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THE CARDIOVASCULAR EFFECTS OF THE TREATMENTS FOR DIABETES MELLITUS

‘The effect of SIRT-1 activation on markers of endothelial function, arterial stiffness and thrombosis; and the effect of hypoglycaemia on myocardial perfusion and biomarkers of ischaemia.

Thesis for Doctor of Medicine

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The University of Edinburgh

2019
Lay Summary of Thesis

‘Type 1 diabetes mellitus’ is a condition where the body does not produce enough insulin. Insulin is essential for the body to absorb its main source of energy, which is glucose. ‘Type 2 diabetes mellitus’, by contrast, is a condition where the body is resistant to the effects of insulin. Both these chronic conditions are on the rise, and the main cause of death in this group of individuals is heart problems. Treatment of type 1 diabetes with insulin, has over the years, improved the outcomes of people with type 1 diabetes. However, a very common side effect is low blood sugar. Treatment of type 2 diabetes includes a variety of tablet treatments, and sometimes insulin. The treatments of type 2 diabetes mellitus itself have complex, sometimes adverse, effects on the heart.

The purpose of this body of research is to identify whether a new treatment for type 2 diabetes, SRT2104, has any benefits to the heart and blood vessels, as well as the clotting system, in people who have type 2 diabetes, and in a group of smokers who are otherwise healthy. In addition, the effect of low blood sugar to the heart muscles, and its circulation, is examined in two groups of volunteers, with and without type 1 diabetes.

At the end of the research programme, it appears that SRT2104 has a predominantly neutral effect to the heart, and markers of clotting. It appears to be potentially beneficial in lowering cholesterol, and possibly have a beneficial effect on the stiffness of blood vessels. During the course of the study however, we observed an unexpected rise in blood sugar, and weight loss. Although the use of
SRT2104 as an agent to control diabetes may be problematic, its effect on weight and cholesterol merits further study.

Low blood sugar may be potentially harmful to the heart muscles, but this is not conclusive and merits further exploration. These results may prompt clinicians to avoid causing low blood glucose in treating people with diabetes at high risk of heart disease.
Abstract

Background. Both type 1 and type 2 diabetes mellitus are increasing in incidence and prevalence. Cardiovascular disease remains the number one cause of co-morbidity and mortality in this group of individuals. Intensive glucose control has been shown to be beneficial to cardiovascular outcomes in people with type 1 diabetes, but low blood sugar remains a problematic limiting factor. Intensive glucose control in people with type 2 diabetes and cardiovascular disease may cause harm, but the exact mechanism which mediates the poorer outcomes is unknown. Tight glycaemic control is associated with increased risk of hypoglycaemia. This increased risk of hypoglycaemia may have various effects on the blood supply to the heart, and the myocardium. Various treatments of diabetes also have complex effects on the heart, the vascular tree, and markers of thrombosis. The aim of this thesis was to firstly examine the cardiometabolic effects of a novel oral agent for the treatment of diabetes. Secondly, the thesis examines whether hypoglycaemia, a common side effect of insulin therapy, exerts an effect on myocardial perfusion and to markers of myocardial damage.

Methods. Studies 1, 2 and 3 were conducted as Phase 1 cross-over randomised controlled trials of people with type 2 diabetes (n=15; Study 1) and people without diabetes but a cardiovascular risk factor (otherwise healthy smokers, n=24; Study 2), examining the effect of a novel sirtuin agonist on endothelial function, platelet-monocyte aggregation, and metabolic markers. In the cohort of patients with type 2 diabetes, the markers of glucose control were measured. The volunteers underwent 28 days of treatment with the novel agent SIRT2104 and crossed-over to 28 days of placebo, or vice versa. Three venous-occlusion plethysmography studies were performed at baseline, day 28 and day 56, and similarly platelet-monocyte aggregation studies. Serum triglycerides and cholesterol were measured in both cohorts. In Study 3, the effect of the novel agent treatment on markers of arterial stiffness
(pulse wave velocity and pulse wave analysis) was assessed in both the diabetes and smoker cohorts. Finally, in Study 4, 17 individuals with type 1 diabetes mellitus and 10 controls without diabetes underwent experimentally-induced hypoglycaemia, using the hyperinsulinaemic glucose clamp method. Coronary flow reserve was measured non-invasively via transthoracic echocardiography and adenosine induced coronary vasodilation. The marker of myocardial injury, highly sensitive troponin I, was also measured during a euglycaemic clamp and hypoglycaemic clamp.

