood pressure by the MBF at rest and stress. There was no difference in the coronary vascular resistance at rest between the two groups. However, at peak stress coronary vascular resistance was less in patients with type 1 DM (31±12 mmHg x min x g/ml) in comparison to the controls (54±32 mmHg x min x g/ml, $p<0.05$). This was consistent with previous evidence suggesting impaired myocardial vascular resistance in the patients with DM. However, it is important to note that all the participants in this study were males. Using PET Yokoyama had earlier shown that MBFR was significantly less in male patients with type 2 DM (2.35±0.84) in comparison to age-matched female patients with type 2 DM (3.18±0.79, $p<0.05$).[82] Consistent with earlier studies MBFR was less in patients with type 2 DM (2.77±0.85) vs. controls (3.80±1.00, $p<0.01$). This was secondary to a reduction in peak MBF (184±99 ml/min/100g) in the DM patients in comparison to the controls (262±120 ml/min/100g) as the resting MBF was not different between the groups.

In a separate study, Akasaka investigated the effects of microvascular complications on MBF and MBFR.[83] The study consisted of 18 patients with type 1 DM and retinopathy (background and pre-proliferative), 11 type 1 DM patients without retinopathy and 15 control subjects. Intracoronary Doppler guide wire was introduced into the left anterior descending artery to acquire the data. The resting MBF was increased in the patients with and without

retinopathy (58±16 ml/min and 45±12 ml/min) vs. controls (37±12 ml/min, $p<0.05$ for both comparisons). Also the resting MBF was higher in the group with retinopathy as compared to the group without retinopathy ($p<0.05$). Peak MBF was decreased in the group with and without retinopathy (107±23 ml/min and 116±18 ml/min) vs. controls (136±17 ml/min, $p<0.05$ for both comparisons). This translated into a significant reduction in MBFR in the diabetic group with retinopathy (1.9±0.4, $p<0.01$ vs. controls and diabetics without retinopathy). Also, MBFR was reduced in the group without retinopathy (2.8±0.3) in comparison to controls (3.3±0.4, $p<0.01$). These changes were more marked in the patients with preproliferative retinopathy in comparison to the patients with background retinopathy. For example MBF at rest was higher in the group with preproliferative retinopathy (62±16 ml/min vs. 52±15 ml/min, $p<0.05$) with decreased MBF at peak stress (102±11 ml/min vs. 114±16 ml/min, $p<0.05$) and reduced MBFR (1.6±0.2 vs. 2.3±0.2, $p<0.01$). In fact, MBF (both at rest and peak stress) and MBFR were not different between the patients with background retinopathy and those without retinopathy suggesting that the presence of advanced microvascular complications was associated with altered myocardial vascular reactivity. In a subsequent study, Carli and co-workers demonstrated that MBFR was comparably decreased in patients with type 1 and type 2 DM in comparison to control subjects.[80]

The effects of glycaemic control on MBFR are less clear. Two studies have examined this aspect. Sundell studied nine patients with type 1 DM on two separate occasions using PET.[84] On one occasion the subjects were studied with a strict glycaemic control (fasting glucose 5-7 mmol/L, post-prandial glucose <9mmol/L) and under hyperinsulinemic euglycaemic conditions (5.5

mmol/L). On the other occasion the subjects were studied with a 'relaxed' insulin regime (fasting glucose 10-11 mmol/L and post prandial glucose >12 mmol/L) under hyperinsulinemic hyperglycaemic conditions (10 mmol/L). The sequences of the clamp studies were randomised. In addition, 10 controls subjects were studied during euglycaemia only. Interestingly, resting or peak MBF were not different between the patients with DM and control subjects during euglycaemia. Under euglycaemic conditions, insulin led to a 24% and 20% increase in the peak MBF in patients with type 1 DM and controls respectively ($p<0.05$ vs. baseline values). Furthermore, there was no change in the resting MBF, peak MBF or MBFR between euglycaemia and hyperglycaemia in the patients with DM. The authors concluded that short-term hyperglycaemia did not have a detrimental effect on MBF or MBFR in patients with type 1 DM.

In contrast, Srinivasan and group found that following 90 minutes of hyperglycaemia during a hyperinsulinemic clamp study,[85] MBFR was decreased in patients with type 1 DM. The study included two age- and sex-matched groups of patients with type 1 DM (n=10 each) which were studied either under euglycaemic or hyperglycaemic conditions. Each subject was studied at baseline (without insulin) prior to the commencement of an insulin clamp. Under euglycaemic conditions, insulin did not induce any change in the resting MBF, peak MBF or MBFR. This is in contrast to the results of Sundell et al as discussed above. However, during hyperglycaemia the resting MBF increased by 28% ($p<0.005$ vs. baseline). In addition, there was a 8% decrease in the peak MBF ($p<0.05$ vs. baseline) leading to a 24% decrease in MBFR (p=NS). More importantly, in the study by Srinivasan, MBF and MBFR were quantified

during hyperglycaemia and euglycaemia in two separate groups (and not the same individuals) and therefore, patients could not act as their own controls.

1.14 Insulin and Myocardial Blood Flow Reserve

Insulin is a peptide hormone composed of 51 amino acids and weighs 5808 daltons. It is secreted from the beta cells that reside in the pancreas. In addition to its metabolic properties it has important actions on the vascular function.[86] In healthy subjects, insulin increases the blood flow in the skeletal muscle.[87] The effects of insulin on myocardial vasculature were poorly understood until recently. Sobrevia performed the first in vitro study to examine this association using human umbilical vein endothelial cells (HUVECs) and analysing L-arginine transport into the HUVECs.[88] L-arginine has been shown to act as the precursor for nitric oxide production via nitric oxide synthase.[89] The HUVECs were incubated with insulin under euglycaemia (5 mmol/L) and hyperglycaemia (25 mmol/L). The investigators demonstrated that under euglycaemia, insulin led to a 2.5-fold increase in the transport of the amino acid L-arginine into the HUVECs. This led to a 3-fold increase in intracellular cyclic guanosine monophospate (cGMP) concentrations which serves as an index of nitric oxide synthesis. The data demonstrated that insulin led to an endothelium-dependent release of nitric oxide and suggested that this was the mechanism behind insulin-induced vasodilatation. More importantly, these effects were abolished by exposing the HUVECs to hyperglycaemia.

Professor Knuuti's group were the first to demonstrate that insulin under euglycaemic conditions led to a significant increase in peak MBF as compared

to peak MBF without insulin in healthy humans.[90] Dexamethasone (2mg per day) was given for 2 days to inhibit the effects of the sympathetic nervous system on the myocardial vasculature. The PET studies were repeated under euglycaemic conditions and it was shown that this manoeuvre did not inhibit the vasodilatory effect of insulin on the myocardial vasculature. In another study by the same group, nine type 1 DM patients had MBF quantified using PET.[91] Under euglycaemic conditions, insulin led to a 24% and 20% increase in the peak MBF in patients with type 1 DM and controls respectively ($p<0.05$ vs. baseline values for both groups). In a subsequent study by the same group, the effects of increasing dose of insulin were studied on the MBF of 10 healthy humans using PET.[92] They had resting baseline MBF assessed followed by an adenosine infusion and the measurement of peak MBF. Following this, 1-hour of euglycaemia was maintained using 1 mU/kg/min (physiological insulinemia) and peak MBF was measured again. Finally, euglycaemia was maintained for a further 1-hour, however, the insulin infusion rate was increased to 5 mU/kg/min (supraphysiological insulinemia) at the end of which peak MBF was assessed for the third time following the infusion of adenosine. Physiological insulinemia increased the peak MBF 3.92±1.17 ml/g/min (at baseline) to 4.72±0.96 ml/g/min representing 20% increase ($p<0.05$). In addition, supraphysiological doses of insulin further increased the peak MBF to 5.61±1.03 ml/g/min ($p<0.05$ for comparison with peak MBF at baseline and physiological insulinemia). Coronary vascular resistance decreased by 16% ($p<0.05$) under physiological insulin concentrations. More importantly, the coronary vascular resistance reduced by a further 12% ($p<0.05$) under supraphysiological insulin concentrations.

The above mentioned studies all investigated the effects of insulin in subjects with no coronary disease. Therefore, Lautamäki and co-workers investigated the effects of physiological dose of insulin (1 mU/kg/min) on myocardial blood flow in patients with type 2 DM and coexistent coronary artery disease (CAD).[93] Forty three subjects with angiographic evidence of CAD were included in the study (one-vessel disease 53%, two-vessel disease in 33% and three vessel disease in 14% of patients). All participants had myocardial perfusion assessed using a single photon emission computed tomogram (SPECT). A large proportion of angiographic lesions were flow limiting as the average number of myocardial segments with ischemic defects were 8.3±2.9 (out of a 20 segment model). Insulin induced a significant increase in the resting MBF in both the ischemic (0.98±0.30 ml/g/min vs. 1.07±0.35 ml/g/min, p=0.043) and non-ischemic regions (1.09±0.36 ml/g/min vs. 1.28±0.39 ml/g/min, p=0.003). However, the resting MBF was significantly less in the ischemic regions in comparison to the non-ischemic regions at baseline (p<0.0001) and following insulin infusion (p=0.0005). Furthermore, insulin increased the adenosine-induced peak MBF by 20% in the ischemic regions (3.34±0.93 to 3.83±1.06 ml/g/min, p=0.018) and by 18% in the non-ischemic regions (3.82±0.90 to 4.30±1.07 ml/g/min, p=0.045). In addition, the peak MBF was significantly less in the ischemic regions in comparison to the non-ischemic regions without (p<0.0001) and with insulin (p=0.017).

In summary, these studies demonstrate that insulin has a vasodilatory effect on normal coronary arteries and in the presence of flow-limiting CAD. Of note, it was a decade after the publication of the first DIGAMI trial that a direct link had been established between insulin and the myocardial vasculature

in the presence of significant CAD which could explain the observed positive outcome of that trial.[19]

1.15 Possible Mechanisms for Hypoglycaemia-mediated Adverse Effects on the Heart (Figures 1.7 and 1.8)

When the use of insulin was first implemented in the 1920's there were scattered case reports of it being associated with chest pain, angina and myocardial infarction.[27-31] At the time it was suggested that insulin-induced hypoglycaemia resulted in coronary artery occlusion, myocardial ischemia and death.[94] These conclusions meant that some clinicians were reluctant to commence their patients on insulin therapy unless all other available treatments had failed. Importantly, over the past few decades various mechanisms have been associated with hypoglycaemia-mediated insult on the myocardium. These include the potential to induce cardiac arrhythmias, promotion of a hypercoagulant and pro-thrombotic state, endothelial dysfunction and aggravation of myocardial ischaemia. These have been summarised below.

1.15a Hypoglycaemia and the Risk of Cardiac Arrhythmias

Two decades ago, amidst concerns of increasing number of sudden deaths in the UK (and corresponding media attention), Tattersall and Gill carried out a retrospective analysis of 50 suspected sudden deaths in young patients with type 1 DM.[95] In 22 cases, no cause was found on autopsy. These were subsequently, classed as 'dead in bed syndrome' as strong

circumstantial evidence suggested hypoglycaemia as the most likely cause of death. These patients were young (aged between 12-43 years), and a majority of the cases had had severe nocturnal hypoglycaemic attacks in the preceding six months. Only four of these cases had been diagnosed with microvascular complications in the preceding year. Furthermore, 20 cases had been alone at the time of death and were discovered in an undisturbed bed. One individual was found in a disarrayed room with glucose tablets strewn all over the floor while in another case, the bedside table had been knocked over.

The relation between hypoglycaemia and cardiac arrhythmias is recognised albeit poorly understood.[96, 97] Over the last twenty years, several studies have examined this association and some of these are summarised below. Prolonged QT syndrome is a well recognised condition and individuals with either the acquired or congenital variety of this syndrome are at much higher risk of sudden cardiac death (SCD) due to ventricular fibrillation.[98] Rossing examined the predictive power of QT interval (QTc) prolongation (>440 milliseconds) in an observational study including 697 patients with type 1 DM over 10 years.[99] A total of 61 deaths attributable to cardiovascular causes occurred with 38 and 23 occurring in the groups with prolonged and normal QTc respectively ($p=0.02$). Furthermore, prolonged QTc was more prevalent in the subjects dying from all-cause mortality ($p=0.0016$). In addition, prolonged QTc emerged as an independent predictor of all-cause mortality following multivariable adjustment in the entire cohort ($p<0.05$). Importantly, prolonged QTc emerged as an independent predictor of cardiovascular mortality in the group with macrovascular complications following multivariable adjustment with a 12% increase in relative risk ($p<0.05$). Subsequently, the

EURODIAB Prospective Complications Study looked at the cumulative prevalence of prolonged QTc in 1415 patients with type 1 DM.[100] It showed that one in five patients developed a persistently prolonged QTc during a seven-year follow up with an annual incidence of around 3%. Important multivariable predictors of prolonged QTc were poor glycaemic control, (66% increased risk with an HbA1C >7.7%) and female sex (2-fold increased risk of prolonged QTc, $p<0.0001$).

A recent study explored the relationship between QTc and blood glucose concentrations through continuous glucose monitoring in a young age-group (9-19 years).[101] This study demonstrated that severe QTc prolongation (>550 milliseconds) was identified in four patients. A significant correlation between QTc and low glucose concentrations was found ($r=0.672$, $p=0.003$). In addition, it showed that the QTc intervals were longest during the night and early hours of the morning and corresponded with the lowest glucose levels. The authors postulated that their findings could provide with a possible mechanism for increased cardiovascular mortality due to hypoglycaemia.

Marques and co-workers investigated the effects of insulin-induced hypoglycaemia on the QTc.[102] They studied eight patients with type 1 DM and seven patients with type 2 DM. Each individual was studied on two separate occasions (in random sequence) under euglycaemic conditions (5.0 mmol/L) and hypoglycaemic conditions (3.0 mmol/L) using hyperinsulinemic hypoglycaemic clamps. They demonstrated that there was no change in the QTc during euglycaemic conditions. The insulin concentrations were comparable between euglycaemia and hypoglycaemia for each group. More importantly, during hypoglycaemic conditions QTc increased by 35% ($p<0.01$) in the patients with

type 1 DM and almost by a similar extent in patients with type 2 DM, 28% ($p<0.01$). In addition, there was a 15-fold increase in the plasma adrenaline levels during hypoglycaemia. There was a significant relationship between peak increase in the QTc during hypoglycaemia and adrenaline concentrations with a correlation coefficient (Spearman rank) of 0.73, ($p<0.0001$). Of note, the plasma potassium concentrations decreased with the commencement of insulin clamps, however, there was no difference between the potassium levels between the euglycaemic or hypoglycaemic phase for any group. The investigators suggested that the QTc prolongation was secondary to hypoglycaemia-induced adrenaline release. This was supported by the observation that patients with an attenuated adrenaline response showed minimal QTc increase. In an earlier paper, Lindström had investigated the effects of acute insulin-induced hypoglycaemia (median of 2.0 mmol/L for 60 minutes) on six patients with type 2 DM with no antecedent history of CAD.[103] One patient developed a severe nodal bradycardia with a corresponding loss of consciousness lasting a few seconds with full recovery following glucose administration. Another patient developed symptomatic frequent ventricular ectopics while 5 patients had marked ST depression in the chest leads without concomitant chest pain. Furthermore, the serum adrenaline concentrations increased by 17-fold ($p<0.001$) while there was a small but significant decrease in the serum potassium levels from 4.1±0.3 mmol/L to 3.5±0.2 mmol/L ($p<0.001$).

In an elegant series of experiments, Robinson and co-investigators studied the mechanisms leading to QTc prolongation during hypoglycaemia.[104] They studied two groups of 10 patients with type 1 DM (one patient was

studied as part of both groups) either with or without beta-blockade (achieved by administering atenolol 100 milligrams once a day for a week) or potassium replacement. Each study was repeated during hyperinsulinemic euglycaemic (5.0 mmol/L) and hyperinsulinemic hypoglycaemia (2.5 mmol/L).

Potassium replacement studies (n=10): Each individual was studied on 4 occasions in a random sequence comprising a total of 40 clamp studies. During hyperinsulinemic euglycaemia QTc did not vary significantly with or without concomitant potassium infusion ($p=0.56$). Furthermore, during hypoglycaemia each individual was again studied in the presence or absence of potassium infusion. It was shown that in the absence of potassium replacement QTc was prolonged during hypoglycaemia by a mean of 67 milliseconds ($p<0.0001$ vs. corresponding euglycaemic values). In comparison, with potassium replacement the QTc was still prolonged by a mean of 46 milliseconds ($p=0.02$, vs. euglycaemia). In addition, potassium replacement had no effect on QTc during hypoglycaemia as the increase in QTc with or without potassium infusion were not significant ($p=0.16$).

Beta-blockade studies (n=10): The effects on QTc were also studied with and without concomitant beta blockade. During hyperinsulinemic euglycaemia QTc did not vary significantly with or without concomitant beta-blockade ($p=0.23$). However, during hypoglycaemia QTc was significantly prolonged by 55 milliseconds, ($p<0.0001$ vs. corresponding euglycaemic values). More importantly, beta-blockade prevented any increase in QTc during the hypoglycaemic phase, ($p=0.98$ vs. corresponding euglycaemic values). In addition, QTc was significantly prolonged during hypoglycaemia without concomitant beta-blockade in comparison to with beta-blockade ($p<0.05$).

In conclusion therefore, accumulating evidence suggests that hypoglycaemia indirectly induces electrical instability in the myocardium through an adrenaline surge, with a subsequent prolongation of QTc. This is of clinical relevance has prolonged QTc has been shown to be a marker of CVM.

1.15b Hypoglycaemia and the Risk of Thrombosis

There is a growing body of evidence that suggests that acute hypoglycaemia can lead to changes in the platelet function and clotting factors that can induce a pro-coagulant and pro-thrombotic state. This has been put forward as another mechanism that could accelerate endothelial damage and myocardial injury.

Corrall and co-workers examined the effect of acute insulin-induced hypoglycaemia on clotting by measuring the levels of factor VIII (a key clotting factor in the coagulation cascade) in response to hypoglycaemia.[105] Their study included 9 healthy volunteers who were given 0.15 units/kilogram bodyweight to induce hypoglycaemia. Mean plasma glucose was decreased down to a nadir of 0.9 mmol/L. They observed a 2-fold significant increase in the serum factor VIII levels within 30 minutes of acute hypoglycaemia. The investigators repeated the studies in volunteers following non-selective beta blockade (propranolol, n=4) and selective beta blockade (metoprolol, n=4). Volunteers who had non-selective beta blockade had almost complete suppression in the rise in factor VIII levels post hypoglycaemia. In contrast, volunteers receiving selective beta blockade recorded a 90% increase in their factor VIII levels following hypoglycaemia ($p<0.01$). The authors concluded

that acute insulin-induced hypoglycaemia led to a significant increase in the clotting factor VIII, an effect mainly mediated by beta 2 receptors.

In a subsequent study, Dalsgaard-Nielsen investigated the effects of acute insulin-induced hypoglycaemia on platelet function and clotting factors.[106] Seven healthy controls and 8 patients with type 1 DM participated in the study. The investigators observed that following acute hypoglycaemia, the threshold concentrations of adenosine diphosphate (ADP) at which platelet aggregation would occur, decreased in both groups ($p<0.05$ for controls and $p<0.02$ for type 1 DM). The decrease in the ADP threshold was more pronounced in the patients with type 1 DM (3-fold) in comparison to controls (2-fold) which suggested increased platelet aggregation. This was mirrored by a 20% decrease in the platelet count in the patients with DM ($p<0.01$) in comparison to controls which recorded no change. Finally, both groups demonstrated a 50% increase in factor VIII concentrations following hypoglycaemia. In summary, this study demonstrated that acute hypoglycaemia led to a pro-coagulant and pro-thrombotic state that could be potentially detrimental to patients with established coronary artery disease.

Another study investigated the effects of acute hypoglycaemia on haemostatic parameters such as von Willebrand factor (vWF) and blood viscosity.[107] vWF is a 2050 amino acid protein that is released from endothelial cells and platelets. It has important actions which include prolonging the circulation life-time of the coagulation factor VIII and promoting platelet aggregation. Once factor VIII is unbound, it is unstable and degrades very rapidly. Six healthy volunteers and 6 patients with type 1 diabetes participated in the study. Acute hypoglycaemia was induced by a 0.15

unit/kg body weight bolus of insulin. Blood glucose levels decreased to a mean nadir of 1.0 mmol/L. The investigators found that baseline vWF levels were 45% higher in the subjects with DM in comparison to controls. Furthermore, both groups recorded a significant increase in vWF levels following hypoglycaemia (20% for DM) and (31% for controls). However, following hypoglycaemia, the vWF levels were significantly increased in the patients with DM in comparison to controls ($p<0.02$). In addition, plasma viscosity increased significantly in both groups and remained so for 15 minutes following acute hypoglycaemia.

In a series of elegantly designed experiments, Trovati and colleagues investigated the mechanisms behind hypoglycaemia-induced platelet activation.[108] Sixteen healthy volunteers participated in the study. A 1.5mU/kg/min hyperinsulinemic clamp was used to induce hypoglycaemia without a concomitant glucose infusion. Approximately, 45 minutes into the clamp, hypoglycaemia occurred with a mean nadir of 2.7±0.1 mmol/L. A specific measure of platelet aggregation was measured, known as ED_{50} which represents a concentration of each aggregating agent necessary to induce maximal aggregation of 50% of platelets. The investigators noted a significant decrease in the ED_{50} of key platelet aggregating agents ADP (30%, $p<0.02$), thrombin (30%, $p<0.005$) and platelet activating factor (40%, $p<0.002$). These results suggested that the sensitivity of platelets to agents promoting platelet aggregation increased dramatically. Importantly, the authors also reported a 2.5-fold increase in the circulating β-thromboglobulin levels, which is a marker of platelet activation and degranulation, following hypoglycaemia ($p<0.025$). It was not clear if there was a change in the platelet levels as these were not

reported. These changes were accompanied with a 5-fold increase in plasma adrenaline levels during hypoglycaemia. Seven in-vitro studies were carried out by exposing the plasma of volunteers to the levels of counter-regulatory hormones (adrenaline, cortisol, growth hormone and glucagon) typically encountered during hypoglycaemia. Platelet sensitivity to platelet aggregating agents increased significantly when exposed to increased concentrations of adrenaline however, the remaining hormones had no effect on this function. Finally, hyperinsulinemic clamp studies were repeated in four volunteers following concomitant blockage of alpha receptors by an infusion of phentolamine. A marked decrease in the response of platelets to aggregating agents was seen (with a resulting increase in ED_{50}) suggesting that hypoglycaemia-induced platelet aggregation was an adrenaline-mediated response carried out through alpha receptors.

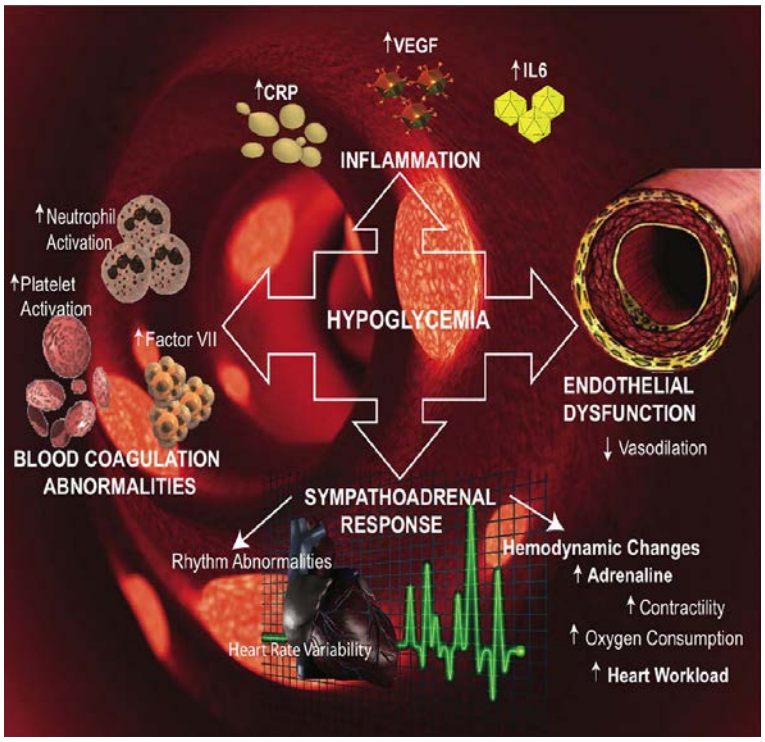
The evidence from afore-mentioned studies suggested that the changes in platelet function and coagulation system were hypoglycaemia-induced however, it was not clear if insulin had any effect on these parameters. Therefore, Mauricio-Leguizamo investigated the effects of insulin under euglycaemic conditions (5.0 mmol/L) on various haemostatic parameters.[109] Seven healthy volunteers participated in the study. Insulin was infused at a constant rate of 0.7 mU/kg.min for eight hours with a subsequent 8-fold increase in serum insulin levels. Blood samples were drawn at various time intervals to measure several haemostatic parameters. The investigators noted that under euglycaemic conditions, insulin did not cause any change in platelet function (platelet count or β-thromboglobulin levels). In addition, there was no change in factor VIII, fibrinogen or plasminogen levels. Finally, as expected, there was no change in

plasma adrenaline levels. The authors concluded, that insulin under euglycaemic conditions did not have any effect on haemostatic parameters and that earlier results had indeed been due to hypoglycaemia-induced changes.

Just as the human body has platelets and coagulation factors to prevent increased bleeding, there coexists a fibrinolytic system which prevents excessive clotting which could potentially block blood vessels and prevent vital blood supply to human tissue. Professor Frier's group from Edinburgh studied the effects of acute hypoglycaemia on the fibrinolytic system.[110] Six patients with type 1 DM and 11 healthy subjects participated in their study. A 2.5 mU/kg/min hyperinsulinemic clamp was initiated and all parameters were measured during euglycaemia (5.0 mmol/L). Thereafter, hypoglycaemia was induced by stopping the dextrose infusion in both groups to a nadir of 1.5 mmol/L (controls) and 1.2 mmol/L (DM). There was a significant increase in vWF levels in both groups (50%) following hypoglycaemia. At baseline the fibrinolytic system was impaired in the patients with DM in comparison to controls. Following hypoglycaemia, both groups exhibited an increase in the fibrinolytic system however, the magnitude of increase was less in the patients with DM in comparison to controls (44% vs. 100%, $p<0.05$). More impotantly, tissue plasminogen activator (tPA) levels (starts the fibrinolytic cascade) increased by 60% in the control population within 10 minutes of hypoglycaemic reaction. However, this response was both attenuated and delayed in the patients with DM as demonstrated by a 20% increase in tPA levels 20 minutes after the hypoglycaemic reaction. In addition, tPA activity (denoting function) were 7-fold higher in the control group in comparison to the DM group within 5 minutes of acute hypoglycaemia ($p<0.05$).

In summary, hypoglycaemia induces several changes in the haemostatic parameters which include an increase in platelet aggregation, factor VIII and vWF levels and fibrinolysis. More importantly, the activation in the fibrinolytic system is impaired in patients with DM with no impairment in the activation of pro-thrombotic pathways in comparison to healthy controls. These data suggest that patients with diabetes are more susceptible to potential microthrombi formation in response to acute hypoglycaemia which could be detrimental to the myocardial circulation.

Figure 1.7. Putative Pathological Mechanisms Linking Acute Hypoglycaemia with Adverse Cardiovascular Events. Reproduced with permission.[24]



CRP= C reactive protein, VEGF=Vascular endothelial derived growth factor, IL-6=Interleukin 6.

1.15c Hypoglycaemia and Endothelial Dysfunction

Over the past few decades, several anecdotal case reports have associated hypoglycemia with episodes of angina and myocardial infarction.[29-31] While a direct causal link has not been established, animal studies have demonstrated that hypoglycemia can increase myocardial infarct size by over 40%.[94] Furthermore, in patients with DM and coexisting CAD, hypoglycemia was associated with a third of all episodes of angina and corresponding ischemic ECG changes.[32]

The endothelium is a highly biologically active single cell layer responsible for the release of several substances the most important of which are nitric oxide (NO) and endothelin-1 (ET-1).[111, 112] NO is produced by the endothelium via an L-arginine pathway through endothelial nitric oxide synthase (eNOS) and mediates the vasodilatation of blood vessels. NO is also important in preventing platelet activation, leucocyte adhesion, vascular smooth muscle (VSM) proliferation and oxidative stress.[89]

ET-1 is a 21-amino acid peptide and the most potent vasoconstrictor yet identified in man with a plasma half life of 4-7 minutes.[113, 114] ET-1 induces its predominant vasoconstrictive effect by acting on receptors located on vascular smooth muscle cells and fibroblasts. This reduces NO bioavailability by either decreasing its production (caveolin-1-mediated inhibition of eNOS activity) or by increasing its degradation (via formation of oxygen radicals).[115] One recent study demonstrated that direct infusion of ET-1 into the coronary sinus of six humans decreased the coronary blood flow in a dose-related manner by up to 25%.[116] ET-1 levels have also been shown to be the strongest

predictor of no-reflow following primary angioplasty.[117] Several disease states have been shown to be associated with endothelial dysfunction (an imbalance between the bioavailability of NO and ET-1). Examples of these include atherosclerosis, pulmonary arterial hypertension, DM and myocardial ischemia.[118-122]

To explore the effects of ET-1 on the coronary circulation, a Japanese group examined the effects of intracoronary administration of increasing doses of ET-1 in 23 dogs.[123] At the highest dose ET-1 caused maximal vasoconstriction within 30 seconds leading to reduced MBF. Almost 20% dogs exhibited complete cessation of MBF with corresponding ST-segment elevation on ECG. In a subsequent study in 6 human volunteers, ET-1 induced a 23% decrease in MBF at a plasma level 4 times that of baseline levels.[116] In a more recent study, Wright investigated the effect of symptomatic hypoglycaemia on ET-1 levels in 20 patients with Type 1 DM.[124] The plasma concentration of ET-1 increased by 70% above baseline values one hour following insulin-induced hypoglycaemia. The authors used an insulin infusion for rapid induction of hypoglycaemia following baseline ET-1 sampling without a glucose infusion. Hence, a euglycaemic stage was not included as a comparator. Therefore, it was not clear whether this response was induced by insulin or the counter-regulatory mechanisms secondary to hypoglycaemia. Furthermore, the study did not include a control group of healthy volunteers therefore the association between hypoglycaemia and ET-1 in the non-diabetic population remains unknown. Finally, the level of hypoglycaemia induced was quite severe (1.9 mmol/L).

In addition to ET-1, other biomarkers may be of clinical importance. One such biomarker is high sensitivity C-reactive protein (hs-CRP) which is an acute phase reactant that is produced by the hepatocytes. Longitudinal studies in both women and men have shown that elevated baseline hs-CRP values are associated with adverse cardiovascular outcomes in patients with and without coronary disease.[125-127] Infact, hs-CRP has been shown to be a more reliable predictor of future cardiovascular events than LDL cholesterol levels.[128] Of note, there is accumulating evidence that hs-CRP is a key moderator of atherosclerosis.[129] For example, hs-CRP has been detected in the intimal layer of coronary arteries prepared from autopsied human hearts.[130] There is also a growing consensus that hs-CRP may induce endothelial dysfunction as hs-CRP has been shown to inhibit in vitro NO production by decreasing eNOS synthesis.[131] Conversely, Verma and colleagues demonstrated that hs-CRP led to a four-fold increase in the production of ET-1 from incubated human endothelial cells.[132] Furthermore, hs-CRP has also been shown to activate the coagulation cascade (by increasing tissue factor production), increase LDL-cholesterol uptake by macrophages, increase the endothelial expression of adhesion molecules on human endothelial cells such as intracellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1) and E-selectin.[129, 132-134] These adhesion molecules promote the extravasation of monocytes into the extra-vascular space where these engulf LDL cholesterol to become foam cells. Galloway and co-investigators induced acute symptomatic hypoglycaemia in 6 patients with DM and healthy male subjects.[135] They demonstrated that acute symptomatic hypoglycaemia was associated with a 3-fold increase in the median baseline hs-CRP values in diabetic and non

diabetic subjects, 24 hours after the hypoglycaemic episode. hs-CRP values at 4 hours post hypoglycaemia did not change. The authors suggested that acute hypoglycaemia could induce a sub-clinical inflammatory response in the endothelium and such episodes in repetition could be detrimental in the long-term.

More recently, Nematollahi and co-investigators looked at the effect of acute severe hypoglycaemia (<2.0 mmol/L) at several cytokines and markers of inflammation including interleukins 6 and 8 (IL-6, IL-8), tumor necorsis factor (TNF-α) and reactive oxygen species (ROS).[136] Thirteen non-diabetic healthy men took part in their study. Acute severe hypoglycaemia (<1 hour duration), led to a 35% increase in TNF-α, 2-fold increase in IL-6 and 50% increase in IL-8 levels. In addition, their was a two-fold elevation in the levels of detected ROS. Moreover, these changes lasted for up to two hours. The authors concluded that the release of pro-inflammatory cytokines in response to acute hypoglycaemia could induce endothelial dysfunction and be detrimental to patients.

1.16 Conclusion

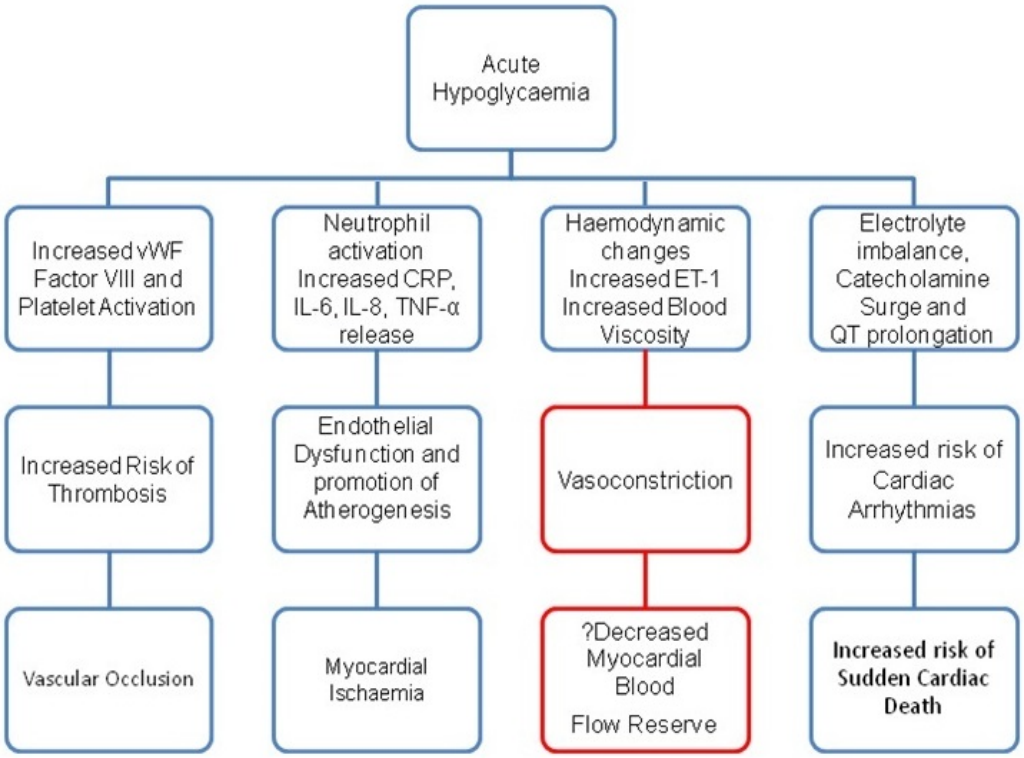
1.16a What is already known?

Hypoglycaemia is a frequent complication of aggressive glycaemic therapy in Type 1 DM. It has shown to be strongly and independently associated with adverse cardiac outcome in several studies in patients with and without coronary disease. The mechanisms by which this association can be linked are heterogeneous and unclear but hypoglycaemia can potentially induce electrophysiological disturbances through QTc prolongation and electrolyte imbalance thereby providing a substrate for cardiac arrhythmias. In addition, hypoglycaemia has the potential to induce endothelial dysfunction by promoting inflammation as well as preferential release of ET-1 and hs-CRP which could play an important role in the development and progression of atherosclerosis. Furthermore, hypoglycaemia can also induce coronary ischemia, by inducing a pro-thrombotic state leading to myocardial damage.

1.16b What this study aimed to add?

We set out *a priori* to look at the effects of hypoglycaemia on MBFR, our main outcome variable, using myocardial contrast echocardiography. We hypothesized that acute hypoglycaemia was associated with a reduction in MBFR in comparison to baseline values.

Figure 1.8 Putative Mechanisms Involved in the Association Between Hypoglycaemia and Increased Cardiovascular Mortality. The pathway in red was Explored in our Study.



Chapter 2 Methods

2.1 Methods for Selection

This section describes the methods involved in patient population selection.

2.1.1 Study Population

The study population comprised of two groups.

1. Healthy volunteers acting as controls.
2. Patients with Type 1 DM without microvascular disease (MVD).
3. Patients with Type 1 DM with MVD.

2.1.2 Definition of Microvascular disease

Microvascular disease was defined as the presence of 2 out of the following 3 features:

1. Diabetic retinopathy was defined as either preproliferative or proliferative retinopathy with no history of laser treatment within the preceding 6 months.
2. Diabetic neuropathy was diagnosed on the basis of clinical examination and a vibration perception threshold score of >12 (measured on the Great Hallux using a Bio-thesiometer [Biomedical Instrument, Newbury, Ohio, USA]).[137]
3. Diabetic nephropathy was defined as an albumin/creatinine ratio of >2.5 mg.mmol/L for men and >3.5 mg.mmol/L for women.[138]

2.1.3 Confidentiality

The individuals were assigned codes to anonymise personal details. The following coding system was incorporated.

C denoted healthy controls.

DM- denoted patients with Type 1 DM without MVD.

DM+ denoted patients with Type 1 DM with MVD.

2.1.4 Consent

Written informed consent from the subjects was obtained prior to the implementation of study procedures.

2.1.5 Ethical Approval

The study was approved by the local research ethics committee (LREC number 08/H0201/22).

2.1.6 Inclusion Criteria

The following inclusion criteria were used

• Male or female aged 18-50 years of age.

AND

• Subject was able to provide written informed consent and was willing to comply with the protocol.

AND

• Healthy control.

OR

• Type 1 DM with or without MVD.

2.1.7 Exclusion Criteria

The following exclusion criteria were applied

• Ischemic heart disease.

• Chronic respiratory disease.

• Hypertension.

• Smoker.

• Renal impairment (creatinine >110 μmol/L).

• Hypoglycaemic episode 48 hours prior to study.

• Dyslipidaemia.

• Left ventricular impairment.

• Congenital heart disease.

• Cerebrovascular disease.

• Peripheral vascular disease.

• Severe cardiac rhythm disorders.

• Pregnant or lactating female.

• Had received an investigational product within 30 days before admission into this study.

• Had any contraindications to dipyridamole for example, asthma, chronic obstructive airways disease.

2.1.8 Screening of Subjects for Medical Safety.

All subjects had the following baseline investigations done as part of routine for medical safety and to exclude any undiagnosed and potentially confounding medical conditions.

• Full blood count.

• Renal profile.

• Liver function tests.

• Thyroid function tests.

• Fasting lipid profile (LDL, HDL, TG).

• HbA1c.

• 12-lead electrocardiography (ECG).

• Stress echocardiography (To exclude coronary artery disease)

In addition to the above tests, the subjects with Type 1 DM went onto have the following tests:

• Albumin/Creatinine Ratio from first morning urinary sample. (>2.5mg/mmol/L in men and > 3.5mg/mmol/L in women).

•Retinal examination.

• Vibration perception threshold studies to look for peripheral neuropathy.

2.2 Investigational Methods

2.2.1 Hyperinsulinemic Clamp Technique

The hyperinsulinemic glucose clamp technique was developed originally by Defronzo and co-workers.[139] Following an overnight fast, insulin is infused at constant rate (typically between 1.5 to 3 mU/kg/min. This insulin infusion results in a steady-state insulin level which is higher than the conventional fasting insulin levels (hyperinsulinemia). This increases the rate of transfer of glucose into the adipose tissue and skeletal muscles and suppresses the hepatic glucose production (gluconeogenesis). Following the initiation of the insulin infusion, a 'steady-state' is reached during which the coefficient of variation for plasma glucose and plasma insulin levels is <5%. Exogenous glucose is infused by a simultaneous 20% dextrose intravenous infusion. A bed-side glucose analyser is used to measure the plasma glucose levels at 5-minute intervals as in our study.[140] Conventionally, the insulin infusion rate is held constant and the dextrose infusion rate is changed to induce either hyper- or hypoglycaemia.

The difference between arterial and venous glucose levels is determined by the sensitivity to insulin of the patient (which affects the rate of uptake of glucose by the tissues) and the mean transit time (time taken for the blood to pass through any body tissue).[141] This is a potential source of error due to significant difference between the standard arterial and venous samples. This arterio-venous difference can be minimised by placing a retrograde cannula in the dorsum of the hand and placing this hand in a heated box (55-65°C) and

obtaining an ‘arterialised sample’. The fraction of glucose lost due to tissue uptake can be as low as 2% during the euglycaemic state. Furthermore, the difference between arterial and arterialised samples approaches 0% following 50 minutes of insulin infusion in comparison to a 15% difference between a standard arterial and venous sample.

2.2.2 Hyperinsulinemic Clamp Study

All participants were admitted to the Department of Cardiology by 07:30 am on the day of the study after an overnight fast. The overall study scheme is shown in Figure 2.2. Written instructions were provided to avoid caffeine-containing products and alcohol for >12 hours. Two antegrade and one retrograde cannulae were sited after application of a local anaesthetic cream (Ametop gel 4.0% w/w, Smith and Nephew, UK) to minimise discomfort. The antegrade cannulae were inserted into the antecubital fossa on either side. The right antegrade cannula was used for insulin (Actrapid; Novo Nordisk, Copenhagen, Denmark) and 20% dextrose infusions. A retrograde cannula was inserted into the dorsum of the right hand and was kept patent with a slow infusion of 0.9% (w/v) saline to which 1000 units of heparin were added. This hand was placed in a heated box (55-60ºC) to obtain arterialized samples.[141] All studies were performed in a quiet and comfortable room (22-25ºC) with the volunteers resting on a couch in a semi-reclined position. After a 30-40 minute resting period, Baseline plasma glucose was determined and the hyperinsulinemic clamp was commenced. Insulin was infused at 3 mU/kg/min for 4 minutes followed by 2 mU/kg/min for a further 3 minutes after which

the infusion rate was maintained at 1.5 mU/kg/min.[139] Glucose sampling was performed every 3-5 minutes and the 20% dextrose infusion was adjusted accordingly. Hyperinsulinemic euglycemia (HE, 90 mg/dl, 5.0 mmol/L) was maintained for 60 minutes following an initial 30-minute stabilization period. Glucose levels were subsequently reduced over a 30-minute period by decreasing the 20% (w/v) dextrose infusion (Baxter Healthcare, Thetford, Norfolk, UK) rate and symptomatic hypoglycemia (50 mg/dl, 2.8 mmol/L) was induced. Participants were asked to report any symptoms which could be attributed to hypoglycemia which included general symptoms (dry mouth, headache, and weakness), autonomic symptoms (palpitations, trembling, tingling, sweating and feeling hungry) and neuroglycopenic symptoms (poor concentration, dizziness and blurred vision). The glucose concentrations were maintained for a further 60 minutes (hyperinsulinemic hypoglycemia, HH) following which insulin infusion was terminated and normoglycemia was restored. All participants were provided with meals and observed for 1-hour at the end of which plasma glucose was rechecked before allowing them home.

Figure 2.1 Retrograde cannula with Heparinised Saline Infusion and Anterograde Cannula with Insulin and 20% Dextrose Infusions. The Hand is Placed in the Heated Box.

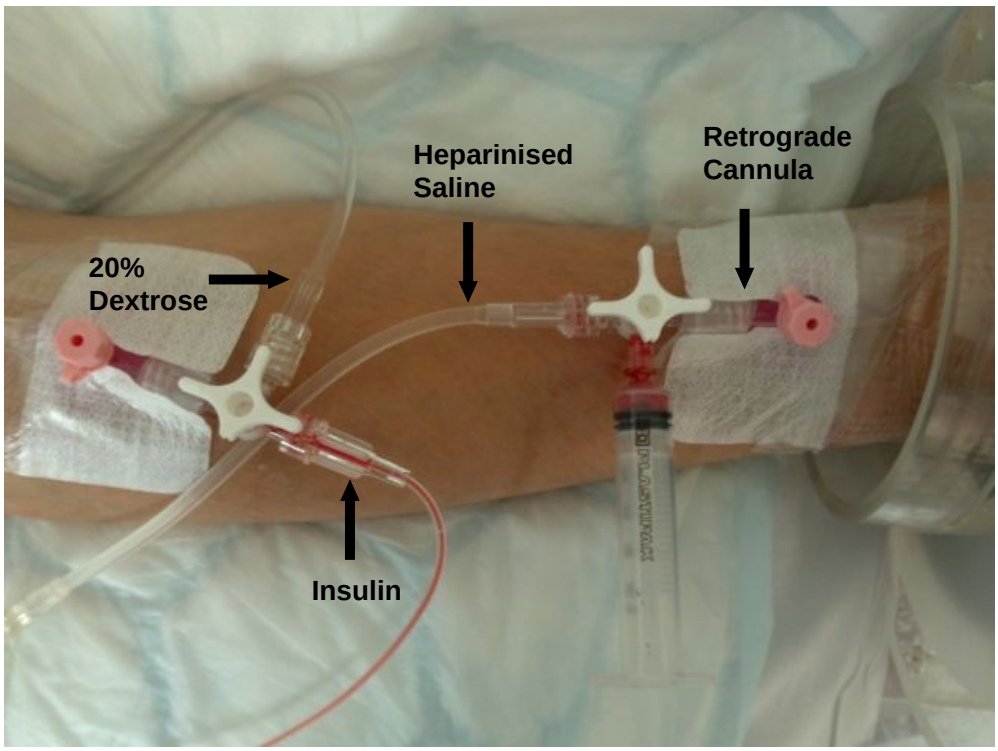


Figure 2.2. Study design showing glucose concentrations and the timings of myocardial contrast echocardiography (MCE) measurements taken at baseline (B), and during hyperinsulinemic euglycaemia (HE) and hyperinsulinemic hypoglycaemia (HH)

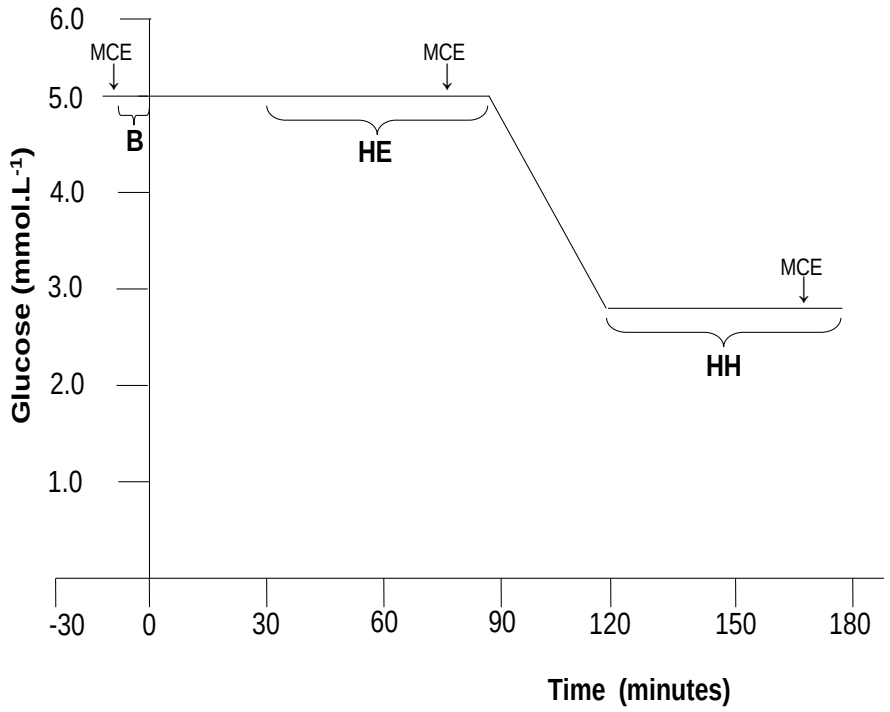
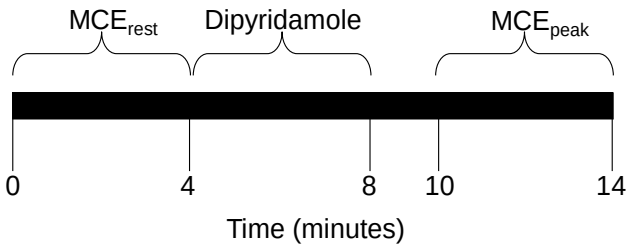


Figure 2.3. Scheme showing sequence of image acquisition during each MCE study at baseline, HE and HH. MCE_{rest}=myocardial contrast echocardiography at rest, MCE_{peak}=myocardial contrast echocardiography at post dypridamole-induced stress



2.2.3 Modifications for patients with Type 1 DM

The following modifications were made to the experiments for patients with Type 1 DM.

1. All volunteers with Type 1 DM were asked to reduce their night time long-acting insulin dosage down to 60% that of their routine dosage. This was to avoid any hypoglycaemic events prior to the study.
2. Once admitted on the study morning, volunteers were commenced on a standard sliding scale intravenous insulin regime to maintain the plasma glucose around 5.0 mmol/L.

2.2.4 Myocardial Contrast Echocardiography (MCE) Studies

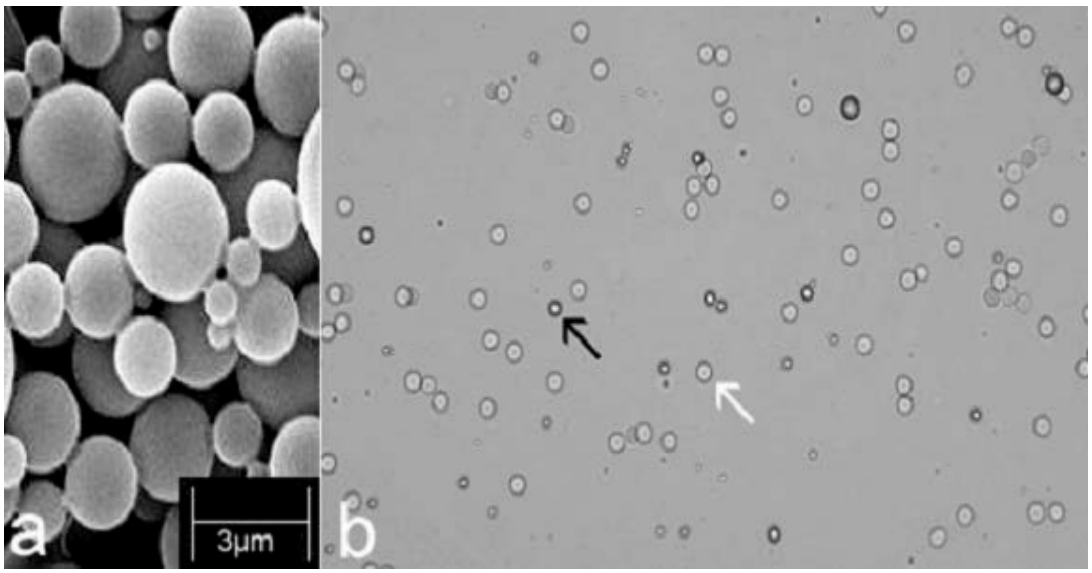
The last 25 years have seen the emergence of myocardial contrast echocardiography (MCE) as an accurate, non-invasive, reliable, cheap and bedside method of assessing myocardial perfusion.[67, 70, 142, 143] This technique has developed so rapidly that it now allows the quantification of myocardial blood volume and myocardial blood flow. There is a substantial amount of evidence that has shown that MCE has an accuracy comparable to positron emission tomography and coronary Doppler flow wire measurements.[70, 144, 145]

2.2.4.a Contrast Agents

The contrast agent comprises of microbubbles which are encapsulated gas-filled spheres. These are similar to red blood cells in rheology and are small enough (<8μM) to pass the pulmonary circulation intact.[142] Furthermore, these can enter the myocardial microcirculation and thereby allow qualitative and quantitative assessment of myocardial perfusion. The microbubbles are 1 x 10^{14} times more reflective of ultrasound than red blood cells (Figure 2.4).

Microbubbles are pure intravascular agents and contain an insoluble gas encapsulated in a phospholipid shell. The insoluble gas provides the microbubble its stability. For example, a soluble gas would diffuse out of the microbubble allowing it to collapse. The shell gives the microbubble its elasticity to reflect ultrasound back and hence generate images. Also, the scatter that a microbubble generates (amount of ultrasound reflected) is directly proportional to its radius. However, a compromise has to be attained as a larger microbubble may reflect more ultrasound but would not be able to traverse the pulmonary circulation and hence, myocardial perfusion could not be assessed.

Figure 2.4 Comparison Between Microbubbles and Red Blood Cells.



A microbubble is pointed out (black arrow) which is smaller than a red blood cell (white arrow)

The contrast agents currently in use in the UK are Sonovue ((Bracco Research SA, Geneva, Switzerland), Optison (Mallinkrodt Medical Inc, St Louis MO, USA) and Luminity (Bristol-Myers Squibb, Pharma, Bruxelles, Belgium) as shown in Table 2.1.[146] We used **Sonovue** as the contrast agent in ours study. Sonovue uses sulphur hexaflourane in a phospholipid shell.