**Results:** In Study 1, SRT2104 had an inconsistent, predominantly neutral effect on endothelial function and platelet-monocyte aggregation studies. It had an unexpected effect in increasing markers of glucose control, and decreasing weight by approximately 1 kilogram over 28 days. Study 2 found that SRT2104 had a neutral effect on endothelial function and platelet-monocyte aggregation studies. A statistically significant reduction in total cholesterol, low density lipoproteins and triglycerides was observed after 28 days of SRT2104. Study 3 found that SRT2104 improved markers of arterial stiffness, with no discernible change to systolic and diastolic blood pressure. Finally, Study 4 found that hypoglycaemia did not have any effect on markers of myocardial injury. Coronary flow reserve was lowest during hypoglycaemia in people with type 1 diabetes, although this trend did not reach statistical significance.

**Conclusions.** SRT2104 was a well-tolerated agent. There is a signal toward benefit in terms of lipids and markers of arterial stiffness. In people with type 2 diabetes, SRT2104 may induce weight loss at a cost of short-term loss of glycaemic control. Although the use of SRT2104 as an anti-diabetes agent may be problematic, its effect of weight and lipids merit further assessment and may provide vital clues to important molecular pathways underpinning lipid metabolism, weight, and cardiovascular disease. Hypoglycaemia in people with type 1 diabetes did not cause direct myocardial injury, but
appeared to be associated with a low coronary flow reserve. This may prompt clinicians to screen for problematic hypoglycaemia during treatment, and to proceed with caution in cohorts of people with diabetes and known cardiovascular disease.
Declaration

This thesis is a presentation of my original research work. The contributions of colleagues in my research group to the body of work, are clearly indicated in the following paragraph. This thesis has not been submitted for any other higher degree or qualification.

Study 1 and 2’s protocol was set up by Drs Langrish and Daga. The recruitment of volunteers, and the conduct of the venous occlusion plethysmography and platelet monocyte aggregation studies were by myself and Dr Subramanian. Analysis and write up of study 1 were conducted predominantly by myself, and study 2 predominantly by Dr Subramanian.

Study 3’s protocol was also set up by Drs Langrish and Daga. and was conducted by myself and Dr Subramanian, with the pulse wave analysis component performed by my research nursing colleagues at the Wellcome Trust Clinical Research Facility in Edinburgh. Analysis and write up was by myself and Dr Subramanian.

Study 4’s protocol was written by Drs Graveling and Lang. The recruitment of the volunteers and hyperinsulinaemic glucose clamp were by myself, and the transthoracic echocardiography was performed by Mrs Audrey White and Dr Ninian Lang. Analysis of the data and write up was by myself.

[Signature]

Dr M Radzi M Noh

Nov 2019
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This thesis would not have been possible without the support of many colleagues, and my friends and family. I am grateful to Drs Alex Graveling, Ninian Lang, Sowmya Venkatasubramanian, and Mrs Audrey White for invaluable help and guidance during my clinical studies. All the nurses at the Wellcome Trust Clinical Research Facility, and the Scottish Diabetes Research Network have been instrumental in the recruitment of volunteers and conduct of said studies. I am grateful to Sirtris and the Chief Scientist Office of Scotland for funding for my research studies. The synthesis of the research was only possible with the counsel and guidance of my research supervisors, Professors Frier and Newby. Finally, I dedicate this thesis to my wife and family for their constant support.
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List of Abbreviations