Table 2.1 Different Contrast Agents Available in UK and Europe.

Agent	**SonoVue** Bracco	**Optison** GE	**Luminity** Lantheus
Gas	Sulphurhex-aflouride	Perfluorop-ropane	Perfluorop-ropane
Size µm	2-8	3.0-4.5	1.1-2.5
Surface coating	Phospholip-ids	Human albumin	Lipids
SE %	11	17	8

µm=micrometre, SE=side effects

2.2.4.b Principles of Myocardial Contrast Echocardiography

Ultrasound waves are transmitted through tissue as pressure fluctuations. These represent waves of alternating compression and rarefaction. Compression exerts positive pressure (positive half cycle) on the microbubble allowing it to contract and rarefaction exerts negative pressure (negative half cycle). on the microbubble thereby allowing it to expand.

When ultrasound waves strike the microbubbles these oscillate (expand and contract) and scatter the reflected ultrasound waves.[147] The manner in which the microbubbles perform this function is intrinsically related to the mechanical index (MI) of the reflected ultrasound wave from the transducer. MI of the transmitted ultrasound refers to the approximate ultrasound pressure

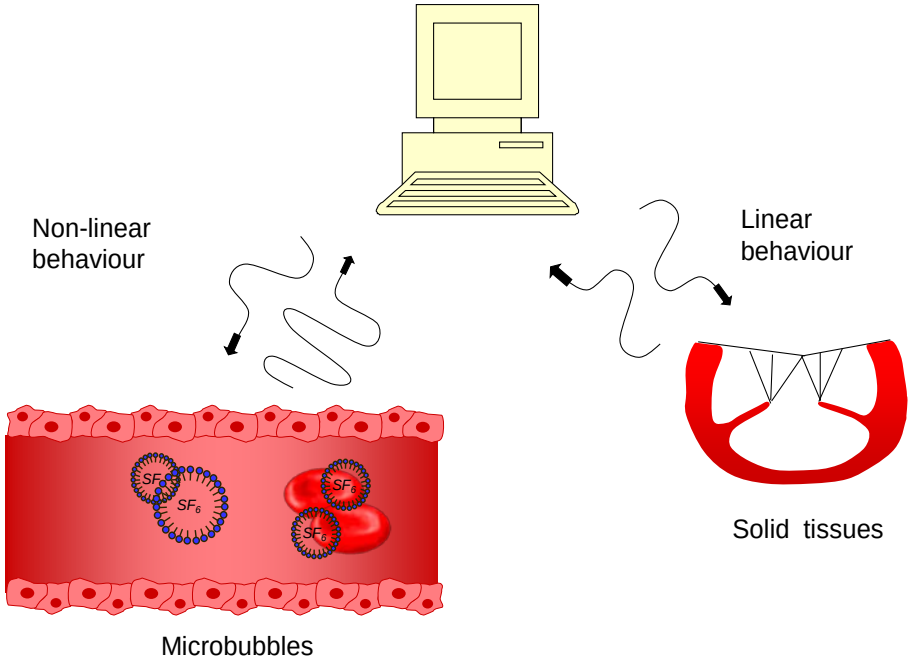
at the focus of the beam in an average tissue. The MI is defined as the peak of negative pressure divided by the square root of the ultrasound frequency. Quite simply, it represents the amount of mechanical work that can be performed on a single microbubble during a single negative half cycle of ultrasound.

Furthermore, microbubbles demonstrate resonance, a property which means that there is a frequency of oscillation at which these will expand and contract with maximum efficiency. At low MI, the degree of contraction is equal to expansion (linear oscillation). However, at high MI the microbubbles expand more than they contract (nonlinear oscillation). For example, if a contrast agent has a fundamental frequency of 3.75 MHz, then linear oscillation will result in a reflected wave of 3.75 MHz. However, nonlinear oscillation will result in a second peak harmonic of 7.5 MHz and third peak harmonic of 11.25 MHz and so on. This unique property allows the suppression of ultrasound waves reflected from the solid tissues (as they exhibit linear oscillation) by the ultrasound machine and hence focussing on data generated from the nonlinear oscillation of the microbubbles (Figure 2.5).

Importantly, microbubbles have different responses depending on the MI. For example, microbubbles exhibit a linear behaviour at a mechanical index of <0.1 (<100 kilo pascals) i.e. expansion of the microbubbles is equal to their contraction. When the mechanical index is between 0.1-1.0 (100 kilo pascals to 1 mega pascal) the microbubbles demonstrate a nonlinear behaviour (expansion is greater than contraction) and produce frequencies that are multiples of the fundamental frequency emitted by the ultrasound transducer which are called

harmonics. When the MI is greater than 1.0 (>1 mega pascal) the microbubbles are destroyed.

Figure 2.5 Nonlinear behaviour of microbubbles



2.2.4.c Methodology of MCE Studies

MCE was performed using a commercial ultrasound machine iE33 (Philips Medical Systems, Best, the Netherlands) and SonoVue (Bracco Research SA, Geneva, Switzerland) as the contrast agent as described previously.[71] Real-

time images were recorded of the 3 apical views (apical 4-chamber, apical 2-chamber and apical 3-chamber) with low-power settings at a mechanical index of 0.1 within 3-4 minutes. The focus was set at the mitral valve level. SonoVue was initially started at 60mL/h through the left antegrade cannula with an infusion syringe pump VueJect (BR-INF 100, Bracco Research, SA), which gently rotates and maintains the contrast agent in a suspension. Thereafter, the rate was set between 48 to 60 mL/h to maximise image quality with minimal attenuation. Once optimized, the machine settings were held constant throughout each participant study.

Flash-impulse imaging at a high mechanical index (1.0) was performed to achieve complete myocardial bubble destruction, after which 10 end-systolic frames were recorded digitally in each apical view. After the resting images were acquired, dipyridamole was infused at 0.56mg/kg over a 4-minute period. Following an interval of 2 minutes, post-stress images were subsequently recorded within 3-4 minutes. This entire sequence took 14 minutes and the MCE studies were performed at baseline, HE and HH as outlined in Figure 2.3. Continuous ECG monitoring was undertaken and blood pressure was recorded pre- and post-stress during each study.

2.3 Analytical Methods

2.3.1 Quantitative Analysis of MCE Studies

Quantitative MCE analysis was performed offline using a standard commercially available software, QLab version 7.0 (Q-Laboratory, Philips

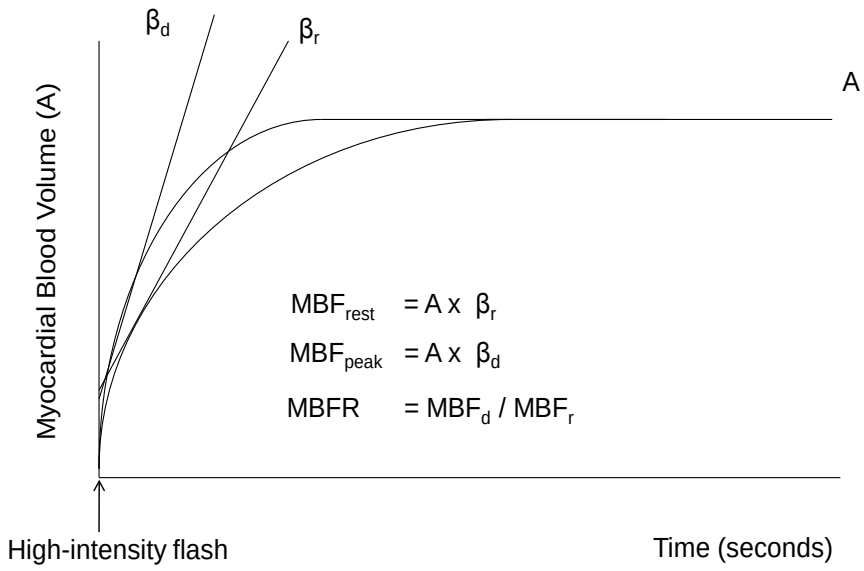
Medical Systems). Quantitative assessment of myocardial perfusion was performed for 10 consecutive end-systolic frames after microbubble destruction. A region of interest (ROI) was placed over the entire thickness of the myocardium and particular care was taken to exclude high-intensity epicardial and endocardial borders by manually moving the ROI between each frame. Background-subtracted plots of peak myocardial contrast intensity (representing myocardial blood volume A, dB), versus pulsing intervals (representing time) were automatically constructed by QLab software to fit the monoexponential function conventional equation $y = A\ (1-e^{-\beta t})$.[70] From these plots the slope of the replenishment curve was determined (representing myocardial blood velocity β, dB/s). The product of A and β yielded resting myocardial blood flow (resting MBF, dB^2/s) and post-dipyridamole myocardial blood flow (peak MBF, dB^2/s) respectively (see Figure 2.6). Myocardial Blood Flow Reserve (MBFR) was calculated by the ratio peak MBF/resting MBF.[70]

Furthermore, MBFR was calculated by dividing the peak MBF with the resting MBF of the same segment at each of the 3 time-frames (baseline, HE and HH). The basal segments were not included in the analysis in view of contrast attenuation.

The remaining 10 mid- and apical cardiac segments were analysed as shown in Figure 2.7.[148] A segment was not included in the analysis if there was artefact, inadequate microbubble destruction, attenuation or a wide variation in contrast intensity to minimise errors. The average number of analyzable segments for baseline, HE and HH were 6 each. All studies were reanalysed blindly for intra-observer variability and for inter-observer agreement 100 myocardial segments were randomly analysed by another observer (K.G) who

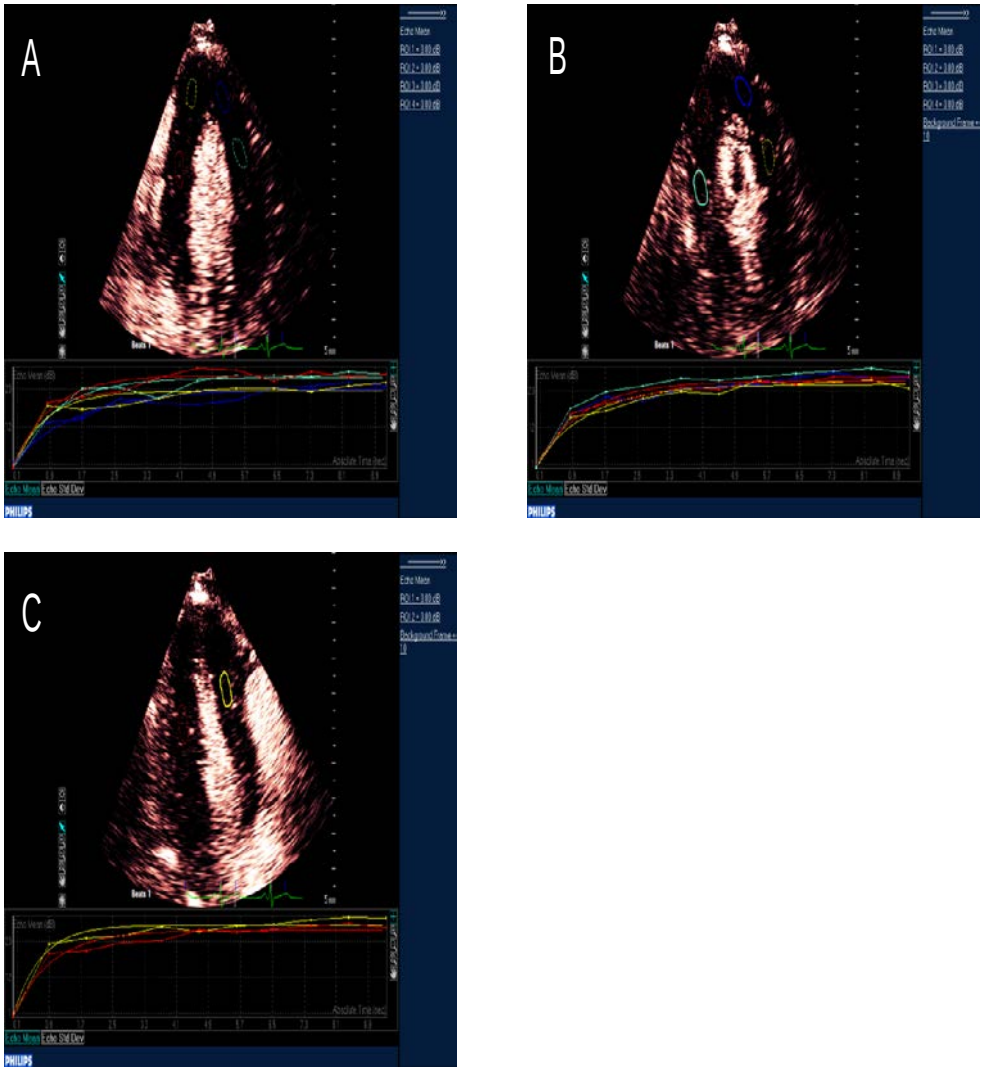
was blinded to the sequence of the studies. The intra-observer and inter-observer variability were 7.7% and 8.2% respectively.

Figure 2.6 Measurement of Myocardial Blood Flow Reserve using Flash Impulse Imaging.



A=myocardial blood volume, β_r=myocardial blood velocity at rest, β_d=myocardial blood velocity at peak, MBF_{rest}=myocardial blood flow at rest, MBF_{peak}=myocardial blood flow at peak, MBFR=myocardial blood flow reserve.

Figure 2.7. Model used for quantitative analysis of myocardial segments. (A) Apical 4 chamber, (B) Apical 2 chamber, (C) Apical 3 chamber



2.3.2 Plasma Glucose

Plasma glucose was determined every 3-5 minutes from arterialized blood samples with the glucose oxidase method (YSI 2300 STAT Plus, Yellow Springs, OH, USA) as shown in Figure 2.8.[140] The inter-assay coefficient of variation (CV) is <2% while the calibration of the analyzer was checked at 30-minute intervals with a glucose standard (10mmol/L). Volunteers were not informed of their glucose levels during the study.

Figure 2.8. YSI 2300 Stat Plus Glucose Analyzer

2.3.3 Laboratory Tests

All biochemical tests were performed by Dr Soha Zouwail, in duplicate, who was blinded to the MCE and haemodynamic data. Blood samples were drawn for the following tests:

1. Plasma endothelin-1 (ET-1) levels
2. Plasma adrenaline levels.
3. Serum insulin levels.
4. Serum high sensitivity CRP (hs-CRP).

Venous samples were taken at baseline and every 30 minutes thereafter **(7 samples in total)** for determination of ET-1, hs-CRP, adrenaline and insulin levels. All assays were performed in duplicate by a single observer (S.Z) who was blinded to the haemodynamic and MCE data.

2.3.3.1 Plasma Endothelin-1 Measurements

2.3.3.1a Sample Storage

Peripheral venous samples were collected in EDTA vacutainer tubes. Samples were subsequently centrifuged at 1000 x g for 15 minutes and the plasma was stored immediately at -20ºC. Plasma ET-1 levels were measured with by a specific enzyme-linked immunosorbent assay (ELISA) technique (QuantiGlo; R&D Systems).

2.3.3.1b Principle of the Assay

The assay employed a quantitative 'sandwich' enzyme immunoassay technique. A microplate coated with a rat monoclonal antibody specific for ET-1 was used. Standards and samples were pipetted into the wells following which any ET-1 present was bound by the immobilised antibody. Subsequently any unbound substances were washed away and an enzyme-linked mouse monoclonal antibody specific for ET-1 (conjugate) was added to the wells. Wells were washed to remove any unbound antibody-enzyme reagent, an enhanced luminal/peroxide substrate solution was added to the wells. This caused light production in proportion to the amount of ET-1 bound in the initial steps. A microplate luminometer was finally used to measure the intensity of the light emitted and relative light units (RLU) were recorded.

2.3.3.1c Sample Analysis

All reagents and samples were brought to room temperature before use. Standard kits provided by R&D Systems were used. One hundred µL of diluent assay containing a buffered protein base with preservatives followed by 100 µL of plasma sample or standard (2500 pg/ml of ET-1) were added to each of the 96 wells in a black polystyrene microplate provided by the manufacturer (12 strips, 8 wells). The microplate was covered by adhesive strip and incubated for 90 minutes at room temperature on a horizontal orbital microplate shaker set at 500 revolutions per minute (rpm). Each well was then

aspirated and washed using wash buffer provided. This process was repeated three times (for a total of four washes). Complete removal of liquid was ensured after the last wash and 200 µL ET-1 Conjugate (containing mouse monoclonal antibody against ET-1 conjugated to peroxidase) was added in each well. The microplate was covered with adhesive tape and incubated for 3 hours on the microplate shaker at 500 rpm. At the same time Working Glo reagent was prepared by mixing 1-part of Reagent A (luminal) and 2 parts of Reagent B (hydrogen peroxide). The wells were then aspirated and washed for a total of four washes, any remaining Wash buffer was removed by decanting the plate. 100 µl of Working Glo reagent was added to each well and the microplate was protected from light and incubated at room temperature for 5 minutes on a tabletop. The RLU were recorded from each microplate well containing the standards and a curve was drawn with the human ET-1 concentration (pg/ml) on the x-axis and the RLU on the y-axis. To determine the concentration of each sample, first the corresponding RLU was located on the y-axis and a horizontal line was extended to the linear curve. At the point of intersection, a vertical line was extended to the x-axis and the corresponding ET-1 concentration was noted.

2.3.3.1d Sensitivity of the Assay

The reported intra-assay and inter-assay coefficient of variation (CV) were ≤3.4% and ≤8.9% respectively with cross-reactivity of 0.02% for human big ET, 9.0% for ET-3 and 51% for ET-2 (product literature).

2.3.3.2 Plasma Adrenaline Measurements

2.3.3.2a Sample Storage

Peripheral venous samples were collected in EDTA vacutainer tubes. Samples were subsequently centrifuged at 1000 rpm and plasma was stored immediately at -20ºC. Plasma adrenaline levels were measured using ELISA (Labor Diagnostika Nord, Nordhorn, Germany).

2.3.3.2b Test Principle

During competitive enzyme immunoassay, the antigen (adrenaline) which is already bound to the solid phase of the microplate, competes with acylated adrenaline from the sample for a fixed number of antiserum binding sites. When equilibrium point is established, free antigen and free antigen-antiserum complexes are removed by washing. The anibody (from the sample) bound to the solid phase is detected by an anti-rabbit IgG conjugated with peroxidase using tetramethylbenzidine (TMB). The latter acts as a chromogenic substance and gives the precipitate a colour which can be detected by the spectrophotometer at 450nm. The amount of antibody bound to the solid phase adrenaline is inversely propotional to the antigen concentration of the sample (competitive immunoassay).

2.3.3.2c Sample Analysis

To the microplate, 10 µL of standard solution and 300 µL of plasma sample were added into the designated wells. Following this 250 µL of distilled water, 50 µL each of the assay buffer and extraction buffer were added. The microplate was then covered with adhesive foil and incubated for 30 minutes at room temperature (20-25ºC) on a shaker at 600 rpm. Subsequently, the microplate was washed with 1 µL of wash buffer using a pipette. The microplate was further incubated for 5 minutes on a shaker at 600 rpm. This step was repeated followed by the addition of 150 µL of acylation buffer, 25 µL of acylation reagent and a further incubation period of 15 minutes on the shaker at 600 rpm. The microplate was again washed as above and incubated for 10 minutes on the shaker and at that point 150 µL of hydrochloric acid was added into all the wells.

Following the fixation and acylation of adrenaline into the microplate wells, 25 µL of catechol-O-methyltransferase (COMT) were added to which a further 100 µL of sample was added. The microplate was incubated for 30 minutes on the shaker at 600 rpm and 50 µL of adrenaline antiserum was added into each well. The microplate was covered with adhesive foil and incubated for 2 hours at room temperature on the shaker. Subsequently, the foil was removed and each well was thoroughly washed thrice to remove any unattached antiserum. This was followed by the addition of 100 µL of enzyme conjugate (anti-rabbit IgG conjugated with peroxidase) and the microplate was incubated for a further 30 minutes. All wells were thoroughly washed thrice and 100 µL of the TMB was added to each well. The microplate was

incubated in the dark for a further 30 minutes. Finally, 100 µL of the stop solution was added to each well and the microplate was shaken to ensure homogeneous distribution of the solution. The absorbance of the solution was measured within 10 minutes using a microplate reader set to 450 nm. The read concentrations of the plasma were divided by 30 as advised by the manufacturer.

2.3.3.2d Sensitivity of the Assay

The reported inter-assay and intra-assay CV were <15% and 6.9% respectively. Furthermore, cross-reactivity of the assay with noradrenaline and dopamine was 0.2% and <0.0007% respectively. Comparing this method with another routinely used method, high performance liquid chromatography (HPLC) has a correlation coefficient of r=0.99 (product literature).

2.3.3.3 Serum High Sensitivity CRP Measurements

2.3.3.3a Sample Storage

Peripheral venous samples were collected in sodium citrate vacutainer tubes. Samples were subsequently centrifuged at 1000 x g and serum was stored immediately at -20ºC. hs-CRP was measured using an immunoturbidometric assay (Roche Diagnostics, Mannheim, Germany).

2.3.3.3b Test Principle

The test was based on the principle that by mixing two sizes of microparticles, each coated with different monoclonal antibodies (mAbs) of different reactivity, the accuracy of measuring hs-CRP could be improved. The high-reactivity mAb was carried by large-sized microparticles and reacted strongly with hs-CRP, generating a signal at low analyte concentrations (hs-CRP). The small-sized particles carried the low-reactivity mAb allowing immuno-reaction at high analyte (hs-CRP) concentrations providing a dynamic range to the assay.

2.3.3.3c Sample Analysis

The Roche systems (Roche Diagnostics, Mannheim, Germany) automatically calculate the hs-CRP concentration of each sample.

Conversion factors:

mg/L x 9.52 = nmol/L

2.3.3.3d Sensitivity of the Assay

The inter-assay CV was 5% and the correlation with two other tests, nephelometric assay and a previously used particle-enhanced turbidimetric assay has been shown to be r=0.992 and r=0.995 respectively (product literature).

2.3.3.4 Serum Insulin Measurements

2.3.3.4a Sample Storage

Peripheral venous samples were collected in sodium citrate vacutainer tubes. Samples were subsequently centrifuged at 1000 rpm for 15 minutes and serum was stored at -20ºC. Serum insulin concentrations were measured using electrochemiluminescence immunoassay (ECLIA, Roche Diagnostics, Mannheim, Germany).

2.3.3.4b Test Principle

Insulin from the sample was 'sandwiched' between a biotin-containing insulin-specific antibody and a monoclonal insulin-specific antibody labelled with a ruthenium complex. Application of a voltage to the electrode then induced a chemiluminescent emission which was measured by a photomultiplier.

2.3.3.4c Sample Analysis

A monoclonal insulin-specific antibody and a biotin-containing monoclonal insulin-specific antibody labelled with ruthenium were added to 20 µL of the sample. After the addition of streptavidin-coated microparticles, the complex became bound to the solid phase via interaction of biotin and streptavidin. Subsequently, the reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the

surface of the electrode. Following the removal of unbound substances, the application of a voltage to the electrode then induced a chemiluminescent emission which was measured by a photomultiplier.

The following conversion factors were used:

μU/mL x 6.945 = pmol/L

pmol./L x 0.144 = μU/mL

Normal fasting plasma insulin range is 2.6-24.9 μU/mL (17.8-173 pmol/L).

2.3.3.4d Sensitivity of the Assay

The interassay CV was reported as < 5% and the cross-reactivity with human pro-insulin was 0.05%. Furthermore, the correlation with another commercially available test (Enzyme-Test Insulin) was r=0.958 (product literature).

2.3.4 Haemodynamic Measurements:

Haemodynamic measurements were recorded including

1. Heart rate.
2. Systolic blood pressure (SBP)
3. Diastolic blood pressure (DBP).
4. Rate pressure product (product of heart rate x SBP)

These parameters were recorded pre- and post-dipyridamole at baseline, HE and HH.

2.3.4.1 Heart Rate

The heart rate was continuously monitored using Datascope Accutorr Plus™, (Datascope Corp, Paramus, NJ, USA).

2.3.4.2 Systolic Blood Pressure

The systolic blood pressure was measured at baseline, HE and HH pre- and post-dipyridamole infusion. Each time the systolic blood pressure was measured twice and the average of the two readings was taken. The systolic blood pressure was measured using an automated sphygmomanometer, Datascope Accutorr Plus™, (Datascope Corp, Paramus, NJ, USA).

2.3.4.3 Diastolic Blood Pressure

The diastolic blood pressure was measured at baseline, HE and HH pre- and post-dipyridamole infusion. Each time the diastolic blood pressure was measured twice and the average of the two readings was taken. The diastolic blood pressure was measured using Datascope Accutorr Plus™, (Datascope Corp, Paramus, NJ, USA).

2.3.4.4 Rate Pressure Product

The rate pressure product was calculated as the product of the average systolic blood pressure and the heart rate. This was calculated at pre- and post-dipyridamole infusion at baseline, HE and HH.

2.3.5 Electrocardiograms:

Continuous cardiac monitoring was carried out during the study using GE Marquette Case 8000 Exercise Testing System (GE Healthcare, USA). Serial ECGs were taken at baseline, HE and HH pre- and post-dipyridamole infusion.

2.4. Medicinal Products:

2.4.1 Insulin (100 units/ml. 1 Vial contains 10ml).

Soluble insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark) was used for this study and was administered as an infusion.

Hypoglycemia- 2.8mmol/L

Euglycaemia- 5.0 mmol/L

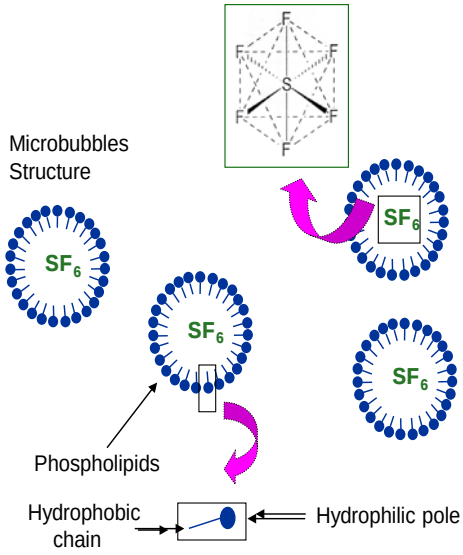
Insulin was constituted according to each participant's weight (see appendix) with 48 mls of 0.9% normal saline, 2 mls of patients own blood and infused via an infusion pump.

All unreconstituted insulin was stored according to the manufacturer's guidelines in a refrigerator at temperatures between 2-8 °C and care was taken not to allow it to freeze. Any unused insulin was discarded within 4 weeks.

2.4.2. SonoVue

Sonovue (Bracco Research SA, Geneva, Switzerland) is formulated as a 25 mg sterile, non-pyrogenic lyophilized powder in a septum-sealed vial. The gas phase in the vial is sulphur hexafluoride (SF_6), an innocuous gas. The lyophilized powder is made of a combination of pharmaceutical grade polyethylene glycol 4000, phospholipids (PLs) and palmitic acid. Phospholipids from chemical synthesis were selected for their higher chemical purity and lower pyrogenic potential. In SonoVue, a mixture of dipalmitoyl phosphatidylglycerol (DPPG.Na) and distearoylphosphatidylcholine (DSPC) is used. Upon reconstitution, 1 mL of the resulting dispersion contains 8 μL SF_6 in the microbubbles (Figure 2.9).

Figure 2.9 Microstructure of the Microbubbles with the Sulphur hexaflouride (SF_6) Core and Phospholipid Shell.

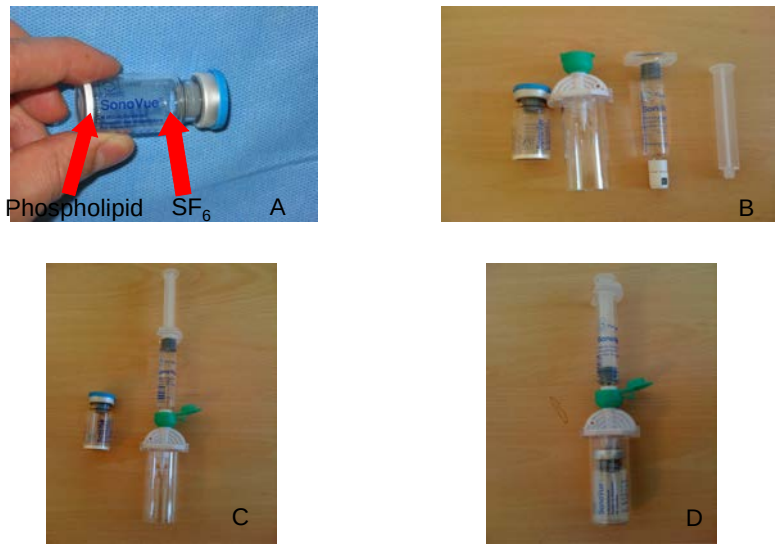


SonoVue was stored at controlled room temperature (20°- 25°C) in a secure area with limited access in standard manufacturer packaging. The shelf-life of any unreconstituted SonoVue at controlled room temperature and away from sun-light is 2 years.

The white, milky suspension of microbubbles was obtained by adding 5.0 mL of saline (USP, 0.9% sodium chloride [preservative-free] to the

lyophilized powder using standard clinical aseptic technique followed by vigorous shaking for 20 seconds (Figure 2.10). Once reconstituted, SonoVue can be left at room temperature.

Figure 2.10. Preparation of SonoVue Contrast Agent.



Phospholipid and sulphur hexaflouride (SF_6) shown (A). Materials provided in a standard SonoVue Kit (B). Prefilled syringe is attached to the plunger (C). The saline from the prefilled syringe is emptied into the vial through the plunger (D).

Although the suspension is useable for up to six hours, upon standing for more than 15 minutes, buoyancy causes some of the larger bubbles to rise to the surface. Therefore, gentle agitation is needed to reconstitute SonoVue in a top-to-bottom manner to suspend the microbubbles before administration. This was achieved by using a Vuejet pump (Bracco Research SA, Geneva, Switzerland).

2.5. Statistical Methods

2.5.1. Sample size

We set out *a priori* to look at the effects of hypoglycaemia on MBFR (our main outcome variable) in accordance with our study objective. Therefore, we understood that the most important and clinically relevant comparison of our results was between baseline and hyperinsulinemic hypoglycaemia (HH). Furthermore, we proposed recruiting n=20 to each group. We calculated that a sample size of 20 would give 85% power to detect standardised effect sizes of 0.70. Standardised effect sizes of 0.7 and 1.0 are considered to be large. There was only limited data in the literature to help interpret and/or justify these effect sizes. Srinivasan et al compared two groups of 10 individuals with type 1 diabetes (hyperglycaemia with euglycaemia).[85] Mean (SD) myocardial blood flow reserves (MBFR) were 1.92 (0.21) and 2.69 (0.41) respectively, equating to an effect size of 2.5, suggesting that effect sizes were likely to be large. We were able to recruit 19 healthy volunteers, 20 patients with Type 1 DM

without MVD and only 8 patients with Type 1 DM and MVD. The results of the healthy volunteers are presented in chapter 3. The results of the Type 1 DM patients without MVD are presented in chapter 4 while the combined data of the entire cohort are presented in chapter 5.

2.5.2 Statistical Analysis

All data are represented as mean±SD except ET-1 and hs-CRP values which are presented as median (interquartile range).

Data were analyzed using a standard commercially available software SPSS version 17.0 (SPSS Inc, Chicago, Ill, USA) and SAS software (Version 9.2). As we set out *a priori* to look at the effects insulin-induced hypoglycaemia on MBFR, therefore, a paired t-test was *only* used to compare MBFR between the three stages, (baseline, HE and HH). A p value of <0.05 was considered significant.

For MBF, β and A, the influence of measurement stage, of stress state, and of the presence of diabetes was assessed via mixed effects regression modelling (to reflect the intra-class correlation resulting from repeated measurements made on each subject). For each of these three outcomes, a mixed effects model was fitted in which the main effects of stage, stress state and diabetes (together with all of their possible interactions) were assessed. Modelling was performed using the MIXED procedure in SAS software (Version 9.2). Interpretation of these models is described below.

In addition to yielding regression parameter estimates, the models were also used to estimate mean values for each combination of effects (via the LSMEANS option in the MIXED procedure), and to test for selected differences in these means. With 12 effects combinations (i.e. three stages X two stress states X two diabetes states [present/absent]), the maximum number of possible between-group differences was 66. It was fully recognised that formal testing of between-group differences under these conditions was justified only (a) where there was *a priori* reason to anticipate the presence of an effect of interest, and (b) under the strict understanding that the primary purpose of such testing was the generation of hypotheses for future research, rather than the drawing of substantive inferences.

For the main outcome (MBFR), the concept of stress state was not applicable. Consequently, this outcome was investigated via a further mixed effects regression model in which MBFR was predicted by the main effects of measurement stage and of diabetes, together with that of their interaction; by age; and by the subject's systolic blood pressure.

2.5.3 Explanation of Mixed Effect Regression Modelling (Chapter 5)

We derived parameter estimates from mixed effects regression models in which the outcome of interest (respectively: myocardial blood volume, myocardial blood velocity and myocardial blood flow) is predicted by the main effects of

i. measurement stage (baseline, during hyperinsulinemic euglycemia and during hyperinsulinemic hypoglycemia)

ii. stress state (at rest and post dipyridamole-induced stress)

iii. diabetes status (controls vs. patients with diabetes)

and by all of their possible interactions: (i) with (ii); (i) with (iii); (ii) with (iii); and the single three-way interaction (i) with (ii) with (iii). Models were fitted using the MIXED procedure in SAS software version 9.2. Interpretation of the results is now described.

MEASUREMENT STAGE is the estimated mean value of the outcome for control subjects, in the rest state, observed at each of the three stages (because models were fitted with the intercept suppressed). These values are identical to those given in the 'Rest' sub-column of the C (Controls) column in the corresponding table of means in the main text.

STATE is the estimated effect of the stress treatment on the outcome for control subjects, at the baseline stage.

INTERACTION (STAGE with STATE) estimates the extent to which the effect of the stress treatment in controls at, respectively, the euglycaemic and hypoglycemic stages varies relative to that observed at the baseline stage.

PRESENCE OF DIABETES estimates the difference in the outcome at the baseline stage, in the resting state, between control subjects and those with diabetes.

INTERACTION (STAGE WITH DIABETES) estimates the extent to which the effect of measurement stage (that is, the change in resting values of the outcome at the euglycemic and hypoglycemic stages relative to the value observed at baseline) differs between control subjects and those with diabetes.

INTERACTION (STATE WITH DIABETES) estimates the extent to which the effect of the stress treatment, at the baseline stage, differs between controls and those with diabetes.

INTERACTION (STAGE with STATE with DIABETES) estimates the additional influence on the outcome of the joint presence of all main and two-way interaction effects. This may be illustrated with reference to Table 5.2b, from which the predicted absolute value of myocardial blood volume under euglycemia, post-stress, in subjects with diabetes is given by:-

20.4 (main effect of euglycemic stage) +

2.7 (main effect of stress) +

0.7 (main effect of diabetes) +

0.7 (euglycemia / stress interaction) +

-0.9 (euglycemia / diabetes interaction) +

-0.4 (stress / diabetes interaction) = 23.2

However, the three-way interaction term indicates that the estimated value of the outcome is 0.5 of a unit lower than that which would be predicted on the above basis (though the interaction is not statistically significant). That is, there is an additional effect arising from the joint presence of the euglycemia stage, the stress state and diabetes.

Chapter 3

Results of Healthy Control Subjects

3.1 Abstract

Background: Hypoglycaemia is a common complication of intensive insulin therapy (IIT) and is associated with increased cardiovascular mortality (CVM). Insulin, during euglycaemia, increases myocardial blood flow reserve (MBFR), but the effect of insulin-induced hypoglycaemia on MBFR is unknown. We tested the hypothesis that MBFR is decreased during insulin-induced hypoglycaemia using myocardial contrast echocardiography (MCE) and simultaneously measured endothelin-1 (ET-1) levels.

Methods and Results: Nineteen healthy volunteers (31.8±8.6 years, mean±SD) were studied. Hyperinsulinemic clamps maintained sequential hyperinsulinemic euglycaemia (HE, plasma glucose 4.9±0.2 mmol/L) and hyperinsulinemic hypoglycaemia (HH, plasma glucose 2.8±0.1 mmol/L) for 60 minutes each. Low-power real-time MCE was performed with flash impulse imaging using low-dose dipyridamole, at baseline, HE and HH to estimate resting and peak MBF. Resting myocardial blood flow (MBF) during HH (22.2±4.3 dB^2/s, 95%CI 20.1, 24.3) was increased compared to resting MBF at baseline (17.7±3.7 dB^2/s, 95%CI 15.9, 19.5). Peak MBF during HE increased in comparison to peak MBF at baseline (57.4±11.2, 95%CI 52.0, 62.8 vs. 44.3±12.1 dB^2/s, 95%CI 38.4, 50.1). Peak MBF during HH (46.7±8.4 dB^2/s, 95%CI 42.6, 50.8) was not different relative to baseline. There was an increase in MBFR during HE (3.1±0.5, 95%CI 2.9, 3.4) in comparison to baseline (2.6±0.3, 95%CI 2.4, 2.7) ($p<0.0001$). However, MBFR was decreased during

HH (2.2±0.2, 95%CI 2.1, 2.3) as compared to baseline ($p<0.0001$) and HE ($p<0.0001$).

Conclusions: In healthy humans insulin-induced hypoglycaemia is associated with a significant decrease in MBFR. This may explain the association between insulin-induced hypoglycaemia and increased CVM.

3.2 Introduction

Increased glucose concentrations in patients with acute coronary syndromes (ACS) and other acute severe illnesses are associated with increased short- and long-term all-cause and cardiovascular mortality (CVM).[34-36, 57] In addition, several studies have shown that the prevention of hyperglycaemia in acute illness through the use of intensive insulin therapy (IIT) reduces mortality.[18-20] However, more recent studies have failed to confirm this benefit, and in some cases IIT was associated with an increased all-cause mortality.[21-23] The reason for these discrepant study results is not clear, although accumulating evidence suggests that the higher prevalence of insulin-induced hypoglycaemia associated with IIT may be responsible.[25,26] This has led to the development of guidelines from the American Heart Association (AHA) and American Diabetes Association (ADA) which recommend IIT for in-patient control of hyperglycaemia but also add the caveat that "care should be taken to avoid hypoglycaemia".[17, 39] However, despite this advice, the rates of hypoglycaemia continue to be as high as 19% during IIT.[23]

The mechanisms by which hypoglycaemia adversely affect the cardiovascular system are unclear. Insulin, under euglycemic conditions, has important beneficial effects on the vascular tone by inducing nitric oxide (NO)-mediated vasodilatation.[149] Studies on healthy control subjects and patients with diabetes show that insulin causes a marked increase in myocardial blood flow (MBF) during euglycaemia.[90-92] In contrast, the effect of insulin-induced hypoglycaemia on MBF is uncertain. Therefore, to begin with, it is essential to establish the effect of insulin-induced hypoglycaemia on MBF in

normoglycemic individuals before studying the effects of insulin-induced hypoglycaemia on MBF in patients with diabetes.

Endothelin-1 (ET-1) is a 21-amino acid peptide mainly synthesised and released from the endothelial cells.[114] It is the most potent vasoconstrictor in man yet identified and may play an important role regulating MBF.[113] Recent investigators have demonstrated that ET-1 levels rise significantly in patients with type 1 diabetes during symptomatic hypoglycaemia.[124]

High sensitivity C-reactive protein (hs-CRP), an acute-phase reactant and marker of underlying inflammation and endothelial dysfunction has been shown to predict future coronary events.[126-128] In addition, hs-CRP levels have been shown to increase significantly during insulin-induced hypoglycaemia.[135]

Myocardial contrast echocardiography (MCE), is an established technique used in the non-invasive quantitative assessment of MBF with an accuracy similar to that of positron emission tomography and coronary Doppler flow wire measurements.[71, 144, 145] We tested the effect of insulin during euglycaemia followed by hypoglycaemia on both MBF and myocardial blood flow reserve (MBFR), calculated as the ratio of peak MBF to resting MBF, using a one-step hyperinsulinemic clamp technique in healthy humans.[139]

3.3 Methods

See Chapter 2 for details on methodology.

3.4 Results

3.4.1 Subject Charactersitics

The baseline characteristics of the 19 (11 males/ 8 females) healthy volunteers are summarized in Table 3.1. The fasting glucose concentrations were 4.9±0.3 mmol/L.

3.4.2 Haemodynamic Data

3.4.2a Heart Rate

There was no significant difference between resting pulse ($pulse_r$, b/min) at baseline (64±8, 95%CI 59, 68) and during HE (66±8, 95%CI 62, 70) as shown in Table 3.2. During HH, the $pulse_r$ was significantly elevated (75±11, 95%CI 70, 81) vs. baseline and HE. The post-dipyridamole pulse ($pulse_d$) was significantly increased in comparison to $pulse_r$ during all stages.

3.4.2b Systolic Blood Pressure

The resting systolic blood pressure did not change significantly (SBP_r, mmHg) at any stage as shown in Table 3.2. The post-dipyridamole systolic blood pressure (SBP_d, mmHg) did not differ between stages.

3.4.2c Diastolic Blood Pressure

Resting diastolic blood pressure (DBP_r, mmHg) did not vary between baseline (79±12, 95%CI 73, 85) and HE (78±9, 95%CI 74, 83) as shown in Table 3.2. However, this was followed by a significant reduction during HH (69±12, 95%CI 64, 75) vs. baseline and HE. Following dipyridamole, DBP_d was significantly reduced in comparison to corresponding DBP_r at baseline (72±9, 95%CI 68, 76) and HE (71±9, 95% CI 66, 75) but not during HH (68±6, 95% CI 65, 70).

3.4.2d Rate Pressure Product

The resting rate-pressure product (RPP_r), calculated as the product of resting systolic blood pressure and heart rate (b/min x mmHg), did not differ between baseline (7739±1533, 95%CI 7065, 8506) and HE (8163±1448, 95%CI 7519, 8860) as shown in Table 3.2. However, there was a significant increase during HH (9454±1692, 95%CI 8781, 10355) vs. baseline and HE. The post-dipyridamole rate-pressure product (RPP_d) did not differ between the three stages.

3.4.3 Myocardial Contrast Echocardiography-Derived Measurements (Table 3.3 and Figure 3.3).

3.4.3a Myocardial Blood Volume

Resting myocardial blood volume (A_r, dB), (19.3±2.4, 95%CI 18.1, 20.3) at baseline and HE (20.4±2.4, 95%CI 19.3, 21.6) did not vary as shown in Table 3.3. There was an increase in A_r during HH (21.7±2.2, 95%CI 20.7, 22.8)

compared to baseline. Post-dipyridamole peak myocardial blood volume (A_d) was increased in comparison to resting myocardial blood volume (A_r) at all three stages.

3.4.3b Myocardial Blood Velocity

Resting myocardial blood velocity (β_r, dB/s) did not vary between baseline (0.9±0.2, 95%CI 0.8, 1.0) and HE (1.0±0.2, 0.9, 1.0) as shown in Table 3.3. During HH, β_r was increased (1.04±0.2, 95%CI 1.0, 1.1) compared to baseline. Post-dipyridamole peak myocardial blood velocity (β_d) was increased compared to resting β_r at baseline (2.0±0.5, 95%CI 1.8, 2.2), HE (2.4±0.4, 95%CI 2.2, 2.6) and HH (2.0±0.3, 95%CI 1.9, 2.2).

3.4.3c Myocardial Blood Flow

There was no difference in the resting MBF (dB^2/s) between baseline (17.7±3.7, 95%CI 15.9, 19.5) and HE (19.3±3.9, 95%CI 17.4, 21.1). However, resting MBF was increased during HH (22.2±4.3, 95%CI 20.1, 24.3) in comparison to baseline and HE as shown in Table 3.3. Peak MBF was significantly elevated at all three stages, baseline (44.3±12.1, 95%CI 38.4, 50.1), HE (57.4±11.2, 95%CI 52.0, 62.8) and HH (46.7±8.4, 95%CI 42.6, 50.8) in comparison to corresponding resting MBF values. Peak MBF recorded a 30% incremental increase with insulin under euglycaemic conditions as compared to baseline. Finally, a 19% reduction in peak MBF was observed during HH in comparison to HE which was not significantly different from baseline.

3.4.3d Myocardial Blood Flow Reserve (Figure 3.3)

Myocardial blood flow reserve (MBFR) increased by 20% ($p<0.0001$) during HE (3.1±0.5, 95%CI 2.9, 3.4) in comparison to baseline (2.6±0.3, 95%CI 2.4, 2.7). However, there was a 29% and 15% reduction in MBFR during HH (2.2±0.2, 95%CI 2.1, 2.3) in comparison to HE and baseline respectively ($p<0.0001$, for both comparisons).

3.4.4 Changes in Concentrations of Plasma Glucose, Endothelin-1, Adrenaline, Serum High sensitivity CRP and Insulin during the Hyperinsulinemic Clamp

Plasma glucose (mmol/L) at baseline was 4.7±0.3, (95%CI 4.6, 4.9) which was maintained at 4.9±0.2, (95%CI 4.7, 4.9) during HE until symptomatic hypoglycaemia (HH) was induced at a nadir of 2.9±0.1, (95% CI 2.8, 2.9) as shown in Table 3.4. Serum insulin concentrations (pmol/L) at baseline were 43±23, (95%CI 31.8, 54.2) and increased significantly after commencing the hyperinsulinemic clamp with levels of 763.8±160, (95%CI 686.2, 840.5) and 714.3±160, (95%CI 637.4, 791.3) during HE and HH respectively. Plasma ET-1 concentrations (pg/ml) represented as median(interquartile range, IQR) at baseline were 0.19(1.04) which remained unchanged during HE 0.5(0.9) and HH 0.5(1.1). There was no significant change in plasma adrenaline concentrations (pg/ml) when comparing baseline (76.3±77, 95%CI 35.7, 111.8) with HE values (96.3±56.7, 95%CI 69.0, 123.6). During HH, adrenaline levels were significantly higher (405.7±310, 95%CI 299.8, 570.9) vs. baseline and HE.

Serum hs-CRP concentrations (mg/L) represented as median(IQR) did not vary significantly between baseline 0.6(0.7), HE 0.6(0.6) or HH 0.5(0.4).

3.5 Discussion

This study has shown for the first time that, in healthy individuals during insulin-induced hypoglycaemia, there is an increase in resting myocardial blood flow and a decrease in dipyridamole-induced peak myocardial blood flow, causing a reduction in the myocardial blood flow reserve. In contrast, hyperinsulinemic euglycaemia induced a marked increase in dipyridamole-induced peak myocardial blood flow and myocardial blood flow reserve, an effect mitigated completely by insulin-induced hypoglycaemia.

Under hyperinsulinemic euglycaemic conditions we recorded a 30% increase in peak MBF in comparison to peak baseline values. These findings are consistent with the results of earlier studies.[90-92] The overall effect of HH was to reduce peak MBF back to baseline values and entirely remove the vasodilatory effect of insulin. This appeared to be due to a reduction in myocardial blood velocity rather than blood volume. Of note, resting MBF during hypoglycaemia was increased in comparison to resting HE and baseline values. This finding, combined with a decrease in the peak MBF, resulted in an overall net reduction in MBFR during HH of 30% and 15% when compared to HE and baseline MBFRs respectively.

The pathophysiological mechanisms by which insulin-induced hypoglycaemia may be associated with increased CVM are unknown but are

likely to be multifactorial. Animal studies have demonstrated that hypoglycaemia increases myocardial infarct size by over 40%.[94] Furthermore, in patients with diabetes and coronary artery disease, hypoglycaemia was associated with up to 30% of all episodes of angina.[32] In addition, low blood glucose also encourages a hypercoagulant state as there is an increase in plasma concentrations of coagulation factors and platelet aggregation.[105, 108] Hypoglycaemia is also associated with prolongation of the QT interval in patients with diabetes and this may increase the risk of sudden cardiac death.[102] Our observation that insulin-induced hypoglycaemia is associated with a marked reduction in MBFR may also explain the increased CVM. Although there was an increase in resting MBF during hypoglycaemia, it is extremely likely that the peak MBF is the more clinically relevant measurement. This is because during severe illness such as septicaemia or acute myocardial infarction, the coronary vasculature is in a maximally vasodilated state. Consequently, any decrease in peak MBF is therefore likely to have important and adverse consequences.

ET-1 is a 21-amino acid peptide mainly synthesised and released from the endothelial cells.[114] It is the most potent vasoconstrictor in man yet identified and may play an important role regulating MBF.[113] Recent investigators have demonstrated that ET-1 levels rise significantly in patients with type 1 diabetes during symptomatic hypoglycaemia.[124] In a study measuring ET-1 levels following primary percutaneous intervention, those patients with elevated plasma ET-1 levels above 0.6 pg/ml had a six-fold increase in 30-day mortality.[117] Following an infusion of ET-1 directly into the coronary sinus, Pernow et al demonstrated that, when exogenously administered,

a ET-1 plasma concentration of 11.8 pg/mL was required to produce a significant reduction in coronary blood flow.[116] This is 15-fold above the highest values measured in our study. However, it is possible that our ET-1 values, taken 60 minutes after the onset of hypoglycaemia, represent pre-peak levels and later measurements may well have been higher. Furthermore, ET-1 is known to be preferentially released abluminally towards the vascular smooth muscle cells (basement membrane side) and not into the vascular lumen.[150] However, in our study there was no change in ET-1 levels during hypoglycaemia. This association should be tested in future studies with longer duration of hypoglycaemia.

High sensitivity C reactive protein (hs-CRP) has emerged as a possible prognostic indicator for primary cardiovascular events in apparently healthy men and women.[125, 126] Verma and co-workers noted that incubating human saphenous vein endothelial cells in a solution of hs-CRP at concentrations similar to those during mild inflammation, led to a 4-fold increase in ET-1 levels.[129] Other investigators have shown that hs-CRP levels rise following insulin-induced hypoglycaemia.[135] In our study there was no demonstrable increase in the hs-CRP concentration after 1-hour of hyperinsulinemic hypoglycaemia which is consistent with the findings of Galloway and associates.

The prevention of hyperglycaemia during acute illness has been shown to improve survival in patients with and without coronary artery disease. In the Diabetes Mellitus Insulin-Glucose Infusion in Acute Myocardial Infarction (DIGAMI) study, patients with DM presenting with acute myocardial infarction were randomly assigned to receive either IIT or conventional treatment for

hyperglycaemia.[18, 19] At 1 and 3.5 years follow-up there was a significant reduction in all-cause mortality in those treated with IIT. Consistent with this, a larger study examined the effect of IIT on patients admitted to surgical intensive care.[20] This also demonstrated a marked relative risk reduction in the total in-hospital mortality by 34%. Subsequently, guidelines were issued by the American Heart and Diabetic Associations recommending IIT for the prevention of in-patient hyperglycaemia and these are now part of standard care.[17]

In contrast, more recent trials have failed to show the beneficial effect of IIT.[21-23] Two studies have shown a 'neutral' effect on survival and two others have actually demonstrated an increase in mortality.[21-23, 52] Van den Berghe and co-workers repeated their work in medical (rather than surgical) intensive care patients and found that IIT did not improve 90-day mortality.[22] The reason for this discrepancy between these two trials (undertaken by the same group) is not clear. An important reason may be the higher rates of hypoglycaemia observed to occur in the IIT groups. For example, the prevalence of IIT-induced hypoglycaemia in the medical ICU trial was much higher than that in the surgical ICU study at 19% vs. 5% respectively. Similarly, in the Efficacy of Volume Substitution and Insulin Therapy in Severe Sepsis (VISEP) trial there was no difference in survival between intensive versus conventional insulin therapy.[52] The VISEP trial had to be terminated prematurely due to a 4-fold increase in hypoglycaemic events in the IIT arm More importantly, hypoglycaemia from any cause was found to be independently associated with a 3-fold increase in all-cause mortality. The NICE-SUGAR investigators, in the largest study to-date, demonstrated a significant increase in the absolute risk of 90-day mortality by 2.6% following

the use of IIT in patients admitted to ICU.[23] More importantly, the investigators reported a 16% increase in the relative risk of CVM in the IIT group in comparison to controls. Although this finding was unexplained, there was a 13-fold increased prevalence of severe hypoglycaemia (<2.2 mmol/L) in the patients on IIT in comparison to patients receiving conventional therapy.

In the light of our findings it is plausible that insulin-induced hypoglycaemia, by causing a decrease in myocardial blood flow reserve, increases the risk of CVM in susceptible individuals. This observed association has been recognised by the AHA and ADA in their recent guidelines and recommend a target glucose level of between 5.0 and 7.8 mmol/L for patients receiving IIT 'as long as hypoglycaemia is avoided'.[17]

3.6 Limitations

Dipyridamole was used three times in succession with our study protocol (Figure 3.1a and b). However, dipyridamole has a short half-life of 8-12 minutes and the time period between each dipyridamole infusion in our study was 76 minutes. Dipyridamole-induced changes in left ventricular ejection fraction, end-systolic volume, heart rate and diastolic blood pressure have previously been shown to return to baseline after a 60-minute period using a high-dose (0.76 mg/kg) protocol.[151] Furthermore, in our study a low-dose (0.56 mg/kg) protocol was used. Finally, the effects of administering dipyridamole three times in succession was tested in a healthy individual over the same time-course and there was no change in MBF, MBFR or other haemodynamic

parameters. Thus we consider that the repeated use of dipyridamole did not artefactually influence our results.