**ACCORD** Action to Control Cardiovascular Risk in Diabetes

**ACEi** Angiotensin Converting Enzyme inhibitors

**ADA** American Diabetes Association

**ADDITION** Anglo Danish- Dutch Study of Intensive Treatment in People with Screen Detected Diabetes in Primary Care

**ADP** Adenine Diphosphate

**ADVANCE** Action in Diabetes and Vascular disease: PreterAx and Diamicron MR Controlled Evaluation

**AGEs** Advanced glycation end-products

**AIx** Augmentation Index

**AMP-K** Activating 5-Adenine Mono Phosphate-Activated Protein Kinase

**ANCOVA** Analysis of covariance

**ANOVA** Analysis of variance

**ApoB** Apolipoprotein B-100

**ATP** Adenine triphosphatase

**CACTI** The Coronary Artery Calcification in T1DM

**CAN** Cardiac Autonomic Neuropathy
**CANVAS** Canagliflozin cardiovascular Assessment study

**CFR** Coronary Flow Reserve

**CFV** Coronary Flow Velocity

**CHD** Coronary Heart Disease

**cm/s** centimetres per second

**C\text{max}** mean maximum plasma concentration

**C-myC** Cardiac myosin-binding protein C

**CONSORT** Consolidated Standards of Reporting Trials

**COX-2** Cyclo-oxygenase-2

**CT-CA** Computed Tomography Coronary Angiography

**CV** Coefficient of variation

**CVD** Cardiovascular disease

**CVOTs** Cardiovascular outcome trials

**DC** Diabetic cardiomyopathy

**DCCT** Diabetes Control and Complications Trial

**DECODE** Diabetes Epidemiology: Collaborative analysis of Diagnostic Criteria in Europe

**DIAD** Detection of Ischaemia in Asymptomatic Diabetics

**DiRECT** Diabetes Remission Clinical Trial

**DPP-4** Dipeptidyl Peptidase 4
DPP-4 Dipeptidyl Peptidase-4 Inhibitors

EASD European Association of the Study of Diabetes

ECG Electrocardiograph

EDC Pittsburgh Epidemiology of Diabetes Complications study

EDIC Epidemiology of Diabetes Interventions and Complications

EDTA Ethylene Diamine Triacetic acid

ELISA Enzyme linked immunosorbent assay

ELIXA Evaluation of Lisexenatide in Acute Coronary Syndrome

EMPRA-REG OUTCOME Empagliflozin CV event outcome in T2DM patients

eNOS endothelial nitric oxide synthase

EURODIAB The Epidemiology and Prevention of Diabetes study

EXAMINE Examination of Cardiovascular outcomes in Alogliptin in patients with T2DM and acute coronary syndrome

EXSCEL Exenatide Study of Cardiovascular Event Lowering

FACS Fluorescence-activated cell sorting

Factor-64 Screening For Asymptomatic Obstructive Coronary Artery Disease Among High-Risk Diabetic Patients Using CT Angiography, Following Core 64