Hypoglycaemia has shown to be associated with increased cardiovascular mortality in a heterogeneous group of patients suffering from a wide range of conditions.[54-57] For example, a recent study showed that patients with acute ST-elevation myocardial infarction and a high thrombolysis in myocardial infarction (TIMI) risk score, suffering from hypoglycaemia had a 10-fold increased 30-day mortality in comparison to patients who did not experience hypoglycemia.[55] Therefore, our group felt it important to investigate the physiological effects of insulin-induced hypoglycaemia in healthy volunteers first, in order to provide a mechanistic basis for this observed association. It is possible that this study will enable future research to look at the effects of insulin-induced hypoglycaemia in other clinical conditions.

The authors decided not to calculate absolute myocardial perfusion values. This was because all settings and infusion parameters, once optimised at the start of each patient study, were kept constant for the rest of that individual procedure. Each patient acted as their own control and all changes in myocardial blood flow were relative to that patient's baseline values and have been analysed using statistical tests for paired data. Furthermore, we achieved homogenous opacification of the left ventricular blood pool and the signal intensity received was consistently between 34-36 dB. Calculation of absolute myocardial blood flows to take into account regional blood flow variations that occur within individuals would have introduced an additional potential source of error.

3.7 Conclusion

This study has shown that in healthy individuals during insulin-induced hypoglycaemia there is an increase in resting myocardial blood flow but a marked reduction in peak myocardial blood flow and myocardial blood flow reserve. We suggest that alterations in myocardial blood flow reserve may provide an explanation for the observed association between insulin-induced hypoglycaemia and increased CVM in susceptible individuals.

Table 3.1. Baseline Characteristics of the Healthy Humans

Variable	Mean±SD
Age years	31.8±9
Male/Female	11/8
BMI kg/m^2	24.9±2.6
SBP mmHg	115±10
DBP mmHg	73±7
Heart rate b/min	79±16
Fasting glucose mmol/L	4.9±0.3
TC mmol/L	4.3±0.8
LDLc mmol/L	2.2±0.8
HDLc mmol/L	1.5±0.3
TG mmol/L	1.0±0.4

BMI=body mass index, SBP=systolic blood pressure, DBP=diastolic blood pressure, TC=total cholesterol, LDLc=low density lipoprotein cholesterol, HDLc=high density lipoprotein cholesterol, TG=triglycerides.

Table 3.2. Haemodynamic Data (Pulse rate, systolic blood pressure, diastolic blood pressure and rate pressure product) at rest and after dipyridamole-induced stress during the hyperinsulinemic clamp

Variable	Baseline	Hyperinsulinemic Euglycaemia (HE)	Hyperinsulinemic Hypoglycaemia (HH)
$Pulse_r$ b/min	63±9	66±8	75±11
$Pulse_d$ b/min	90±11	93±14	97±10
SBP_r mmHg	121±14	124±13	124±13
SBP_d mmHg	125±15	125±11	121±11
DBP_r mmHg	79±12	78±9	69±12
DBP_d mmHg	72±9	71±9	68±6
RPP_r b/min.mmHg	7739±1533	8163±1448	9454±1692
RPP_d b/min.mmHg	11375±2399	11533±1839	11712±1616

Pulse rate, systolic blood pressure, diastolic blood pressure and rate pressure product are presented as mean±SD.

$Pulse_r$: resting pulse, $Pulse_d$: post-dipyridamole pulse, SBP_r: resting systolic blood pressure, SBP_d: post-dipyridamole systolic blood pressure, DBP_r: resting diastolic blood pressure, DBP_d post-dipyridamole diastolic blood pressure, RPP_r: resting rate pressure product, RPP_d: post-dipyridamole rate pressure product.

Table 3.3. Myocardial blood volume (A), myocardial blood velocity (β), myocardial blood flow (MBF) at rest and peak stress.

	Baseline		**Hyperinsulinemic Euglycaemia**		**Hyperinsulinemic Hypoglycaemia**	
	Rest	Peak	Rest	Peak	Rest	Peak
A dB	19.3±2.4	22±2.0	20.4±2.4	23.9±1.9	21.7±2.2	23±2.3
β dB/s	0.91±0.19	2.0±0.5	0.96±0.15	2.4±0.4	1.04±0.15	2.0±0.3
MBF dB^2/s	17.7±3.7	44.3±12.1	19.3±3.9	57.4±11.2	22.2±4.3	46.7±8.4

All values are presented as mean±SD.

A=(myocardial blood volume), β=(myocardial blood velocity), MBF= (myocardial blood flow).

Table 3.4. Endothelin-1, hs-CRP, adrenaline, and insulin concentrations during the hyperinsulinemic clamp.

Variable	Baseline	Hyperinsulinemic Euglycaemia			Hyperinsulinemic Hypoglycaemia		
	0 mins	30 mins	60 mins	90 mins	120 mins	150 mins	180 mins
Glucose mmol/L	4.7±0.3	4.9±0.2			2.9±0.1		
ET-1 pg/ml	0.2(1.0)	0.0(0.7)	0.0(0.8)	0.6(0.9)	0.2(0.7)	0.4(0.8)	0.5(1.1)
hs-CRP mg/L	0.6(0.7)	0.6(0.6)	0.6(0.6)	0.6(0.6)	0.6(0.5)	0.6(0.6)	0.5(0.4)
Adrenaline pg/ml	76.3±77	77.7±79	91.6±108	96.3±56.7	106.7±84.4	347±199	405.7±310
Insulin pmol/L	43±23	741±180	728±222	763±160	643±167	683±133	714±160

Glucose, adrenaline and insulin values are presented as mean±SD. Endothelin-1 and high sensitivity C reactive protein are shown as median(IQR).

ET-1:endothelin-1, hs-CRP:high sensitivity C reactive protein.

Figure 3.1a. Overall study scheme

B=baseline, HE=hyperinsulinemic euglycaemia, HH=hyperinsulinemic hypoglycaemia, MCE=myocardial contrast echocardiography

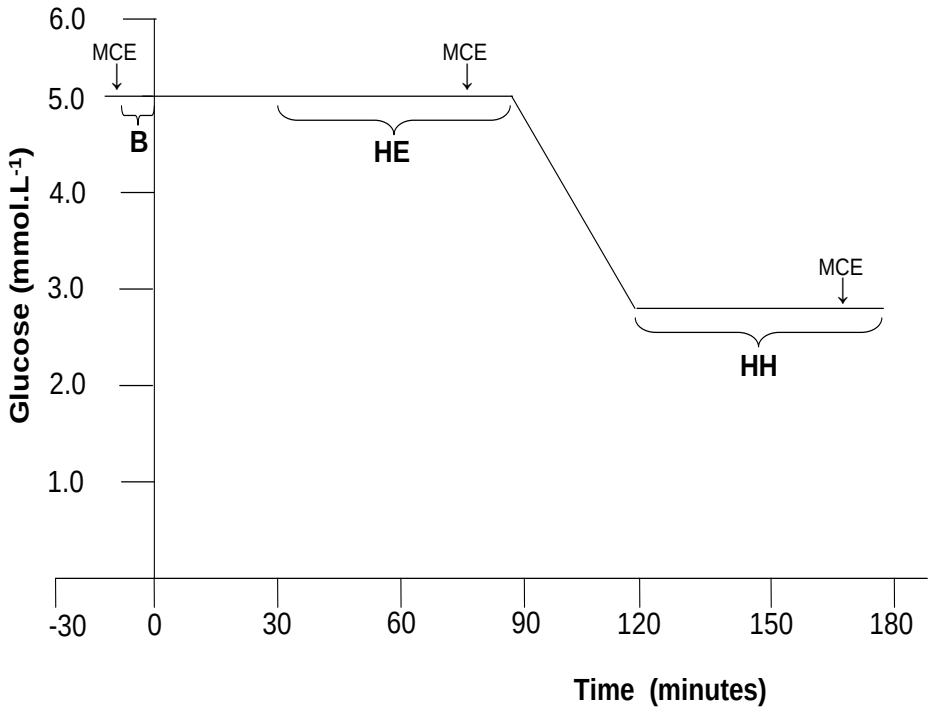


Figure 3.1b. Scheme showing sequence of image acquisition during each MCE study at baseline, HE and HH. MCE_{rest}=myocardial contrast echocardiography at rest, MCE_{peak}=myocardial contrast echocardiography at peak stress

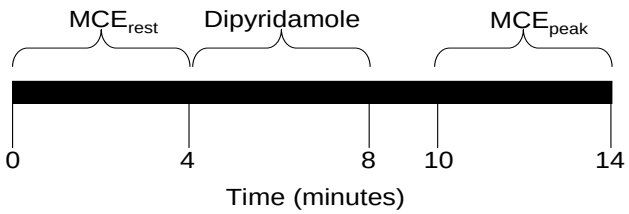


Figure 3.2. Model used for quantitative analysis of myocardial segments. (A) Apical 4 chamber, (B) Apical 2 chamber, (C) Apical 3 chamber

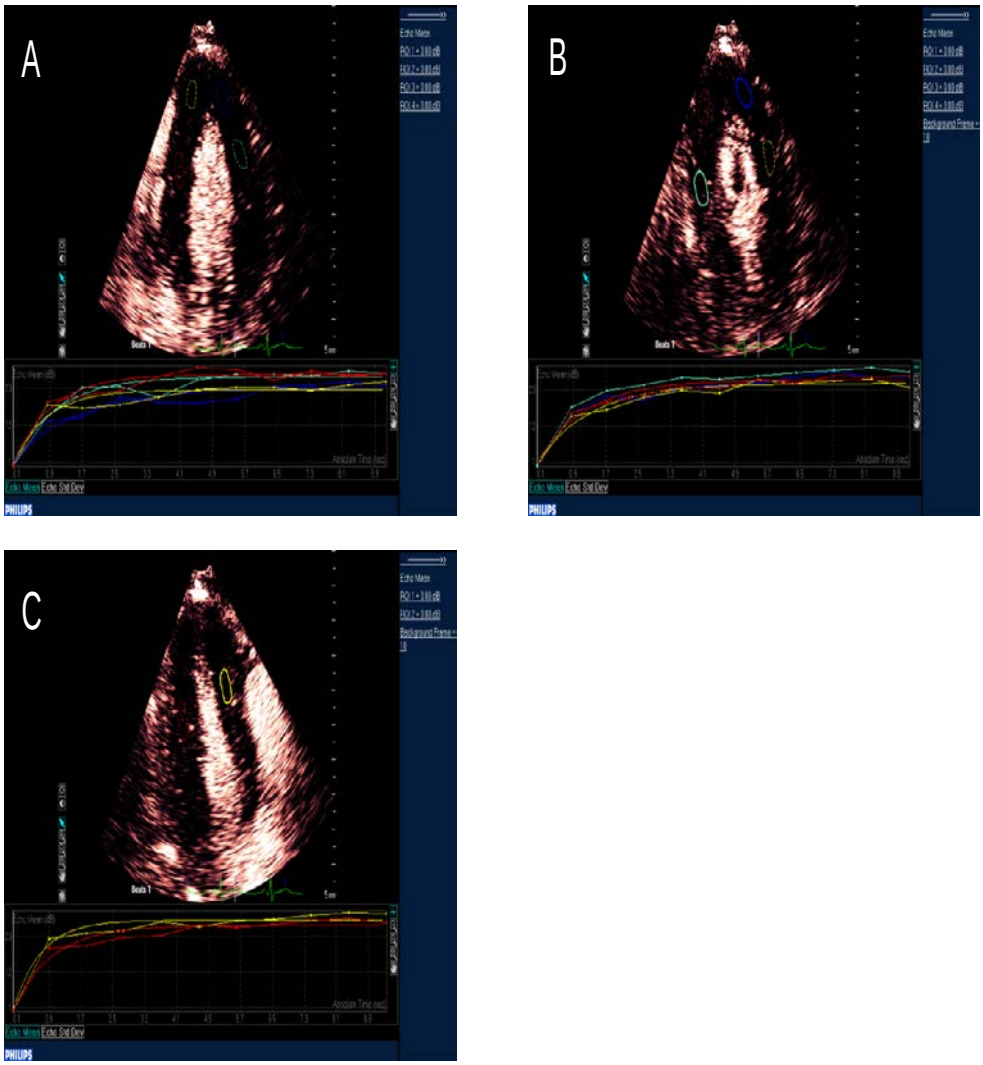
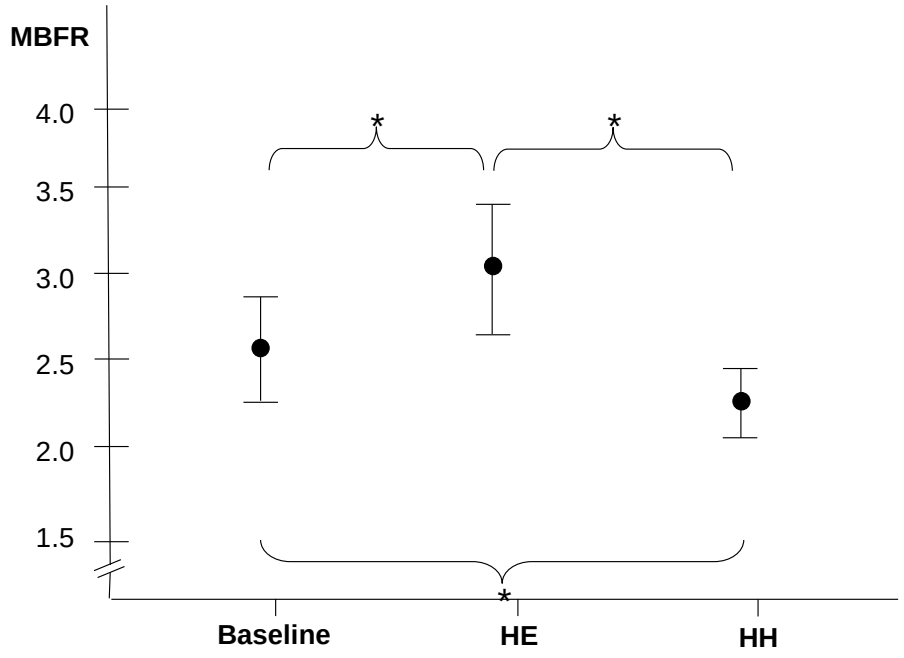


Figure 3.3. Myocardial blood flow reserve (MBFR) at baseline, hyperinsulinemic euglycaemia (HE) and hyperinsulinemic hypoglycaemia (HH) in the Healthy Humans Group



Values are represented as (mean±SD). *p<0.0001.

Chapter 4

Results of Patients with Type 1 Diabetes Mellitus without Microvascular Complications

4.1 Abstract

Background: Earlier studies showed a beneficial effect of intensive glycaemic therapy (IGT) on patients with diabetes mellitus (DM). However, more recent studies with more strict glycaemic targets have failed to reaffirm this benefit and in fact some have suggested increased harm. This association between IGT and increased adverse events is poorly understood although hypoglycaemia has emerged as a putative cause.

Methods and Results: Twenty patients with DM and no evidence of microvascular complications underwent hyperinsulinemic clamps with maintained sequential hyperinsulinemic euglycaemia (HE, plasma glucose 5.0 mmol/L) followed by hyperinsulinemic hypoglycaemia (HH, plasma glucose 2.8 mmol/L) for 60 minutes each. Low-power real-time MCE was performed with flash impulse imaging using low-dose dipyridamole stress, at baseline, and during HE and HH. Resting myocardial blood flow (MBF) during HH (23.9±3.7 dB^2/s, 95%CI 22.2, 25.6) was increased compared to resting MBF at baseline (19.7±3.3 dB^2/s, 95%CI 18.2, 21.2). Peak MBF during HE increased in comparison to peak MBF at baseline (57.4±10.6, 95%CI 52.4, 62.3 vs. 42.7±6.8 dB^2/s, 95%CI 39.5, 45.9). Peak MBF during HH (47.4±6.0 dB^2/s, 95%CI 44.6, 50.1) was not different relative to baseline. There was an increase in MBFR during HE (2.7±0.4, 95%CI 2.5, 2.9) in comparison to baseline (2.2±0.2, 95%CI 2.1, 2.4) ($p<0.0001$). However, MBFR was decreased during

HH (2.0±0.3, 95%CI 2.0, 2.2) as compared to baseline ($p=0.041$) and HE ($p<0.0001$).

Conclusions: Hypoglycaemia decreases MBFR in patients with DM. This finding may explain the association between hypoglycaemia and increased CVM in susceptible individuals.

4.2 Introduction

The Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) showed that intensive glycaemic therapy (IGT) was associated with a decrease in the development of microvascular complications by an average of 57% and 25% respectively.[9, 10] Subsequent follow-up of DCCT showed that IGT was associated with a 57% reduction in the cumulative incidence of macrovascular complications and cardiovascular mortality in patients with type 1 DM.[11] A more recently published follow-up of the UKPDS study showed that IGT was associated with a 22% and 20% reduction in the development of macrovascular complications and all-cause mortality respectively in patients with type 2 DM.[12] More importantly, the glycosylated haemoglobin (HbA1c) was 6.9% in the IGT group (vs. 8.5% in the conventional therapy group) in the DCCT trial and 7.0% in the IGT group (vs. 7.9% in the conventional therapy group) in the UKPDS trial. Therefore, these observations led to subsequent studies exploring the effects of a 'tighter' glycaemic control on patients with DM by aiming for a target HbA1c of <6.0%. The Action in Diabetes and vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) trial failed to demonstrate any benefit of IGT on patients with type 2 DM with a 2-fold increase in hospitalizations secondary to severe hypoglycaemia.[14] On the contrary, Action to Control Cardiovascular in Diabetes (ACCORD) trial recorded an absolute increase of 1.05% (relative increase of 26.5%) in all-cause mortality with IGT which led to the premature closure of the trial.[15] Although the mechanism was not clearly identified, hypoglycaemia emerged as

a putative cause. For example, in the ACCORD study, 1 in 9 patients in the IGT group experienced severe hypoglycaemic episodes requiring medical assistance.

Insulin, under euglycaemic conditions, has important beneficial effects on the vascular tone by inducing nitric oxide (NO)-mediated vasodilatation.[149] Studies on healthy humans and patients with diabetes show that insulin causes a marked increase in myocardial blood flow (MBF) and myocardial blood flow reserve (MBFR) during euglycemia.[90, 91] In contrast, the mechanisms by which hypoglycaemia adversely affects the cardiovascular system are unclear. Hypoglycaemia has been associated with angina and importantly, has been shown to increase the size of a myocardial infarct.[32, 94] Furthermore, low blood glucose also encourages a hypercoagulant state due an increase in plasma concentrations of coagulation factors and by promoting platelet aggregation.[105, 108]

Myocardial contrast echocardiography (MCE), is an established technique used in the non-invasive quantification of myocardial blood flow reserve (MBFR) with an accuracy similar to that of positron emission tomography and coronary Doppler flow wire measurements.[70, 144, 145] MBFR is calculated as the ratio of peak myocardial blood flow (MBF) to resting MBF. In the absence of flow-limiting CAD, a MBFR <2.0 is indicative of underlying endothelial dysfunction.[74] Furthermore, MBFR has been shown to be an independent predictor of CVM in diabetic and non-diabetic patients with normal stress echocardiograms, as well as post-ACS.[152-155]

We hypothesised that hypoglycaemia would decrease the MBFR (measured by MCE) using a one-step hyperinsulinemic clamp technique to induce hypoglycaemia, in patients with type 1 DM.

4.3 Methods

See Chapter 2 for details.

4.3.1 Subjects

This Chapter includes results from the 20 patients with Type 1 DM without MVD.

4.3.5 Statistical Analysis

All data are represented as mean±SD except ET-1 and hs-CRP values which are presented as median (interquartile range).

Data were analyzed using a standard commercially available software SPSS version 17.0 (SPSS Inc, Chicago, Ill, USA) and SAS software (Version 9.2). As we set out *a priori* to look at the effects insulin-induced hypoglycaemia on MBFR, therefore, a paired t-test was *only* used to compare MBFR between the three stages, (baseline, HE and HH). A p value of <0.05 was considered significant.

4.4 Results

4.4.1 Subject Charactersitics

The baseline characteristics of the 20 (15 males/ 5 females) patients with type 1 DM (without MVD) are summarized in Table 4.1. The fasting glucose concentrations were 9.5±3.6 mmol/L.

4.4.2 Haemodynamic Data

4.4.2a Heart Rate

There was no significant difference between resting pulse ($pulse_r$, b/min) at baseline (66±11, 95%CI 61, 71) and during HE (70±14, 95%CI 64, 77) as shown in Table 4.2. During HH, the $pulse_r$ was significantly elevated (77±17, 95%CI 69, 85) vs. baseline and HE. The post-dipyridamole pulse ($pulse_d$) was significantly increased in comparison to $pulse_r$ during all stages.

4.4.2b Systolic Blood Pressure

The resting systolic blood pressure did not change significantly (SBP_r, mmHg) at any stage as shown in Table 4.2. The post-dipyridamole systolic blood pressure (SBP_d, mmHg) did not differ between stages.

4.4.2c Diastolic Blood Pressure

Resting diastolic blood pressure (DBP_r, mmHg) did not vary between baseline (71±11, 95%CI 66, 76) and HE (70±11, 95%CI 65, 75) as shown in Table

4.2. However, this was followed by a significant reduction during HH (63±8, 95%CI 59, 67) vs. baseline and HE. Following dipyridamole, DBP_d was significantly reduced in comparison to corresponding DBP_r at HE only (61±10, 95%CI 57, 66) and not during baseline (71±8, 95%CI 67, 75) or HH (61±7, 95%CI 57, 64).

4.4.2d Rate Pressure Product

The resting rate-pressure product (RPP_r), calculated as the product of resting systolic blood pressure and heart rate (b/min x mmHg), did not differ between baseline (7851±1695, 95%CI 7058, 8644) and HE (8375±2162, 95%CI 7363, 9387) as shown in Table 4.2. However, there was a significant increase during HH (9310±2377, 95%CI 8198, 10422) vs. baseline and HE. The post-dipyridamole rate-pressure product (RPP_d) did not differ between the three stages.

4.4.3 Myocardial Contrast Echocardiography-Derived Measurements (Table 4.3 and Figure 4.3).

4.4.3a Myocardial Blood Volume

Resting myocardial blood volume (A_r, dB), (20.2±2.0, 95%CI 19.2, 21.1) at baseline and HE (20.4±2.4, 95%CI 19.2, 21.5) did not vary as shown in Table 4.3. There was an increase in A_r during HH (21.2±2.2, 95%CI 20.1, 22.2) compared to baseline. Post-dipyridamole peak myocardial blood volume (A_d) was increased in comparison to resting myocardial blood volume (A_r) at all three stages. Finally, A_d was significantly increased during HH (24.0±1.9,

95%CI 23.1, 24.9) in comparison to baseline (23.3±1.8, 95%CI 22.5, 24.2) and HE (23.3±1.8, 95%CI 22.5, 24.2).

4.4.3b Myocardial Blood Velocity

Resting myocardial blood velocity (β_r, dB/s) did not vary between baseline (1.0±0.2, 95%CI 0.9, 1.1) and HE (1.1±0.2, 95%CI 1.0, 1.2) as shown in Table 4.3. During HH, β_r was increased (1.2±0.2, 95%CI 1.1, 1.2) compared to baseline. Post-dipyridamole peak myocardial blood velocity (β_d) was increased compared to corresponding resting β_r at all stages i.e. baseline (1.9±0.3, 95%CI 1.8, 2.0), HE (2.5±0.4, 95%CI 2.3, 2.7) and HH (2.0±0.3, 95%CI 1.8, 2.1). More importantly, B_d was significantly elevated at HE in comparison to baseline and HH.

4.4.3c Myocardial Blood Flow

There was no difference in the resting MBF (dB^2/s) between baseline (19.7±3.3, 95%CI 18.2, 21.2) and HE (22.0±4.8, 95%CI 19.8, 24.3). However, resting MBF was increased during HH (23.9±3.7, 95%CI 22.2, 25.6) in comparison to baseline and HE as shown in Table 4.3. Peak MBF was significantly elevated at all three stages, baseline (42.7±6.8, 95%CI 39.5, 45.8), HE (57.4±11.0, 95%CI 52.4, 62.3) and HH (47.4±6.0, 44.6, 50.1) in comparison to corresponding resting MBF values. Peak MBF recorded a 34% incremental increase with insulin under euglycaemic conditions as compared to baseline. Finally, a 17% reduction in peak MBF was observed during HH in comparison to HE which was not significantly different from baseline.

4.4.3d Myocardial Blood Flow Reserve (Figure 4.3)

1. Patients without Microvascular Complications:

Myocardial blood flow reserve (MBFR) increased by 19% ($p<0.0001$) during HE (2.7±0.4, 95%CI 2.5, 2.9) in comparison to baseline (2.2±0.2, 95%CI 2.1, 2.4) However, there was a 23% ($p<0.0001$) and 9% ($p=0.041$) reduction in MBFR during HH (2.0±0.3, 95%CI 2.0, 2.2) in comparison to HE and baseline respectively.

2. Patients with Microvascular Complications:

MBFR increased by 17% ($p=0.018$) during HE (2.03±0.3, 95%CI 1.8, 2.3) in comparison to baseline (1.72±0.2, 95%CI 1.6, 1.9). Importantly, there was a 28% ($p=0.002$) and 9% decrease in MBFR during HH (1.46±0.1, 95%CI 1.3, 1.6) in comparison to HE and baseline respectively.

4.4.4 Changes in Concentrations of Plasma Glucose, Endothelin-1, Adrenaline, Serum High sensitivity CRP and Insulin during the Hyperinsulinemic Clamp

Plasma glucose (mmol/L) at baseline was 5.3±0.3, (95%CI 5.2, 5.4) which was maintained at 5.0±0.2, (95%CI 4.9, 5.1) during HE until symptomatic hypoglycaemia (HH) was induced at a nadir of 2.8±0.1, (95% CI 2.8, 2.9) as shown in Table 4. Serum insulin concentrations (pmol/L) at baseline were 221.1±210, (95%CI 122.9, 319.3) and increased significantly after commencing the hyperinsulinemic clamp with levels of 639.2±219.9, (95%CI 536.3, 742.1)

and 620.0±269.5, (95%CI 493.9, 746.1) during HE and HH respectively. Plasma ET-1 concentrations (pg/ml) represented as median(interquartile range, IQR) at baseline were 1.5(0.5) which remained unchanged during HE 1.5(0.6) and HH 1.5(0.8). There was no significant change in plasma adrenaline concentrations (pg/ml) when comparing baseline (76.9±44.0, 95%CI 56.8, 98.9) with HE values (122.5±111.7, 95%CI 69.6, 175.8). During HH, adrenaline levels were significantly higher (382.8±379, 95%CI 205.1, 560.4) vs. baseline and HE. Serum hs-CRP concentrations (mg/L) represented as median(IQR) did not vary significantly between baseline 0.7(1.9), HE 0.6(1.2) or HH 0.6(0.9).

4.5 Discussion

We have shown for the first time that insulin-induced hypoglycaemia (HH) decreases the MBFR in patients with type 1 diabetes. We have demonstrated that in patients with type 1 diabetes during HE, insulin induced a marked increase in peak MBF and MBFR whereas hypoglycaemia led to a decline in peak MBF and a consequent decrease in MBFR (Figure 4.3). The reduction in peak MBF during HH appeared to be primarily due to a decrease in myocardial blood velocity. Therefore, the overall effect of hypoglycaemia during HH is to mitigate the vasodilatory action of hyperinsulinemia that occurs during physiological glucose concentrations leading to a decrease in peak MBF and MBFR.

The Diabetes Control and Complications Trial (DCCT) was a landmark multi-centre trial which examined the effect of IGT on patients with type 1 DM aged 13-39 years with no history of coronary artery disease.[9] This study

demonstrated for the first time that strict glycaemic control lowered the risk of developing microvascular complications in type 1 DM significantly (retinopathy by 54%, neuropathy by 60% and nephropathy by 39%.) There was a non-significant 41% relative risk reduction in the development of macrovascular complications. A subsequent analysis of the DCCT (after an extended follow-up of the original cohort) recorded a 57% relative risk reduction in non-fatal myocardial infarction, stroke and cardiovascular mortality (CVM).[11] Similarly, a follow-up analysis of UKPDS was published which examined the effect of IGT on long-term MACE in the original cohort (after an extended 10-year follow-up) following the cessation of the randomized interventions.[12] It recorded a reduction in the rate of myocardial infarction and all-cause mortality by 15% and 13% respectively in the group receiving IGT in comparison to the conventional group. However, it was noted in both studies that IGT was associated with a 3-fold increased risk of severe hypoglycaemic episodes. The rate of hypoglycaemia-induced coma or seizure was 1.1 per patient in the IGT group in comparison to 0.35 episodes per patient. It was estimated that during one year 27% of IGT group would experience at least one episode of severe hypoglycaemia in comparison to 10% of patients receiving conventional therapy. It is important to note that the target HbA1c (<6.05%) was only ever maintained in <5% of the IGT group. The final mean HbA1c in the IGT group was 7.4% and 7.0% in the DCCT and UKPDS trials respectively.

Following on from these encouraging results, two major trials ADVANCE and ACCORD were conducted to explore the effects of a stricter IGT on macrovascular complications.[14, 15] The first of these was the ADVANCE study which included 11,140 patients with type 2 DM aged

≥55years (1 in 6 patients had established macrovascular complications) with a median follow-up of five years. Both groups had a mean HbA1c of 7.5% at randomization. Patients in the IGT group had a target HbA1c of <6.5%. It took two years for the IGT group to achieve a mean HbA1c of 6.5%. There was no difference in the rate of macrovascular complications, all-cause or CV mortality between the IGT or conventional therapy group. Of note 2.7% and 1.5% of patients experienced severe hypoglycaemia in the IGT and conventional therapy group respectively. Subsequently, the ACCORD study also investigated the effects of a stricter IGT on rates of major cardiovascular events in patients with type 2 DM. It included 10251 patients with a mean age of 62 years with either established coronary disease or 2 or more risk factors for coronary disease. Of note, the study excluded patients with a history of frequent or recent severe hypoglycaemic events. The median HbA1c levels were 8.1% in both groups at randomization and rapidly declined to a median of 6.5% within six months in the IGT group (in contrast to the ADVANCE study where it took 2 years for a similar decrease). The ACCORD study recorded a 44% increase in the rate of CVM in comparison to the conventional group with an absolute increase of 1.0% in all-cause mortality (representing a 25% relative increase). More importantly, the rates of death began to separate as early as one year after the trial was commenced. Almost 11% of the IGT group suffered severe hypoglycaemic events requiring medical assistance which was four-fold higher than those of the ADVANCE study. Interestingly, the rates of severe hypoglycaemic events were also five-fold higher in the conventional study group in the ACCORD study as compared to the ADVANCE study. Subsequent retrospective analyses of both studies found

severe hypoglycaemia to be independently associated with an increase all-cause mortality rates.[47-49] For example, in the ACCORD study, the mortality rate was highest in the IGT group patients who had experienced three or more episodes of severe hypoglycaemia requiring medical intervention.

The mechanistic association between hypoglycaemia and increased cardiovascular mortality is complex and at present, poorly defined.[25] It is likely that the pathological processes involved are multiple and may vary. The endothelium is a highly biologically active single cell layer responsible for the release of several substances, the most important of which are nitric oxide (NO) and endothelin-1 (ET-1).[111] Endothelin-1 is a 21-amino acid peptide and the most potent vasoconstrictor yet identified in man with a plasma half life of 4-7 minutes.[113, 114] ET-1 induces its predominant vasoconstrictive effect by acting on receptors located on vascular smooth muscle cells and fibroblasts. This reduces NO bioavailability by either decreasing its production (caveolin-1-mediated inhibition of eNOS activity) or by increasing its degradation (via formation of oxygen radicals).[115] One recent study demonstrated that direct infusion of ET-1 into the coronary sinus of six humans decreased the coronary blood flow in a dose-related manner by up to 25%.[116] ET-1 levels have also been shown to be the strongest predictor of no-reflow following primary angioplasty.[117] Several disease states have been shown to be associated with endothelial dysfunction (an imbalance between the bioavailability of NO and ET-1). Examples of these include atherosclerosis, pulmonary arterial hypertension, DM and myocardial ischaemia.[118, 120, 121]

Acute hypoglycaemia has also been shown to increase ET-1 concentrations.[124] Wright and co-workers demonstrated that ET-1 levels in

patients with type 1 DM rose by almost 70% above baseline values one hour following insulin-induced hypoglycemia. Although the baseline levels of ET-1 were 7-fold higher in the patients with type 1 DM in comparison to healthy controls, these did not change during hypoglycaemia. We suggest further work is needed specifically to elucidate the effects of more prolonged periods of hypoglycaemia on ET-1 expression in patients with type 1 DM.

It is also plausible that there are other effects of hypoglycaemia that may have a deleterious impact on MBFR. Hypoglycaemia induces a hyper-coagulant state in humans via increased platelet aggregation and changes in plasma concentrations of coagulation factors.[105, 108] For example, it has been shown that factor VIII was increased two-fold following 30 minutes of hypoglycaemia.[105] Hypoglycaemia may also be responsible for initiating an inflammatory response. In one study, hypoglycaemia was associated with a three-fold increase in the neutrophil count and also an elevation in neutrophil elastase, a potent proteolytic enzyme.[156] Long-QT syndrome is well recognised as being associated with an increased risk of sudden cardiac death.[98] More worryingly, acute hypoglycaemia has been demonstrated to produce prolongation of the corrected QT interval by up to 35% in patients with type 1 DM with values reaching >550 milliseconds.[101, 102] Interestingly, this change seems to be predominantly attributed to a surge in catecholamine levels and independent of electrolyte imbalance.[104] Finally, prolonged hypoglycaemia can have a detrimental effect on cardiac metabolism due to the inability of the heart to utilise glucose, the preferred substrate instead of fatty acids (during acute myocardial ischemia) following exhaustion of myocardial glycogen reserves.[157]

4.6 Limitations

It is important to note that our study population consisted of patients with type 1 diabetes. Although the DCCT study only included patients with type 1 diabetes, to date no trial has shown a detrimental effect of strict IGT (aiming for HbA1c <6.0%) in patients with type 1 diabetes exclusively. Patients included in the two landmark studies, ADVANCE and ACCORD had type 2 diabetes. A significant proportion of patients had established coronary artery disease unlike our study population. Therefore, although our results should be interpreted with caution, we believe that in the absence of large studies exploring the effects of strict IGT on patients with type 1 diabetes, our findings can be extrapolated to patients with type 2 diabetes. We hope that our work will encourage future research to explore the mechanistic effects of hypoglycaemia on myocardial blood flow reserve in patients with type 2 diabetes.

4.7 Conclusion

This study has shown that insulin-induced hypoglycaemia is associated with a decrease in MBFR in patients with type 1 diabetes due to a reduction in peak MBF. In contrast, insulin infusion at normal plasma glucose concentrations is associated with an increase in MBFR due to an increase in peak MBF. We speculate from our results that alterations in MBFR may provide an explanation for the observed association between hypoglycaemia and increased CVM in susceptible individuals.

Table 4.1 Baseline characteristics of patients with Type 1 diabetes mellitus without microvascular complications

Variable	DM (n=20)
Age years	36.4±10
Males (%)	15 (75)
BMI kg/m^2	25.1±3
SBP mmHg	123±12
DBP mmHg	77±7
Heart rate b/min	78±13
Fasting glucose mmol/L	9.5±3.6
Duration of diabetes mellitus years	18±12
Glycosylated haemoglobin HbA1c %	8.4±1.3
Albumin creatinine ratio mg/mmol/L	0.5±0.4
VPT score	5±3
TC mmol/L	4.8±0.9
LDLc mmol/L	2.4±0.8
HDLc mmol/L	1.9±0.7
TG mmol/L	1.1±0.7

Data are presented as mean±SD.

BMI=body mass index, SBP=systolic blood pressure, DBP=diastolic blood pressure, HE=hyperinsulinemic euglycaemia, HH=hyperinsulinemic hypoglycaemia, VPT score=vibration perception threshold score, TC=total cholesterol, LDLc=low density lipoprotein cholesterol, HDLc=high density lipoprotein cholesterol, TG=triglycerides.

Table 4.2

Haemodynamic Data (Pulse rate, systolic blood pressure, diastolic blood pressure and rate pressure product) at rest and after dipyridamole-induced stress during the hyperinsulinemic clamp.

Variable	Baseline	Hyperinsulinemic Euglycaemia (HE)	Hyperinsulinemic Hypoglycaemia (HH)
$Pulse_r$ b/min	66±11	71±14	77±17
$Pulse_d$ b/min	94±15	94±16	96±14
SBP_r mmHg	119±13	119±11	117±11
SBP_d mmHg	125±17	119±12	121±11
DBP_r mmHg	71±11	70±11	63±8
DBP_d mmHg	71±8	61±10	61±7
RPP_r b/min.mmHg	7851± 1695	8375± 2162	9310± 2375
RPP_d b/min.mmHg	11839±3005	11151±2756	11602±2220

Pulse rate, systolic blood pressure, diastolic blood pressure and rate pressure product are presented as mean±SD.
$Pulse_r$=resting pulse, $Pulse_d$=post-dipyridamole pulse, SBP_r=resting systolic blood pressure, SBP_d:=post-dipyridamole systolic blood pressure, DBP_r=resting diastolic blood pressure, DBP_d=post-dipyridamole diastolic blood pressure, RPP_r= resting rate pressure product, RPP_d= post-dipyridamole rate pressure product.

Table 4.3 Myocardial blood volume (A), myocardial blood velocity (β), myocardial blood flow (MBF) at rest and peak stress.

	Baseline		**Hyperinsulinemic Euglycaemia**		**Hyperinsulinemic Hypoglycaemia**	
	Rest	Peak	Rest	Peak	Rest	Peak
A dB	20.2±2.0	23.3±1.8	20.4±2.4	23.3±1.8	21.2±2.2	24±1.9
β dB/s	1.0±0.2	1.9±0.3	1.1±0.2	2.5±0.4	1.2±0.2	2.0±0.3
MBF dB^2/s	19.7±3.3	42.7±6.8	22±4.8	57.4±10.6	23.9±3.7	47.4±6.0

All values are presented as mean±SD.

A=(myocardial blood volume), β=(myocardial blood velocity), MBF=(myocardial blood flow).

Table 4.4

Endothelin-1, hs-CRP, adrenaline, and insulin concentrations during the hyperinsulinemic clamp.

Variable	Baseline	Hyperinsulinemic Euglycaemia			Hyperinsulinemic Hypoglycaemia		
	0 mins	30 mins	60 mins	90 mins	120 mins	150 mins	180 mins
ET-1 pg/ml	1.5(0.5)	1.3(0.6)	1.3(0.7)	1.4(0.6)	1.3(0.8)	1.5(0.8)	1.6(0.8)
hs-CRP mg/L	0.7(1.2)	0.6(1.0)	0.6(1.1)	0.6(1.2)	0.6(1.0)	0.6(0.9)	0.6(0.9)
Adrenaline pg/ml	76.9±44	97.1±69.6	109.9±124.5	122.5±111.7	194.1±219.8	344.3±326	382.8±379
Insulin pmol/L	221.1±210	688.6±213	679.1±228	639.2±220	651.3±201.4	621±280	620±269.5

Values are presented as median(interquartile range) for ET-1 and hs-CRP. Values are presented as mean±SD for adrenaline and insulin.
ET-1=endothelin-1, hs-CRP= high sensitivity CRP.
.

Figure 4.1.a. Study design showing glucose concentrations and timing of myocardial contrast echocardiography (MCE) at baseline (B), hyperinsulinemic euglycaemia (HE) and hyperinsulinemic hypoglycaemia (HH)

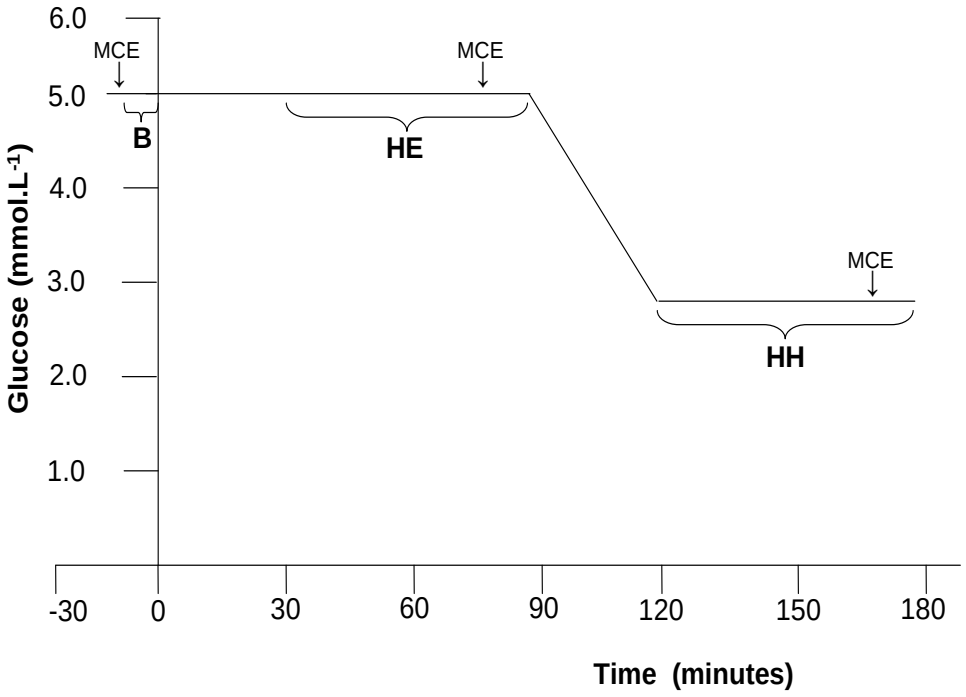


Figure 4.1.b. Sequence of image acquisition during each MCE study at baseline, HE and HH. MCE_{rest}=myocardial contrast echocardiography at rest, MCE_{peak}=myocardial contrast echocardiography at post dypridamole-induced stress

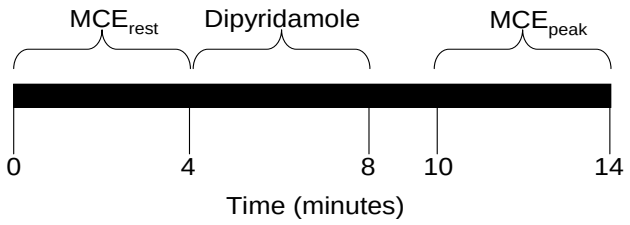


Figure 4.2 Model used for quantitative analysis of myocardial segments. (A) Apical 4 chamber, (B) Apical 2 chamber, (C) Apical 3 chamber

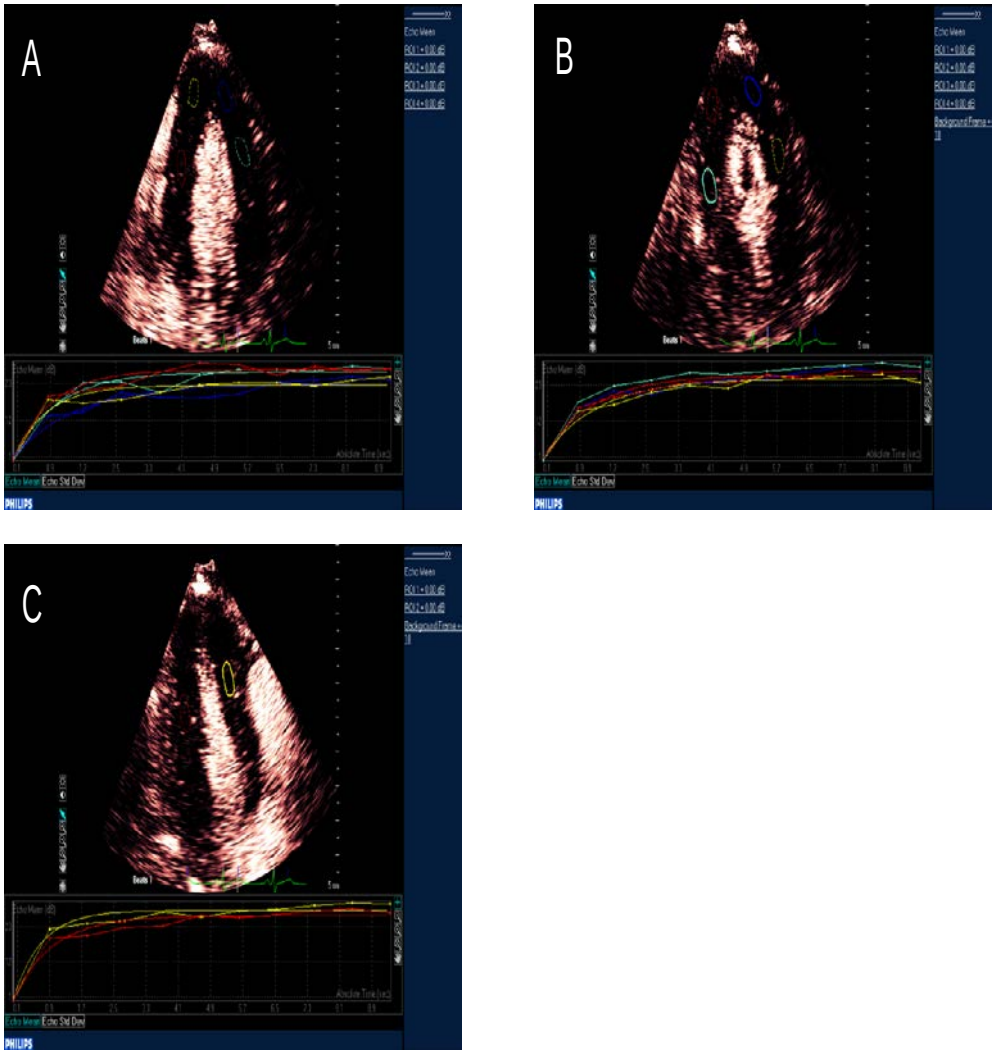
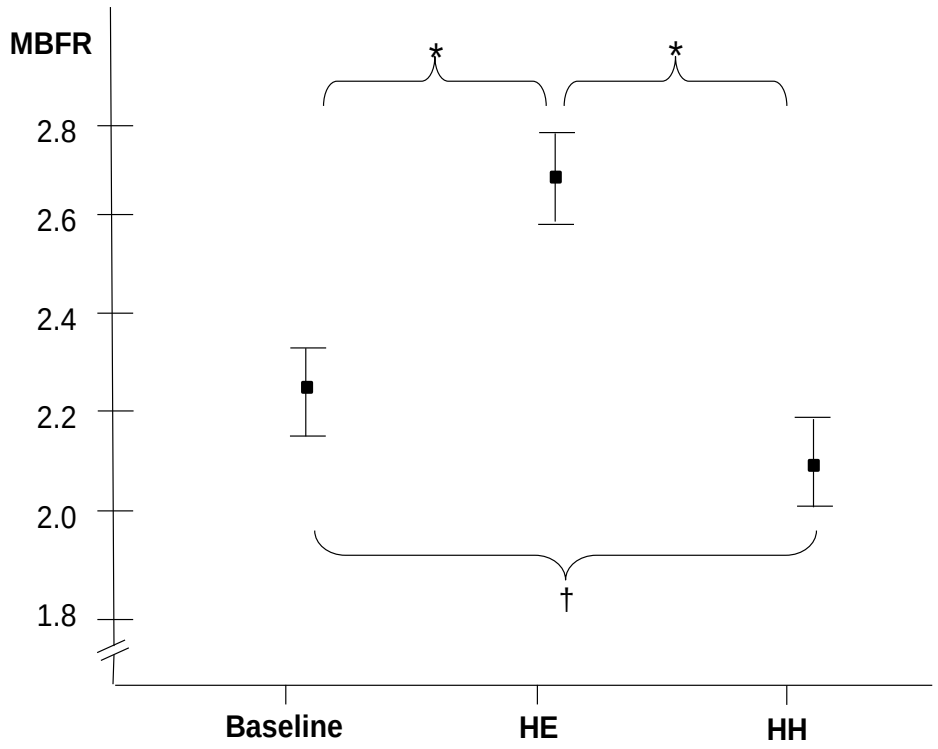


Figure 4.3 Myocardial blood flow reserve (MBFR) at baseline, hyperinsulinemic euglycaemia (HE) and hyperinsulinemic hypoglycaemia (HH) in patients with Type 1 diabetes mellitus without microvascular complications (n=20). Data are presented as means±SD.



*p<0.0001, †p=0.041

Chapter 5

Combined Data Analysis of Healthy Control Subjects and all Patients with Type 1 Diabetes Mellitus using Mixed Effect Regression Modelling

5.1 Abstract

Background: Hypoglycaemia is associated with increased cardiovascular mortality (CVM) but the reason for this is poorly understood. We tested the hypothesis that the myocardial blood flow reserve (MFBR) is decreased during hypoglycaemia using myocardial contrast echocardiography (MCE) in patients with type 1 diabetes mellitus (DM) and in healthy controls.

Methods and Results: Twenty-eight volunteers with DM (patients with and without MVD) and 19 controls underwent hyperinsulinemic clamps with maintained sequential hyperinsulinemic euglycaemia (HE, plasma glucose 90 mg/dl, 5.0 mmol/L) followed by hyperinsulinemic hypoglycaemia (HH, plasma glucose 50 mg/dl, 2.8 mmol/L) for 60 minutes each. Low-power real-time MCE was performed with flash impulse imaging using low-dose dipyridamole stress, at baseline, and during HE and HH. In control subjects MBFR increased during HE by 0.57 units (22%) above baseline, (B coefficient 0.57, 95%CI 0.38, 0.75, $p<0.0001$) and decreased during HH by 0.36 units (14%) below baseline values (B coefficient -0.36, 95%CI -0.50, -0.23, $p<0.0001$). Although MBFR was lower in patients with DM at baseline by 0.37 units (14%), (B coefficient -0.37, 95%CI -0.55, -0.19, $p=0.0002$) when compared to baseline controls the subsequent changes in MBFR during HE and HH in DM patients were similar to that observed in control subjects. Finally, the presence of microvascular complications in the patients with DM was associated with a reduction in MBFR of 0.52 units (24%), (B coefficient -0.52, 95%CIs -0.70, -0.34, $p<0.0001$).

Conclusions: Hypoglycaemia decreases MBFR in both healthy humans and in DM. This finding may explain the association between hypoglycaemia and increased CVM in susceptible individuals.

5.2 Introduction

Several studies have shown that hypoglycaemia is associated with an increase in cardiovascular mortality (CVM).[23, 52, 53, 55-57] This association has been demonstrated in people with and without established coronary artery disease (CAD).[53, 55, 56] Importantly, patients with acute coronary syndromes (ACS) appear to have a worse short- and long-term outcome if they experience hypoglycaemia in the acute phase of their presentation.[55-57] For example, in patients with diabetes mellitus (DM) and ACS, hypoglycaemia within 48 hours of their admission was associated with a 2-fold increase in all-cause mortality over a 2-year follow-up.[56] Similarly, Pinto showed that patients with ST-elevation myocardial infarction (STEMI) and an admission blood glucose <4.5 mmol/L had a 3-fold increased rate of adverse outcomes (defined as 30-day mortality and myocardial infarction).[55] Furthermore, in the same study patients with DM had an 18-fold increased risk of adverse cardiac outcomes. Subsequently, a more recent study showed that in patients following myocardial infarction, spontaneous hypoglycaemia was associated with a 2-fold increase in in-hospital mortality.[57]

Insulin, under euglycaemic conditions, has important beneficial effects on the vascular tone by inducing nitric oxide (NO)-mediated vasodilatation.[149] Studies on healthy humans and patients with diabetes show that insulin causes a marked increase in myocardial blood flow (MBF) and myocardial blood flow reserve (MBFR) during euglycemia.[84, 90] In contrast, the mechanisms by which hypoglycaemia adversely affects the cardiovascular system are unclear. Hypoglycaemia has been associated with angina and importantly, has been shown to increase the size of a myocardial infarct.[32, 94] Furthermore, low blood

glucose also encourages a hypercoagulant state due an increase in plasma concentrations of coagulation factors and by promoting platelet aggregation.[105, 108]

Myocardial contrast echocardiography (MCE), is an established technique used in the non-invasive quantification of MBFR with an accuracy similar to that of positron emission tomography and coronary Doppler flow wire measurements.[70, 144] MBFR is calculated as the ratio of peak MBF to resting MBF.[70] In the absence of flow-limiting CAD, a MBFR <2.0 is indicative of underlying endothelial dysfunction.[74] Furthermore, MBFR has been shown to be an independent predictor of CVM in diabetic and non-diabetic patients with normal stress echocardiograms, as well as post-ACS.[152, 153, 158]

We hypothesised that hypoglycaemia would decrease the MBFR (measured by MCE) using a one-step hyperinsulinemic clamp technique to induce hypoglycaemia, in patients with type 1 DM and in healthy control subjects.

5.3 Methods

5.3.1 Subjects

This chapter presents the results of 28 patients with type 1 diabetes mellitus (group DM) and 19 healthy volunteers (group C) acting as controls. All participants were included in the study after approval of the local research ethical committee. As we were unable to recruit 20 patients with MVD, we could not justify a third group of only 8 patients with Type 1 DM and MVD (see supplemental material). All partcipants underwent assessment of MBF by MCE. Assessment of MBF was undertaken using an insulin clamp at three stages:

(1) Baseline

(2) During hyperinsulinemic euglycaemia (HE)

(3) During hyperinsulinemic hypoglycaemia (HH), using an insulin clamp.

During each stage all volunteers underwent measurement of MBF during two states

(1) At rest

(2) Following dypridamole-induced stress.

None of the volunteers were active smokers, had a history of hypertension, CAD or underlying lipid disorders. All volunteers had normal exercise stress echocardiograms. All volunteers provided written informed consent.

5.3.2 Hyperinsulinemic Clamps

See Chapter 2 for details.