FBF Forearm blood flow

FDA Food and Drug Administration
FFAs free fatty acids

FinnDiane The Finnish Diabetes Nephropathy Study

FITC Fluorescein Isothiocyanate

GFR glomerular filtration rate

GLP1-RA Glucagon- Like Peptide 1 Receptor Agonists

GLUT-4 glucose transporter 4

GPIIa/IIIb Glycoprotein IIa/IIIb

GRADE Glycaemia Reduction Approaches in Diabetes

HbA1c glycated haemoglobin

HCG human chorionic gonadotropin

HDL- High Density Lipoprotein

HDL-C high-density lipoprotein cholesterol

hs-cTnI High-sensitivity cardiac troponin I

HSP hexosamine pathway

IFG fasting glucose

IGT impaired glucose tolerance

IL-6 Interleukin-6

IR insulin resistance
IRS-1 insulin receptor substrate 1

LAD left anterior descending

LEADER Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results

LKB1 liver kinase B1

LXRs liver X receptor proteins

Look-AHEAD Action for Health in Diabetes trial

LYDIA Liraglutide in Young Adults with Type 2 Diabetes

Mac-1/CD11b monocyte CD11b expression

μg/min Microgrammes per minute

MHRA Medicines and Healthcare products Regulatory Agency

MI myocardial infarction

mmol/mol milimoles per mole

mmol/L millimoles per litre

MRFIT Multiple Risk Factor Intervention Trial

MRI Magnetic Resonance Imaging

NAD Nicotinamide Adenine Dinucleotide

NADPH nicotinamide adenine dinucleotide phosphate

NCEP National Cholesterol Education Programme

NEFAs Non-esterified fatty acids (free fatty acids)
NF-\(\kappa B\) nuclear factor kappa B

**NICE** National Institute for Clinical Excellence

**NICE-SUGAR** Normoglycaemia in Intensive Care Evaluation and Surviving Using Glucose Algorithm Regulation

**NO** nitric oxide

**OGTT** oral glucose tolerance test

**p66Shc** protein 66 SiroHydroChlorin

**pAI-1** plasminogen activator inhibitor 1

**PE** Phycoerythrin

**PGC-1\(\alpha\)** PPAR gamma coactivator 1-alpha

**PI3K-Akt** phosphatidylinositol-3-kinase-protein kinase-B

**PKC** protein kinase C

**pmol/min** picomoles / minute

**PMA** platelet–monocyte aggregation

**PPACK** D-phenylalanine-L-arginine chloromethyl ketone

**PPAR\(\gamma\)** peroxisome proliferator-activated receptor gamma

**PROACTIV** PRospective PiOglitAzone Clinical Trial in macroVascular events

**PWV** Pulse Wave Velocity

**RAAS-** Renin Angiotensin Aldosterone System
RAGEs  receptors of advanced glycation end-products

RCT  randomised controlled-trial

ROS  reactive oxygen species

SAVOR-TIMI  Saxagliptin Assessment of Vascular Outcomes in Recorded in patients with T2DM-Thrombolysis in Myocardial Infarction

sCD40L  soluble CD40 ligand

SCI-DC  Scottish Care Information- Diabetes Collaboration

SDS  Scottish Diabetes Survey

SIGN  Scottish Intercollegiate Guideline Network

SIR2  Silent mating-type Information Regulation 2

SNP  sodium nitroprusside

SNS-  Sympathetic Nervous System

SOS  Swedish Obese Subjects

SR-B  scavenger receptor B

SUSTAIN-6  Trial to Evaluate Cardiovascular and Other Long-Term Outcomes with Semaglutide in Type 2 Diabetes.

T1DM  Type 1 diabetes mellitus

T2DM  Type 2 diabetes mellitus

TECOS  Trial Evaluating Cardiovascular Outcomes in Sitagliptin
TGs triglycerides

$T_{\text{max}}$ mean time at which the maximum plasma concentration was observed

TNF-α tumour necrosis factor alpha

TOR target of rapamycin,

tPA- tissue Plasminogen Activator

TZD thiazolidinedione

UCP2 uncoupling protein 2

UGDP The University Group Diabetes Programme

UKGPRD United Kingdom General Practice Research Database

UKHGS United Kingdom Hypoglycaemia Study

UKPDS United Kingdom Prospective Diabetes Study

VA-CSDM Veteran Affairs Cooperative Study in T2DM

VADT Veteran Affairs Diabetes Trial

VLDL very low-density lipoprotein

V$\text{max}$ maximum coronary flow velocity

WESDR The Wisconsin Epidemiologic Study of Diabetic Retinopathy

WHO World Health Organisation
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Diabetes mellitus (DM) is a complex, heterogenous, chronic condition characterised by hyperglycaemia, and microvascular and cardiovascular complications which increase morbidity and mortality as well as impairing quality of life (World Health Organisation, 2006). Type 1 diabetes mellitus (T1DM) is a condition where auto-immune destruction of pancreatic beta-cells results in insulin deficiency and hyperglycaemia. It is typically diagnosed under the age of 30, although it can occur at any age (Bluestone, Herold and Eisenbarth, 2010). Type 2 diabetes mellitus (T2DM) is a progressive metabolic condition characterised by insulin resistance and eventual dysfunction of the pancreatic beta cell. It tends to occur later in life but increasing rates of obesity are contributing to higher rates of diagnosis at ages earlier than 40 (Kahn, Cooper and Del Prato, 2014). Other forms of diabetes mellitus with a genetic cause also exist, but this thesis will focus on type 1 and type 2 DM.

It bears repetition that diabetes mellitus is a worldwide problem. The World Health Organisation (WHO), in its ‘Global Burden of Diabetes Survey’ estimated that in 2014, 422 million people worldwide have diabetes mellitus, compared to 108 million in 1980 (WHO, 2016). Table 1 is reproduced from the same document; it is worth noting the highest absolute numbers of people with diabetes are in South East Asia and the Western Pacific Region but also the prevalence in the Eastern Mediterranean which has almost trebled since 1980.
<table>
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<tr>
<th>WHO Region</th>
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<th>Number (Millions)</th>
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<tr>
<td>Total</td>
<td>4.7%</td>
<td>8.5%</td>
</tr>
</tbody>
</table>

**Table 1: Global Report on the Burden of Diabetes 2016 in Adults Adapted from WHO**

The prevalence of diabetes has also increased faster in lower to middle income countries than in high income countries. These trends mirror the increasing prevalence of obesity worldwide (Non-Communicable Disease Risk Factor Collaboration 2016). The economic implications of the prevalence of diabetes mellitus is substantial. One study estimates that losses in GDP worldwide from 2011 to 2030, including both the direct and indirect costs of diabetes, will total US$ 1.7 trillion, comprising US$ 900 billion for high-income countries and US$ 800 billion for low- and middle-income countries (Bloom DE, 2011).
More locally, the Scottish Diabetes Survey (SDS) in 2016 reported that there were 291,981 people with diabetes in Scotland, representing 5% of the total population (Scottish Diabetes Survey Monitoring Group, 2016a). Of those registered in the survey, 3.5% of people with T1DM are recorded as having a previous myocardial infarction, with 9.7% of individuals with T2DM for the same parameter. Others will have had a myocardial infarction but not survived. 20,032 (6.9%) of people included in the survey have a record of having undergone cardiac revascularisation, with a breakdown of 2.6% of people with T1DM and 7.5% of people with T2DM Scotland-wide (Group, 2016a). Cardiovascular disease remains the main cause of death in people with diabetes in Scotland.

The relationship between diabetes and cardiovascular disease (CVD) is well established. Diabetes mellitus confers a two to threefold risk of cardiovascular disease and premature cardiovascular mortality (Sarwar et al., 2010). The important association between diabetes and CVD is highlighted by the results of the Framingham Heart Study and the Multiple Risk Factor Intervention Trial (MRFIT). In the Framingham Heart Study, the presence of diabetes doubled the age-adjusted risk for cardiovascular disease in men and tripled it in women (Kannel and McGee, 1979). Diabetes remained a major independent cardiovascular risk factor even when adjusting for advancing age, hypertension, smoking, hypercholesterolemia, and left ventricular hypertrophy. In MRFIT, of 5163 men who reported taking medications for diabetes (mostly T2DM), 9.7 percent died from cardiovascular disease over a 12-year period; the comparable cardiovascular death rate in the 342,815 men not taking medications for diabetes was 2.6 percent. This difference was independent of age, ethnic group, cholesterol level, systolic blood pressure, and tobacco use. However, among men with diabetes, the increase in cardiovascular risk rose more steeply than in people without diabetes with the addition of each of these risk factors (Stamler et al., 1993). The Emerging Risk Factors Collaboration group performed a meta-analysis of 102 studies that included 530,083 patients with no history of myocardial
infarction (MI), angina, or stroke at the initial study visit (Sarwar et al., 2010). After adjusting for other risk factors, patients with diabetes had an overall risk of CVD twice that of patients without diabetes (HR 2.0, 95% CI 1.8-2.2), with a similarly higher risk of cardiac death (HR 2.3, 95% CI 2.1-2.6) and non-fatal MI (HR 1.8, 95% CI 1.6-2.0).