5.3.3 Myocardial Contrast Echocardiography (MCE)

See Chapter 2 for details.

5.3.4 Analytical Methods

See Chapter 2 for details.

5.3.5 Statistical Analysis

All data are represented as mean±SD except ET-1 and hs-CRP values which are presented as median (interquartile range).

For MBF, β and A, the influence of measurement stage, of stress state, and of the presence of diabetes was assessed via mixed effects regression modelling (to reflect the intra-class correlation resulting from repeated measurements made on each subject). For each of these three outcomes, a mixed effects model was fitted in which the main effects of stage, stress state and diabetes (together with all of their possible interactions) were assessed. Modelling was performed using the MIXED procedure in SAS software (Version 9.2). Interpretation of these models is described in the supplemental material.

In addition to yielding regression parameter estimates, the models were also used to estimate mean values for each combination of effects (via the LSMEANS option in the MIXED procedure), and to test for selected

differences in these means. With twelve effects combinations (i.e. three stages X two stress states X two diabetes states [present/absent]), the maximum number of possible between-group differences was 66. It was fully recognised that formal testing of between-group differences under these conditions was justified only (a) where there some *a priori* reason to anticipate the presence of an effect of interest, and (b) under the strict understanding that the primary purpose of such testing was the generation of hypotheses for future research, rather than the drawing of substantive inferences. For further detailed explanation see supplemental material.

For the main outcome (MBFR), the concept of stress state was not applicable. Consequently, this outcome was investigated via a further mixed effects regression model in which MBFR was predicted by the main effects of measurement stage and of diabetes, together with that of their interaction; by age; and by the subject's systolic blood pressure.

5.4 Results

Data are presented for the healthy volunteers and all patients with Type 1 DM.

5.4.1 Subject Characteristics

The baseline characteristics of the 19 healthy volunteers (C) and 28 patients with DM are summarized in Table 5.1.

5.4.2 Hemodynamic Data

Throughout the clamp, resting heart rate, resting systolic blood pressure, resting diastolic blood pressure and resting rate pressure product were similar in groups C and DM (supplemental material Table 5.6).

5.4.3 Myocardial Contrast Echocardiography-Derived Measurements

5.4.3a Myocardial Blood Volume

Mean myocardial blood volumes and 95%CIs at rest (A_r) and during dipyridamole-induced stress (A_d) are shown at baseline, during HE and HH in Table 5.2.a. Table 5.2.b shows the mixed effect regression modelling testing the effect of stage, state, diabetes and their interactions on A_r and A_d.

There was a significant increase in A_d in group C at baseline by 2.7 dB ($p<0.0001$) as shown in Table 2b. In addition, there was marginal evidence that A_d was decreased during HH by 1.4 dB in comparison to baseline ($p=0.068$). Furthermore, the presence of diabetes did not affect either A_r or A_d

at the baseline stage (p=0.25 and p=0.65, respectively). However, there was a suggestion that A_d was increased in group DM during HH vs. C by 1.9 dB (p=0.056).

5.4.3b Myocardial Blood Velocity

Mean myocardial blood velocity and 95%CIs at rest (β_r) and during dipyridamole-induced stress (β_d) are shown at baseline, during HE and HH in Table 5.3.a. Table 5.3.b shows the mixed effect regression modelling testing the effect of stage, state, diabetes and their interactions on β_r and β_d.

There was a significant increase in β_d in group C at baseline by 1.08 dB/s (p<0.0001) as shown in Table 5.3.b. During HE β_d was further increased in group C in comparison to baseline values by 0.37 dB/s (p<0.0001). However, during HH β_d declined and was not different from baseline stress values (p=0.28). In group DM, β_r was significantly elevated as compared to group C at baseline by 0.11 dB/s (p=0.035). Importantly, at baseline, β_d was significantly decreased in group DM in comparison to group C by 0.27 dB/s (p=0.005).

In group DM during HE and HH a similar effect on β_d was observed as compared with group C with no significant differences between the two groups at

each stage.

5.4.3c Myocardial Blood Flow

Mean myocardial blood flow and 95%CIs at rest (resting MBF) and during dipyridamole-induced stress (peak MBF) are shown at baseline, during HE and

HH in Table 5.4.a. Table 5.4.b shows the mixed effect regression modelling testing the effect of stage, state, diabetes and their interactions on MBF.

In group C peak MBF was significantly increased in comparison to resting MBF at baseline by 26.5 dB^2/s ($p<0.0001$) as shown in Table 5.4.b. During HE peak MBF was further increased in group C above baseline peak values by 11.6 dB^2/s ($p<0.0001$). However, during HH peak MBF declined and was not significantly different from baseline peak MBF values ($p=0.20$).

The resting MBF was significantly higher in group DM vs. group C at baseline by 2.6 dB^2/s ($p=0.015$). There was no significant difference in the resting MBF values between the two groups at HE or HH. In group DM, peak MBF was significantly decreased in comparison to group C at baseline by 6.0 dB^2/s ($p=0.006$). In group DM during HE and HH a similar effect on peak MBF was observed as compared with group C with no significant differences between the two groups at each stage.

5.4.3d Myocardial Blood Flow Reserve (MBFR)

We tested the effect of measurement stage, age, presence of diabetes and systolic blood pressure (SBP) on MBFR using regression modelling (Table 5.5 and Figure 5.3). In Table 5.5, the intercept of the mixed model was (B coefficient 3.16, 95%CIs, 2.47, 3.85) using baseline as a reference point. In group C, MBFR increased during HE by 0.57 units (2.6±0.3 to 3.1±0.5, $p<0.0001$) (22%) above baseline and decreased during HH by 0.36 units (2.6±0.3 to 2.2±0.2, $p<0.0001$) (14%) below baseline values. Importantly, at baseline, MBFR was significantly lower in group DM as compared to group C by 0.37 units (2.6±0.3 vs. 2.1±0.3, $p=0.0002$). In group DM during HE

(2.5±0.5) a similar effect on MBFR was observed as compared with group C however, there was a suggestion that in group DM, there was a smaller decrease in MBFR during HH (1.9±0.4), compared to the decrease in MBFR observed in group C (p=0.05). Although there was a highly significant (p=0.003) and independent negative effect of age on MBFR, the B coefficient (-0.01) shows that the magnitude of this effect for each year of age was small. Finally, there was no independent effect of SBP on MBFR.

5.4.3e Effect of microvascular complications on MBFR

The mixed model method was applied to explore whether the presence of microvascular complications in people with diabetes were predictive of a decreased MBFR. A mixed model was fitted (using data for subjects with diabetes only) in which MBFR was predicted by stage; the presence of microvascular complications; and a term representing the stage/complications interaction. This mixed model (supplemental material Table 5.7) indicated that the presence of microvascular complications in people with diabetes was associated with a reduction in MBFR of 0.52 units (B coefficient -0.52, 95%CIs -0.70, -0.34), p<0.0001) at baseline. There was no significant interaction of complications with the stage of measurement (i.e. HE or HH).

5.4.4 Changes in concentrations of Endothelin-1, high sensitivity CRP, adrenaline and serum insulin during the hyperinsulinemic clamp

To explore further the explanation for the decrease in MBFR during HH, we measured plasma endothelin-1 (ET-1, as a potent vasoconstrictor) and serum high sensitivity C-reactive protein (hs-CRP, as a non-specific marker of inflammation) (supplemental material Table 5.8). Plasma ET-1 concentrations at baseline were 0.19 pg/ml in control subjects and 1.44 pg/ml in the group with diabetes ($p<0.0001$). In the group with diabetes, ET-1 remained markedly increased throughout the whole clamp and there was a suggestion that ET-1 levels increased towards the end of the HH clamp in control subjects. Serum hs-CRP concentrations were not different between the two groups and did not change during the study. We also measured serum insulin levels (in view of the hyperinsulinemic clamp) and plasma adrenaline levels (to assess the counter-regulatory response to hypoglycaemia) in all individuals (supplemental material Table 5.8). Serum insulin concentrations at baseline were 43±23 pmol/L in control subjects and 208.3±207 pmol/L in the group with diabetes. The plasma epinephrine levels were similar between the two groups at all stages.

5.5 Discussion

We have shown for the first time that insulin-induced hypoglycaemia (HH) decreases the MBFR in both patients with type 1 diabetes and in healthy subjects. We have demonstrated that in healthy controls during HE, insulin induced a marked increase in peak MBF and MBFR whereas hypoglycaemia led to a decline in peak MBF and a decrease in MBFR. Importantly, patients with type 1 diabetes behaved in a similar manner to the healthy controls (Figure 5.3) in the presence of HE and HH, although the presence of diabetes was associated with a more marked reduction in MBFR at baseline. The reduction in peak MBF during HH appeared to be due to a decrease in myocardial blood velocity rather than blood volume. We have also shown that the presence of microvascular complications is associated with a decrease in MBFR in patients with type 1 diabetes. Therefore, the overall effect of hypoglycaemia during HH is to suppress peak MBF thereby mitigating the vasodilatory action of hyperinsulinemia that occurs during physiological glucose concentrations.

A significant amount of evidence has associated hypoglycaemia with increased CVM.[23, 53-56, 159] In a study including 40,069 patients, fasting hypoglycaemia was independently associated with a 3-fold increased risk in CVM after a mean follow-up of 8-years.[53] Pinto observed that following STEMI, patients with a Thrombolysis in Myocardial Infarction (TIMI) risk score >4 and concomitant hypoglycaemia had a >11-fold increased risk of death within 30 days vs. those with normal glucose levels.[55] Furthermore, another study including patients with established CAD showed that fasting

hypoglycaemia was associated with a 2-fold increase in all-cause mortality.[54] A subsequent study observed a 16% increase in the relative risk of CVM in the group receiving insulin-therapy upon admission to intensive care.[23] Although this finding was unexplained, there was a 13-fold increased prevalence of severe hypoglycaemia in the patients on insulin-therapy in comparison to patients receiving conventional therapy. More recently, another study has demonstrated that fasting hypoglycaemia was associated with a 33% increase in three-year mortality rates in a cohort of 1854 elderly patients following an acute myocardial infarction.[159] This negative impact on survival was more pronounced in the subgroups with DM and those requiring coronary artery bypass grafting with a two- and three-fold increase in three-year mortality rates respectively. This evidence suggests that hypoglycaemia is associated with short- and long-term adverse outcomes, however, the pathophysiological mechanisms are yet ill-defined and may vary.

Over the past few decades, several anecdotal case reports have associated hypoglycaemia with episodes of angina and myocardial infarction.[29-31] While a direct causal link has not been established, animal studies have demonstrated hypoglycaemia can increase myocardial infarct size by over 40%.[94] Furthermore, in patients with DM and coexisting CAD, hypoglycaemia was associated with a third of all episodes of angina and corresponding ischemic ECG changes.[32]

The endothelium is a highly biologically active single cell layer responsible for the release of several substances, the most important of which are nitric oxide (NO) and endothelin-1 (ET-1).[111, 160] Endothelin-1 is a 21-amino acid peptide and the most potent vasoconstrictor yet identified in man

with a plasma half life of 4-7 minutes.[113, 114] ET-1 induces its predominant vasoconstrictive effect by acting on receptors located on vascular smooth muscle cells and fibroblasts. This reduces NO bioavailability by either decreasing its production (caveolin-1-mediated inhibition of eNOS activity) or by increasing its degradation (via formation of oxygen radicals).[115] One recent study demonstrated that direct infusion of ET-1 into the coronary sinus of six humans decreased the coronary blood flow in a dose-related manner by up to 25%.[116] ET-1 levels have also been shown to be the strongest predictor of no-reflow following primary angioplasty.[117] Several disease states have been shown to be associated with endothelial dysfunction (an imbalance between the bioavailability of NO and ET-1). Examples of these include atherosclerosis, pulmonary arterial hypertension, DM and myocardial ischemia.[117, 119, 121, 160]

Acute hypoglycaemia has also been shown to increase ET-1 concentrations.[124] Wright and co-workers demonstrated that ET-1 levels in patients with type 1 DM rose by almost 70% above baseline values one hour following insulin-induced hypoglycemia.[124] In our study, although baseline ET-1 levels were approximately 7-fold higher in the DM group compared to controls, the effect of HH vs. HE on ET-1 is uncertain. We suggest further work is needed specifically to elucidate the effects of more prolonged periods of hypoglycaemia on ET-1 expression.

It is also plausible that there are other effects of hypoglycaemia that may have a deleterious impact on MBFR, besides increases in ET-1. Hypoglycaemia induces a hypercoagulant state in humans via increased platelet aggregation and changes in plasma concentrations of coagulation factors.[105, 106, 108, 156] For example, it has been shown that factor VIII was increased two-fold

following 30 minutes of hypoglycemia.[105] Hypoglycaemia may also be responsible for initiating an inflammatory response. In one study, hypoglycaemia was associated with a three-fold increase in the neutrophil count and also an elevation in neutrophil elastase, a potent proteolytic enzyme.[156] Long-QT syndrome is well recognised as being associated with an increased risk of sudden cardiac death.[98] More worryingly, acute hypoglycaemia has been demonstrated to produce prolongation of the corrected QT interval by up to 35% in patients with type 1 DM with values reaching >550 milliseconds.[101, 102] Interestingly, this change seems to be predominantly attributed to a surge in catecholamine levels and independent of electrolyte imbalance.[104] Finally, prolonged hypoglycaemia can have a detrimental effect on cardiac metabolism due to the inability of the heart to utilise glucose, the preferred substrate instead of fatty acids (during acute myocardial ischemia) following exhaustion of myocardial glycogen reserves.[157]

In the light of our findings it is plausible to suggest that hypoglycaemia, by causing a decrease in MBFR, may increase the risk of CVM in susceptible individuals.

5.6 Limitations

Although dipyridamole was used three times in succession with our study protocol (Figure 5.1a and b), we consider that the repeated use of dipyridamole was unlikely to artefactually influence our results (see supplemental material). We did not calculate absolute myocardial perfusion values. This was because all settings and infusion parameters, once optimised

at the start of each patient study, were kept constant for the rest of that individual procedure. We deliberately did not randomize the sequence of HE and HH as this allowed individuals to act as their own controls permitting constant insulin levels, contrast infusion rates and ultrasound machine settings.

5.7 Conclusion

This study has shown that insulin-induced hypoglycaemia is associated with a decrease in MBFR in healthy controls due to a reduction in peak MBF and that patients with type 1 DM behave in a similar manner. In contrast, insulin infusion at normal plasma glucose concentrations is associated with an increase in MBFR due to an increase in peak MBF. Exploratory analyses suggest that the presence of diabetes and also microvascular complications are independently associated with MBFR during hyperinsulinemic hypoglycaemia. We speculate from our results that alterations in MBFR may provide an explanation for the observed association between hypoglycaemia and increased CVM in susceptible individuals.

Table 5.1. Baseline characteristics of Subjects

Variable	DM (n=28)	C (n=19)	P value
Age years	38.7±9	31.8±8	0.013
Males (%)	22 (79)	11 (58)	0.13
BMI kg/m^2	25.7±3.5	24.9±2.6	0.39
SBP mmHg	125±13	115±10	0.007
DBP mmHg	77±7	73±7	0.05
Heart rate b/min	78±14	79±16	0.71
Fasting glucose mmol/L	10.3±3.9	4.9±0.3	<0.0001
Duration of diabetes mellitus years	19.2±12	-	-
Glycosylated haemoglobin HbA1c %	8.9±1.5	-	-
Albumin creatinine ratio mg/mmol/L	4.3±9.4	-	-
VPT score	8.0±5.6	-	-
TC mmol/L	4.8±0.9	4.3±0.7	0.03
LDLc mmol/L	2.5±0.8	2.1±0.9	0.10
HDLc mmol/L	1.8±0.7	1.5±0.3	0.10
TG mmol/L	1.2±0.7	1.3±1.2	0.81

Data are presented as mean±SD.
C=Controls, DM=group with type 1 diabetes mellitus, BMI=body mass index, SBP=systolic blood pressure, DBP=diastolic blood pressure, HE=hyperinsulinemic euglycaemia, HH=hyperinsulinemic hypoglycaemia, VPT score=vibration perception threshold score, TC=total cholesterol, LDLc=low density lipoprotein cholesterol, HDLc=high density lipoprotein cholesterol, TG=triglycerides.

Table 5.2.a. Myocardial blood volume (A) (means and 95%CIs) at rest and post dipyridamole-induced stress, at baseline, during hyperinsulinemic euglycaemia and hyperinsulinemic hypoglycaemia.

Stages	State			
	DM		C	
	Myocardial Blood Volume (A) dB mean (95% confidence interval)			
	Rest (A_r)	Peak (A_d)	Rest (A_r)	Peak (A_d)
Baseline	20.0 (19.2 to 20.8)	22.4 (21.6 to 23.1)	19.3 (18.4 to 20.2)	22.0 (21.1 to 23.0)
HE	20.2 (19.3 to 21.1)	22.7 (21.9 to 23.5)	20.4 (19.4 to 21.5)	23.9 (22.9 to 24.8)
HH	21.0 (20.2 to 21.9)	23.9 (23.1 to 24.7)	21.7 (20.7 to 22.7)	23.0 (22.1 to 24.0)

Stage = baseline; hyperinsulinaemic euglycaemia (HE) or hyperinsulinemic hypoglycaemia (HH)
State = resting (resting blood volume = A_r), during dipyridamole stress (peak = (A_d)

Table 5.2.b. Mixed effect regression model showing the effect of stage (baseline, hyperinsulinemic euglycaemia and hyperinsulinaemic hypoglycaemia), state (resting and dipyridamole-induced stress i.e. post stress) and diabetes, on myocardial blood volume (A)

variable	estimate	95% CI	*P*
MEASUREMENT STAGE:			
baseline	19.3	18.4 to 20.2	< 0.0001
euglycaemia	20.4	19.4 to 21.5	< 0.0001
hypoglycaemia	21.7	20.7 to 22.7	< 0.0001
STATE (post-stress *vs.* rest)	2.7	1.5 to 3.9	< 0.0001
INTERACTION (STAGE with STATE):			
euglycaemia and post-stress	0.7	-0.5 to 1.9	0.25
hypoglycaemia and post-stress	-1.4	-2.9 to 0.1	0.068
PRESENCE OF DIABETES (yes *vs.* no)	0.7	-0.5 to 1.9	0.25
INTERACTION (STAGE with DIABETES):			
euglycaemia and diabetes present	-0.9	-2.5 to 0.6	0.22
hypoglycaemia and diabetes present	-1.4	-2.9 to 0.1	0.075
INTERACTION (STATE with DIABETES):			
post-stress and diabetes present	-0.4	-1.9 to 1.2	0.65
INTERACTION (STAGE with STATE with DIABETES):			
euglycaemia , post-stress, diabetes present	-0.5	-2.1 to 1.0	0.49
hypoglycaemia , post-stress, diabetes present	1.9	-0.1 to 3.8	0.056

Table 5.3.a. Myocardial blood velocity (β) (means and 95%CIs) at rest and post dipyridamole-induced stress, at baseline, during hyperinsulinemic euglycaemia and hyperinsulinemic hypoglycaemia.

Stages	State			
	DM		C	
	Myocardial Blood Velocity (β) $dB.s^{-1}$ mean (95% confidence interval)			
	Rest (β_r)	Peak (β_d)	Rest (β_r)	Peak (β_d)
Baseline	1.03 (0.96 to 1.09)	1.84 (1.70 to 1.98)	0.92 (0.84 to 1.00)	2.00 (1.83 to 2.17)
HE	1.11 (1.05 to 1.17)	2.37 (2.22 to 2.51)	0.96 (0.88 to 1.03)	2.41 (2.23 to 2.58)
HH	1.18 (1.12 to 1.24)	1.85 (1.74 to 1.97)	1.04 (0.96 to 1.11)	2.03 (1.89 to 2.17)

Stage = baseline; hyperinsulinaemic euglycaemia (HE) or hyperinsulinemic hypoglycaemia (HH)
State = resting (resting blood velocity = β_r), during dipyridamole stress (peak = β_d)

Table 5.3.b. Mixed effect regression model showing the effect of measurement stage (at baseline; during hyperinsulinemic euglycaemia; and during hyperinsulinemic hypoglycaemia), presence of diabetes and stress state (rest *vs.* post dipyridamole-induced stress) on myocardial blood velocity (β).

variable	estimate	95% CI	*P*
MEASUREMENT STAGE:			
baseline	0.92	0.84 to 1.00	< 0.0001
euglycaemia	0.96	0.88 to 1.03	< 0.0001
hypoglycaemia	1.04	0.96 to 1.11	< 0.0001
STATE (post-stress *vs.* rest)	1.08	0.94 to 1.22	< 0.0001
INTERACTION (STAGE with STATE):			
euglycaemia and post-stress	0.37	0.25 to 0.49	< 0.0001
hypoglycaemia and post-stress	-0.08	-0.23 to 0.07	0.28
PRESENCE OF DIABETES (yes *vs.* no)	0.11	0.01 to 0.21	0.035
INTERACTION (STAGE with DIABETES):			
euglycaemia and diabetes present	0.04	-0.05 to 0.14	0.36
hypoglycaemia and diabetes present	0.03	-0.05 to 0.12	0.46
INTERACTION (STATE with DIABETES):			
post-stress and diabetes present	-0.27	-0.45 to -0.08	0.005
INTERACTION (STAGE with STATE with DIABETES):			
euglycaemia , post-stress, diabetes present	0.07	-0.08 to 0.23	0.35
hypoglycaemia , post-stress, diabetes present	-0.05	-0.25 to 0.14	0.58

Table 5.4.a. Myocardial blood flow (MBF) (means and 95%CIs) at rest and post dipyridamole-induced stress, at baseline, during hyperinsulinemic euglycaemia and hyperinsulinemic hypoglycaemia.

Stages	State			
	DM		C	
	Myocardial Blood Flow (MBF) $dB^2.s^{-1}$ mean (95% confidence interval)			
	Rest	Peak	Rest	Peak
Baseline	20.4 (19.0 to 21.7)	40.9 (37.5 to 44.4)	17.7 (16.1 to 19.3)	44.2 (40.0 to 48.5)
HE	22.2 (20.6 to 23.8)	53.5 (49.3 to 57.6)	19.3 (17.3 to 21.2)	57.4 (52.4 to 62.4)
HH	24.5 (23.0 to 26.0)	44.4 (41.5 to 47.3)	22.2 (20.4 to 24.0)	46.7 (43.2 to 50.2)

Stage = baseline; hyperinsulinaemic euglycaemia (HE) or hyperinsulinemic hypoglycaemia (HH)
State = resting MBF, during dipyridamole stress (peak MBF)

Table 5.4.b.
Mixed effect regression model showing the effect of measurement stage (at baseline; during hyperinsulinemic euglycaemia; and during hyperinsulinemic hypoglycaemia), presence of diabetes and stress state (rest *vs.* post dipyridamole-induced stress) on myocardial blood flow.

variable	estimate	95% CI	*P*
MEASUREMENT STAGE:			
baseline	17.7	16.1 to 19.3	< 0.0001
euglycaemia	19.3	17.3 to 21.2	< 0.0001
hypoglycaemia	22.2	20.4 to 24.0	< 0.0001
STATE (post-stress *vs.* rest)	26.5	23.3 to 29.7	< 0.0001
INTERACTION (STAGE with STATE):			
euglycaemia and post-stress	11.6	8.9 to 14.4	< 0.0001
hypoglycaemia and post-stress	-2.0	-5.1 to 1.1	0.20
PRESENCE OF DIABETES (yes *vs.* no)	2.6	0.5 to 4.7	0.015
INTERACTION (STAGE with DIABETES):			
euglycaemia and diabetes present	0.3	-2.1 to 2.6	0.83
hypoglycaemia and diabetes present	-0.4	-2.4 to 1.6	0.71
INTERACTION (STATE with DIABETES):			
post-stress and diabetes present	-6.0	-10.1 to -1.8	0.006
INTERACTION (STAGE with STATE with DIABETES):			
euglycaemia , post-stress, diabetes present	-0.9	-4.4 to 2.6	0.60
hypoglycaemia , post-stress, diabetes present	1.3	-2.7 to 5.3	0.51

Table 5.5. Effect of measurement stage, age, presence of diabetes and systolic blood pressure on myocardial blood flow reserve (MBFR).

variable	B coefficient	95% CI	*P*
INTERCEPT	3.16	2.47 to 3.85	<0.0001
MEASUREMENT STAGE: euglycaemia vs. baseline hypoglycaemia vs. baseline	 0.57 -0.36	 0.38 to 0.75 -0.50 to -0.23	 <0.0001 <0.0001
AGE (+ one year)	-0.01	-0.02 to -0.00	0.003
PRESENCE OF DIABETES	-0.37	-0.55 to -0.19	0.0002
INTERACTION (STAGE with DIABETES): euglycaemia and diabetes present hypoglycaemia and diabetes present	 -0.14 0.17	 -0.38 to 0.09 0.00 to 0.35	 0.24 0.05
SYSTOLIC BLOOD PRESSURE (mm / Hg)	-0.00	-0.01 to 0.00	0.56

Figure 5.1.a. Study design showing glucose concentrations and timing of myocardial contrast echocardiography (MCE) at baseline (B), hyperinsulinemic euglycaemia (HE) and hyperinsulinemic hypoglycaemia (HH)

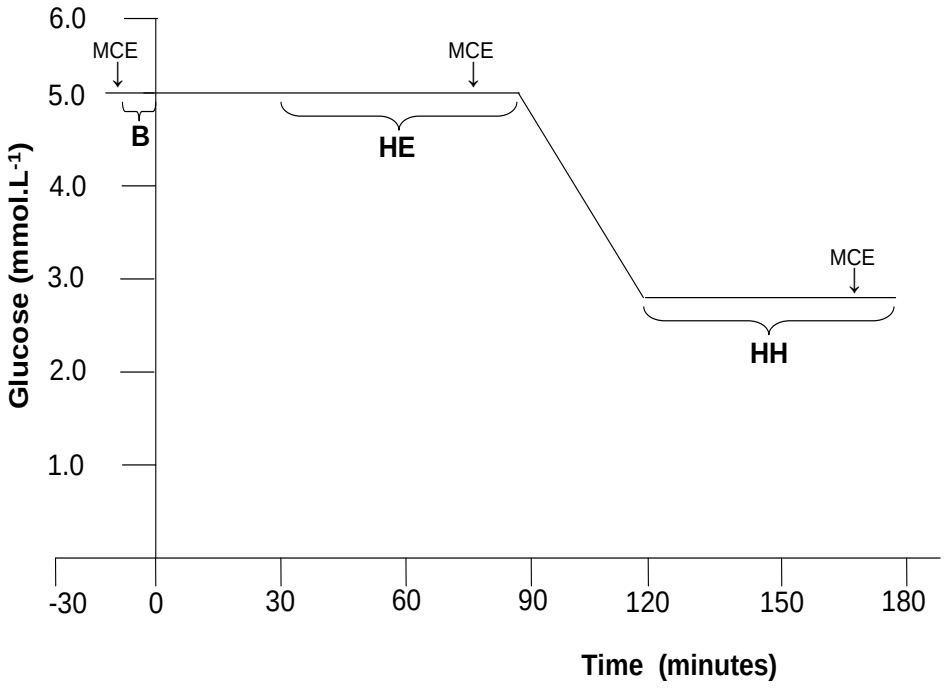


Figure 5.1.b. Sequence of image acquisition during each MCE study at baseline, HE and HH. MCE_{rest}=myocardial contrast echocardiography at rest, MCE_{peak}=myocardial contrast echocardiography at post dypridamole-induced stress