Other disorders of glucose metabolism are also risk factors for CVD. The Diabetes Epidemiology: Collaborative analysis of Diagnostic Criteria in Europe (DECODE) study, analysed several European cohort studies with baseline oral glucose tolerance test (OGTT) data. Increased mortality was observed in people with DM and impaired glucose tolerance (IGT) but not in people with impaired fasting glucose (IFG). A high 2-hour plasma glucose predicted all-cause and CVD mortality after adjustment for other major cardiovascular risk factors including hypertension and dyslipidemia (DECODE study group 2003). Prevalence rates of DM are expected to double by 2030 (Wild et al., 2004), and many people with DM are undiagnosed. Many people who are not categorized as having diabetes will have IGT, which still increases CV risk. With such a close relationship between cardiovascular mortality and DM, the potential impact of the explosion in worldwide numbers of people with diabetes are therefore difficult to overstate.

The next part of the thesis outlines the pathophysiology of cardiovascular disease in T1DM and T2DM, followed by evidence from clinical trials and epidemiological studies to modulate cardiovascular disease.
1.2 The Pathophysiology of Cardiovascular disease in people with Diabetes Mellitus

In order to extract any lessons about the treatments of diabetes and modulation of cardiovascular disease, it is instructive to consider the pathophysiology of cardiovascular disease in T1DM and T2DM separately. Whilst both conditions share the same treatments, particularly insulin, and whilst both conditions share similarities in the way in which cardiovascular risk develops, there are nonetheless very important differences. For example, tight glucose control in people with T1DM appears to be beneficial to cardiovascular health, whilst doing so in counterparts with T2DM appears to be a mixed blessing. These differences may give us a clue as to which pathophysiological pathways and factors which are more important than others in trying to modulate cardiovascular risk.

1.2.1 T2DM, ‘pre-diabetes’, metabolic syndrome and cardiovascular risk

It is impossible to discuss cardiovascular disease without touching upon the ‘metabolic syndrome’, but a full exploration is out with the remit of this thesis. Briefly, the term ‘metabolic syndrome’ itself has been problematic as it is unclear whether it confers any excess cardiovascular risk beyond that of its singular components of obesity, hyperlipidaemia, hypertension and dysglycaemia. Importantly however, metabolic syndrome is associated with progression to T2DM and cardiovascular disease (Reaven, 1988). Typically, patients with T2DM present later in life, and the syndrome of insulin resistance coupled with hypertension and diabetic dyslipidaemia create conditions that promote atheromatous plaques in the coronary circulation and other medium to large calibre vessels. This section gives an overview of the current data on these so-called ‘classical’ risk factors which happen
typically, but not unique to, individuals with type 2 diabetes. Later, pathophysiological factors related to vascular function which precedes atherosclerosis are explored.

1.2.2 Insulin Resistance, and the link between Insulin Receptor Substrate 1, Protein Kinase C, and Nuclear Factor Kappa B.

As explored earlier, IGT confers an increase in risk of cardiovascular disease. The relationship between dysglycaemia, T2DM per se and cardiovascular risk is therefore a continuum. The mechanism of the development of T2DM is complex and still under study. The current consensus is that T2DM develops begins with a state of longstanding insulin resistance (IR) (usually but not necessarily a result of obesity (Hossain, Kawar and El Nahas, 2007) and lack of physical activity, modulated by genetic factors) and compensatory hyperinsulinaemia. Over time, combined with reduction in pancreatic beta-cell function, frank hyperglycaemia develops (American Diabetes Association, 2004).