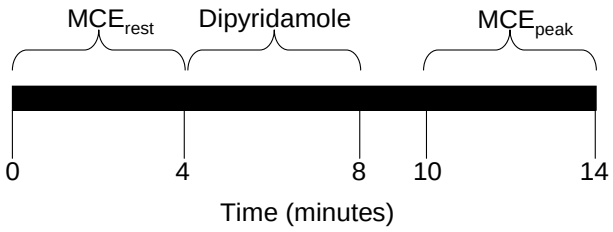


Figure 5.2. Model used for quantitative analysis of myocardial segments. (A) Apical 4 chamber, (B) Apical 2 chamber, (C) Apical 3 chamber

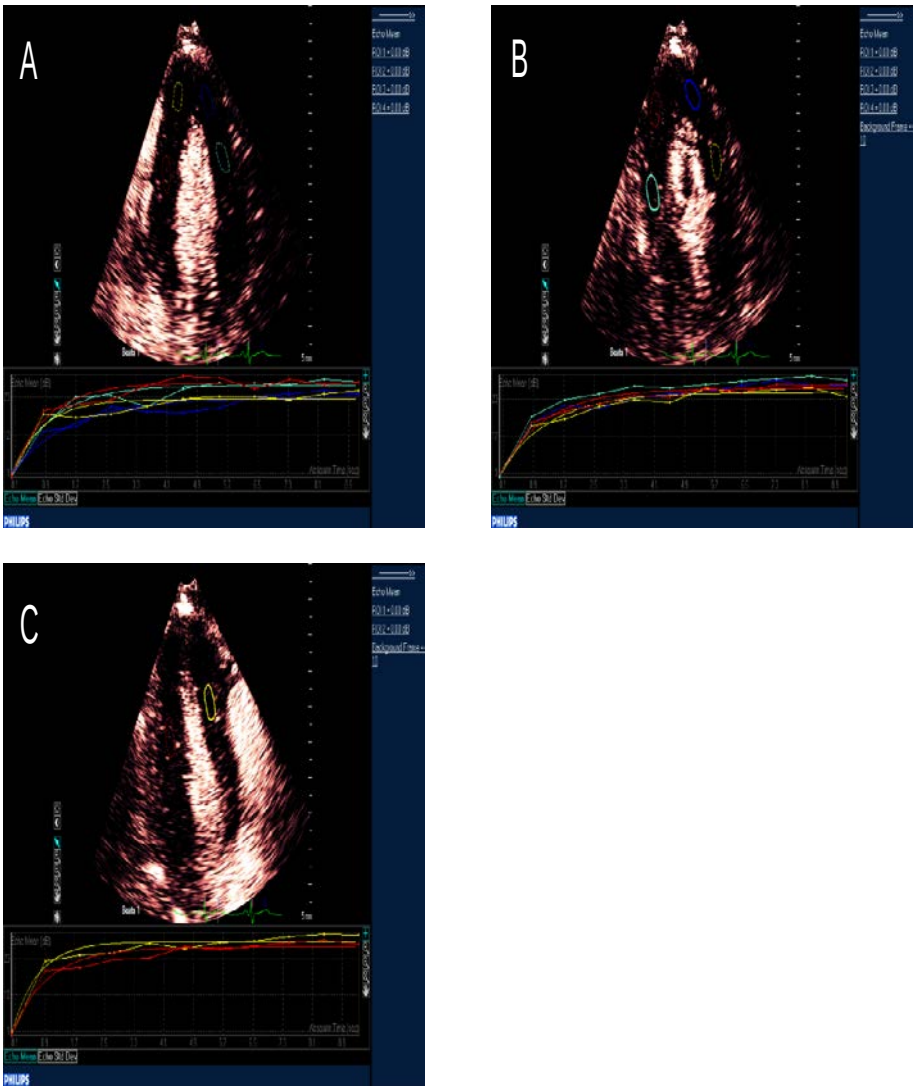
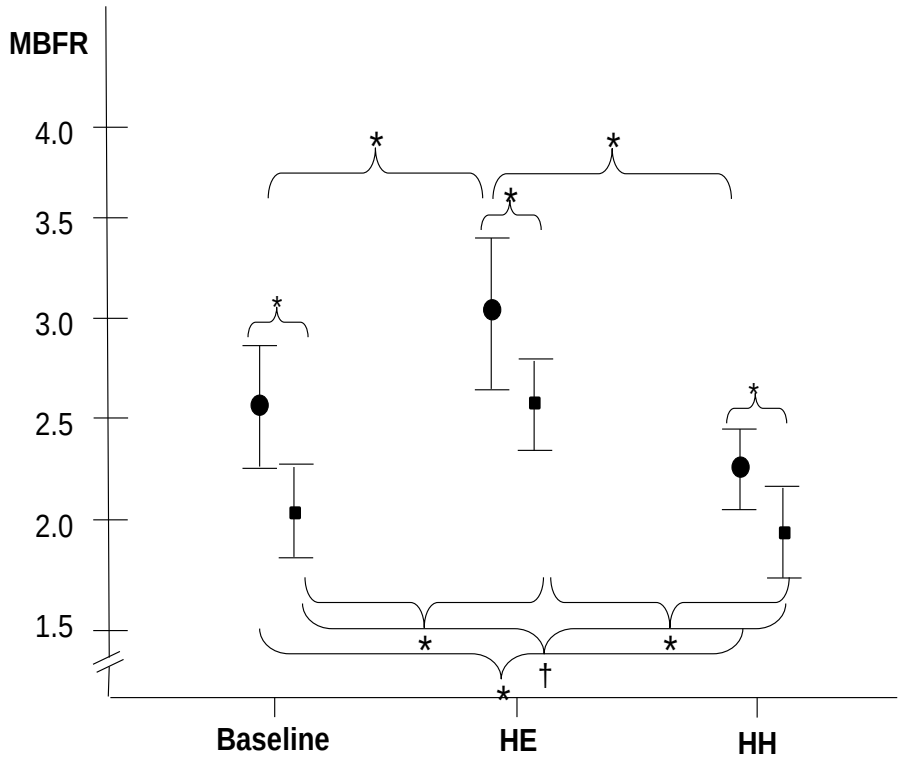


Figure 5.3. Myocardial blood flow reserve (MBFR) at baseline, hyperinsulinemic euglycaemia (HE) and hyperinsulinemic hypoglycaemia (HH). Healthy controls group (n=19) is represented as black circles and Type 1 diabetes mellitus group (n=28) is represented as black squares. Data are represented as means±SD.



*p<0.0001, †p=0.003

5.8 Supplemental Material

5.8.1 Methods

5.8.1 Subjects

Microvascular complications (retinopathy, neuropathy and nephropathy) were defined by the presence of pre- or proliferative diabetic retinopathy, on clinical examination and a vibration perception threshold score of >12 (measured on the Great Hallux using a Bio-thesiometer [Biomedical Instrument, Newbury, Ohio, USA]),[137] and an albumin/creatinine ratio of >2.5 mg/mmol/L for men and >3.5 mg/mmol/L for women.[138] Five patients with type 1 DM and microvascular complications who were taking an angiotensin converting enzyme inhibitor and a statin were instructed not to take their medications 48 hours prior to the study day to rule out any acute effects of medication on myocardial perfusion.[93]

5.8.2 Potential additional limitations

Although dipyridamole was used three times in succession with our study protocol (Figure 5.1a and b), we consider that the repeated use of dipyridamole was unlikely to artefactually influence our results. Dipyridamole has a short half-life of 8-12 minutes and the time period between each dipyridamole infusion in our study was 76 minutes. Furthermore, dipyridamole-induced changes in left ventricular ejection fraction, end-systolic volume, heart rate and diastolic blood pressure have previously been shown to return to

baseline after a 60-minute period using a much higher-dose (0.76 mg/kg) protocol.[151] In addition, we are unable to measure QTc intervals during our experiments.

Finally, the effects of administering dipyridamole three times in succession was tested in a healthy individual over the same time-course and there was no change in MBF, MBFR or other hemodynamic parameters. We did not calculate absolute myocardial perfusion values. This was because all settings and infusion parameters, once optimised at the start of each patient study, were kept constant for the rest of that individual procedure. Furthermore, we achieved homogenous opacification of the left ventricular blood pool and the signal intensity received was consistently between 34-36 dB. Calculation of absolute myocardial blood flows to take into account regional blood flow variations that occur within individuals would have introduced an additional potential source of error.

5.8.3 Explanation of terms used in Tables 5.2b, 5.3b and 5.4b in Chapter 5.

Tables 2b, 3b and 4b in the main text of Chapter 5 present parameter estimates from mixed effects regression models in which the outcome of interest (respectively: myocardial blood volume, myocardial blood velocity and myocardial blood flow) is predicted by the main effects of

i. measurement stage (baseline, during hyperinsulinemic euglycemia and during hyperinsulinemic hypoglycemia)
ii. stress state (at rest and post dipyridamole-induced stress)
iii. diabetes status (controls vs. patients with diabetes)

and by all of their possible interactions: (i) with (ii); (i) with (iii); (ii) with (iii); and the single three-way interaction (i) with (ii) with (iii). Models were fitted using the MIXED procedure in SAS software version 9.2. Interpretation of the results presented in these Tables is now described.

MEASUREMENT STAGE is the estimated mean value of the outcome for control subjects, in the rest state, observed at each of the three stages (because models were fitted with the intercept suppressed). These values are identical to those given in the 'Rest' sub-column of the C (Controls) column in the corresponding table of means in the main text.

STATE is the estimated effect of the stress treatment on the outcome for control subjects, at the baseline stage.

INTERACTION (STAGE with STATE) estimates the extent to which the effect of the stress treatment in controls at, respectively, the euglycaemic and hypoglycemic stages varies relative to that observed at the baseline stage.

PRESENCE OF DIABETES estimates the difference in the outcome at the baseline stage, in the resting state, between control subjects and those with diabetes.

INTERACTION (STAGE WITH DIABETES) estimates the extent to which the effect of measurement stage (that is, the change in resting values of the outcome at the euglycemic and hypoglycemic stages relative to the value observed at baseline) differs between control subjects and those with diabetes.

INTERACTION (STATE WITH DIABETES) estimates the extent to which the effect of the stress treatment, at the baseline stage, differs between controls and those with diabetes.

INTERACTION (STAGE with STATE with DIABETES) estimates the additional influence on the outcome of the joint presence of all main and two-way interaction effects. This may be illustrated with reference to Table 2b, from which the predicted absolute value of myocardial blood volume under euglycemia, post-stress, in subjects with diabetes is given by:-

20.4 (main effect of euglycemic stage) +

2.7 (main effect of stress) +

0.7 (main effect of diabetes) +

0.7 (euglycemia / stress interaction) +

-0.9 (euglycemia / diabetes interaction) +

-0.4 (stress / diabetes interaction) = 23.2

However, the three-way interaction term indicates that the estimated value of the outcome is 0.5 of a unit lower than that which would be predicted on the above basis (though the interaction is not statistically significant). That is, there is an additional effect arising from the joint presence of the euglycemia stage, the stress state and diabetes.

Table 5.6

Haemodynamic Data (Pulse rate, systolic blood pressure, diastolic blood pressure and rate pressure product) at rest and after dipyridamole-induced stress during the hyperinsulinemic clamp.

Variable	Baseline		Hyperinsulinemic Euglycaemia (HE)		Hyperinsulinemic Hypoglycaemia (HH)	
	C	DM	C	DM	C	DM
$Pulse_r$ b/min	63±9	68±9	66±8	71±14	75±11	78±15
$Pulse_d$ b/min	90±11	92±14	93±14	91±15	97±10	94±14
SBP_r mmHg	121±14	123±16	124±13	121±15	124±13	120±14
SBP_d mmHg	125±15	127±18	125±11	120±14	121±11	124±14
DBP_r mmHg	79±12	73±12	78±9	71±12	69±12	65±9
DBP_d mmHg	72±9	72±10	71±9	63±11	68±6	62±7
RPP_r b/min/mmHg	7739± 1533	8394± 1839	8163± 1448	8657± 2114	9454± 1692	9598± 2178
RPP_d b/min/mmHg	11375± 2399	11705±2399	11533± 1839	10929±2534	11712± 1616	11596±2014

Pulse rate, systolic blood pressure, diastolic blood pressure and rate pressure product
are presented as mean±SD.
C=Healthy controls, DM=group with diabetes mellitus, $Pulse_r$=resting pulse, $Pulse_d$=post-dipyridamole pulse, SBP_r=resting systolic blood pressure, SBP_d:=post-dipyridamole systolic blood pressure, DBP_r=resting diastolic blood pressure, DBP_d=post-dipyridamole diastolic blood pressure, RPP_r= resting rate pressure product, RPP_d= post-dipyridamole rate pressure product.

Table 5.7

Effect of measurement stage and presence of microvascular complications on myocardial blood flow reserve (MBFR) in subjects with diabetes.

variable	B coefficient	95% CI	*p*
INTERCEPT	2.24	2.15 to 2.34	< 0.0001
MEASUREMENT STAGE:			
euglycaemia vs. baseline	0.47	0.30 to 0.65	< 0.0001
hypoglycaemia vs. baseline	-0.16	-0.30 to -0.02	0.023
PRESENCE OF COMPLICATIONS	-0.52	-0.70 to -0.34	< 0.0001
INTERACTION (STAGE with COMPLICATIONS):			
euglycaemia and complications present	-0.17	-0.50 to 0.16	0.30
hypoglycaemia and complications present	-0.11	-0.36 to 0.15	0.40

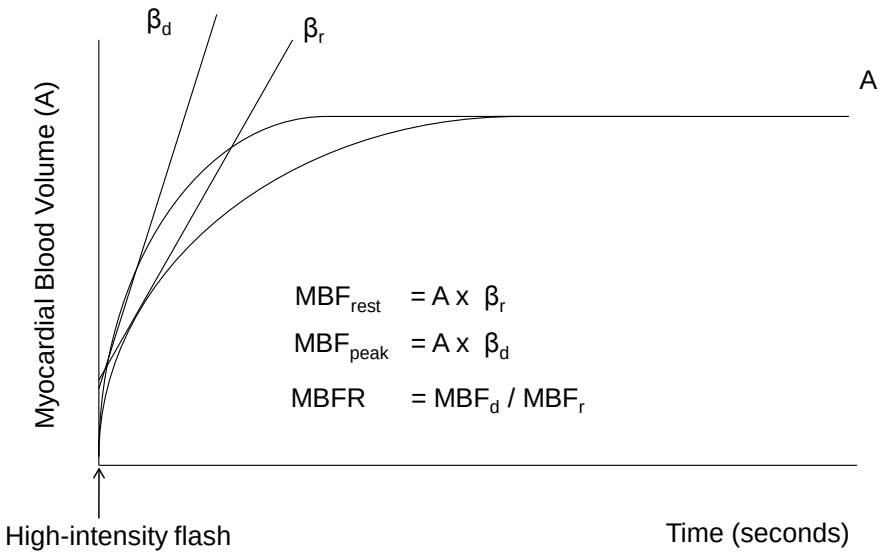
Table 5.8

Endothelin-1, hs-CRP, adrenaline, and insulin concentrations during the hyperinsulinemic clamp.

Variable	Baseline		Hyperinsulinemic Euglycaemia			Hyperinsulinemic Hypoglycaemia		
	Group	0 mins	30 mins	60 mins	90 mins	120 mins	150 mins	180 mins
ET-1 pg/ml	C	0.19(1.0)	0.0(0.7)	0.0(0.8)	0.40(0.9)	0.2(0.7)	0.44(0.8)	0.52(0.8)
	DM	1.44(0.5)	1.30(0.5)	1.32(0.7)	1.49(0.7)	1.44(0.8)	1.45(0.8)	1.49(0.7)
hs-CRP mg/L	C	0.64(0.7)	0.60(0.6)	0.62(0.6)	0.61(0.6)	0.57(0.5)	0.61(0.5)	0.51(0.4)
	DM	1.17(1.9)	0.82(1.9)	1.10(1.8)	0.93±1.9	1.10(1.8)	0.9(1.7)	0.82(1.8)
Adrenaline pg/ml	C	76.3±77	77.7±79	91.6±108	96.3±56.7	106.7±84.4	347±199	405.7±310
	DM	62.2±44.2	98.3±124.4	86.8±81.9	114.7±93.4	150.7±172	294.8±260.4	350.5±260.4
Insulin pmol/L	C	43±23	741±180	728±222	763±160	643±167	683±133	714±160
	DM	208.3±207	736.0±227.4	722.6±232.9	663.3±206.7	685.3±209.3	652.9±274.1	647.3±279.7

Values are presented as median(interquartile range) for ET-1 and hs-CRP.
Values are presented as mean±SD for adrenaline and insulin.
C=healthy controls, DM=group with diabetes mellitus, ET-1=endothelin-1, hs-CRP= high sensitivity CRP.

Figure 5.4 Measurement of Myocardial Blood Flow Reserve using Flash Impulse Imaging.



A=myocardial blood volume, β_{rest}=myocardial blood velocity at rest, β_{peak}=myocardial blood velocity at peak, MBF_{rest}=myocardial blood flow at rest, MBF_{peak}=myocardial blood flow at peak, MBFR=myocardial blood flow reserve.

Chapter 6

Discussion

6.1 What Was Known Before This Study?

Over the last two decades, the importance of improved glycaemic control in achieving a good clinical outcome has become well-established. Studies have shown that a good glycaemic control in the outpatient setting in patients with type 1 and type 2 diabetes (aiming for an HbA1c <7.0%), decreases the development of micro- and macro-vascular complications.[9-12, 33] Of note, a longer follow-up period (of at least 10 years) is required before the beneficial effects of improved glycaemic control become apparent. Similarly, several studies have shown that patients during an acute illness also benefit prognostically from an improved glycaemic control (post-meal glucose readings of 6-10 mmol/L).[18, 19, 20] However, improved glycaemic control is associated with a two- to three-fold increase in severe hypoglycaemic events. More importantly, when a more strict glycaemic regime (post-meal glucose readings of 4-6 mmol/L in patients with an acute illness or an HbA1c <6.0% in the outpatient setting) is not associated with any benefit. In fact, there is strong evidence that such a treatment strategy is associated with increased mortality rates.[23]

Although this association is poorly understood, several potential mechanisms have been hypothesised. Acute hypoglycaemia may induce a pro-thrombotic state by the release of clotting factors and activation of platelets.[105, 108, 156] In addition, acute hypoglycaemia may also be pro-arrhythmic by inducing a prolongation of the QT interval.[101, 102] Finally acute hypoglycaemia could induce a state of endothelial dysfunction by the release of ET-1 in the acute setting and hs-CRP several hours later and thereby may influence

myocardial vascular reactivity.[124, 135] However, despite the evidence from studies in patients with acute coronary syndromes and myocardial infarction the effect of acute hypoglycaemia on myocardial perfusion had not been studied to date. Furthermore, it was not known that patients with advanced diabetes for example, established micro-vascular complications behaved similarly to patients without complications during hypoglycaemia.

It is with this background that we set out to investigate the effects of acute hypoglycaemia on myocardial blood flow reserve (MBFR) in patients with type 1 diabetes and in healthy humans.

6.2 What This Study Has Added?

Our study has shown for the first time that acute hypoglycaemia is associated with a significant decrease in MBFR in healthy humans and patients with type 1 diabetes. This translates into 10% and 14% decrease in MBFR during hypoglycaemia in comparison to baseline values in patients with type 1 diabetes and healthy humans respectively. In addition, our study has shown that patients with type 1 diabetes and microvascular complications had on average a further 25% reduction in MBFR at baseline in comparison to patients without complications. To summarize, our study has shown that:

1. During baseline conditions, MBFR was 14% less in patients with type 1 diabetes in comparison to healthy humans. This was primarily due to a decrease in the relative peak (post-dipyridamole-induced MBF) in patients with type 1 diabetes.

2. Hyperinsulinemic euglycaemia leads to a 22% and 19% increase in MBFR in healthy humans and patients with type 1 diabetes respectively. This again is secondary to a significant increase in the peak MBF.

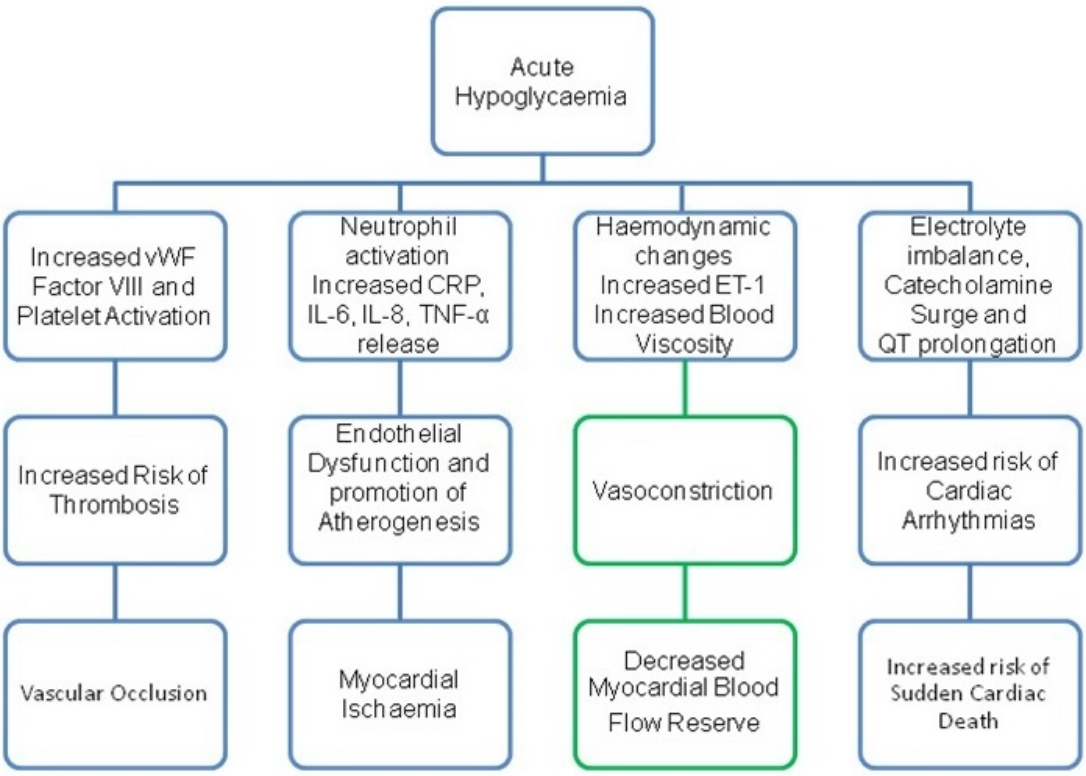
3. During hypoglycaemia, MBFR is significantly decreased in both patients with type 1 DM and healthy humans with values returning back to baseline. These observations suggest that the main action of hypoglycaemia appears to mitigate the vasodilatory effect of insulin.

4. Although patients with microvascular complications have a significantly decreased MBFR at baseline, their MBFR values at euglycaemia and hypoglycaemia are no different from those of without complications.

5. Age appeared to exhibit a significant negative interaction with MBFR albeit the magnitude of effect was extremely small.

6. ET-1 levels were seven-fold higher in patients with type 1 diabetes in comparison to healthy humans at baseline and remained so for the entire study. Although there was a suggestion that ET-1 levels increased in the healthy humans during hypoglycaemia, no such effect was recorded in patients with type 1 diabetes.

Figure 6.1 Mechanisms Likely to be Involved in the Association Between Hypoglycaemia and Increased Cardiovascular Mortality. The Pathway Highlighted in Green was Explored and Proven in Our Study.



6.3 Future Directions

We believe that our study will pave way for future studies to explore the complex association between hypoglycaemia and cardiovascular mortality. We feel that there are other key areas worth investigation which could help further our understanding of this association between hypoglycaemia and myocardial perfusion.

1. The effects of acute hypoglycaemia on myocardial blood flow reserve in patients with type 2 diabetes should be investigated. The study population in the ACCORD and ADVANCE studies was exclusively that of patients with type 2 diabetes.[14, 15] Although the glycaemic targets were aggressive, the rates of severe hypoglycaemia were alarmingly high in the ACCORD study with 1 in 9 patients experiencing severe hypoglycaemia. This led to a 27% relative increase in the all-cause mortality rates thereby necessitating the study to be prematurely stopped. It is plausible that patients with type 2 diabetes may respond differently to hypoglycaemia in the long term. Of note, the patients included in these two studies were older and had either had established coronary disease or strong cardiovascular risk factors. There is accumulating evidence that the endothelium may respond differently to exogenous insulin in patients with type 2 diabetes in comparison to type 1 diabetes patients. In patients with type 2 diabetes, insulin has been shown to facilitate the endothelial release of ET-1, a potent vasoconstrictor, in contrast to patients with type 1 diabetes.

2. Although we demonstrated that the baseline levels of ET-1 were seven-fold higher in patients with type 1 diabetes, these did not change subsequently. The effect of hypoglycaemia on ET-1 levels in patients with type 1 DM has only ever been investigated in one previous study which recorded a significant increase.[124] However, the investigators used a different assay with different cross reactivity between ET-1, ET-2 and ET-3. In addition, their results showed very wide standard deviations. We feel that further studies should include prolonged periods of hypoglycaemia and the measurement of ET-1 a few hours after the cessation of hypoglycaemia. This is because ET-1 is released abluminally and therefore serum concentrations may not reflect those within the smooth muscle layer of arterioles.

3. The effects of acute hypoglycaemia should be studied in patients with angiographically proven moderate coronary artery disease. Wall motion analysis combined with the measurement of myocardial blood flow reserve using myocardial contrast echocardiography should be performed to investigate the effects of hypoglycaemia in such a cohort.

4. The use of endothelin receptor blockers for example, bosentan is another avenue worth exploration. If a decrease in MBFR during acute hypoglycaemia is prevented with the administration of bosentan then this would further confirm our findings and help us understand the role of ET-1 during acute hypoglycaemia.

5. Finally, it is also worth investigating the hemodynamic effects of acute hypoglycaemia on patients with coronary artery disease using invasive assessments such as fractional flow reserve (FFR). It is plausible that patients with anatomically moderate coronary stenoses and an FFR between 0.75-0.85 (below 0.75 is abnormal and warrants percutaneous coronary intervention) may record an acute decrease during hypoglycaemia. This would again help us understand the mechanistic interplay between acute hypoglycaemia and myocardial perfusion in patients with existing coronary artery disease.

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