On a molecular level the syndrome of insulin resistance is explained by obesity and the excess release of free fatty acids (FFAs) and cytokines like tumour necrosis factor alpha (TNF-α) and Interleukin-6 (IL-6) from adipose tissue, which directly impairs the action of insulin. In skeletal muscle and adipose tissue, FFA-induced reactive oxygen species (ROS) production blunts activation of insulin receptor substrate 1 (IRS-1) and phosphatidylinositol-3-kinase-protein kinase B (PI3K-Akt) signalling (Kim et al., 2006), leading to downregulation of insulin responsive glucose transporter 4 (GLUT-4). FFA-induced impairment of the PI3K pathway blunts Akt activity and phosphorylation of endothelial nitric oxide synthase (eNOS) resulting in decreased production of nitric oxide (NO), endothelial dysfunction, and
vascular remodelling (increased intima-media thickness) (Saltiel and Kahn, 2001; Wang, Goalstone and Draznin, 2004). In turn, accumulation of ROS activates transcription factor nuclear factor kappa B (NF-κB), leading to increased expression of inflammatory adhesion molecules and cytokines. Chronic IR stimulates pancreatic secretion of insulin, triggering a complex sequence of events that includes progressive beta cell dysfunction, decreased insulin levels, and eventually frank T2DM and hyperglycaemia. Hyperglycaemia further decreases endothelium-derived NO availability and affects vascular function via a number of mechanisms, mainly involving overproduction of ROS (Cosentino et al., 1997). The mitochondrial electron transport chain is one of the first targets of high glucose, with a direct net increase in superoxide anion formation. This drives a vicious cycle of further superoxide anion production via ROS-induced activation of protein kinase C (PKC) (Cosentino et al., 2003). Activation of PKC by glucose leads to up-regulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, mitochondrial adaptor p66Shc and cyclo-oxygenase-2 (COX-2) as well as thromboxane production and impaired NO release. Mitochondrial ROS, in turn, activate signalling cascades involved in the pathogenesis of cardiovascular complications, including polyol flux, advanced glycation end-products (AGEs) and their receptors (RAGEs), PKC and hexosamine pathway (HSP) (Cosentino et al., 1997; Cosentino et al., 2008; Camici et al., 2007). Figure 1 summarizes the relationship between these various pathways and how it influences endothelial dysfunction and vascular inflammation.
**FIGURE 1:** SUMMARY OF INTERACTING MOLECULAR PATHWAYS AND RISK FACTORS LINKING INSULIN RESISTANCE AND Atherosclerosis

AGE-advanced glycation end-product, eNOS endothelial Nitric Oxide Synthase, FFA- Free Fatty Acids, GPIIa/IIb- Glycoprotein IIa/IIb, GLUT4- Glucose Transporter 4, HDL- High Density Lipoprotein, Insulin Receptor Signalling 1&2, IL-6 Interleukin 6, LDL-Low Density Lipoprotein, NFkB Nuclear Factor Kappa B, pAI-1 plasminogen activator inhibitor 1, PKC Protein Kinase C, RAAS- Renin Angiotensin Aldosterone System, SNS- Sympathetic Nervous System, tPA- tissue Plasminogen Activator, Trigs-Triglycerides.
1.2.3 Hypertension and Insulin Resistance

Hypertension is two to three times more common in people with T2DM than age matched controls, and occurs concurrently with T2DM in 50-75% of cases (Colosia, Palencia and Khan, 2013). Insulin resistance and hypertension share many common genesis pathways including PI3K and its downstream Akt signalling pathways. It is thought that insulin signalling is interfered in these pathways, causing attenuation of vascular vasodilatation (Sowers, 2004). Compensatory hyperinsulinaemia may have direct effects on the distal convoluting tubule via IRS2, promoting salt and water retention and thereby hypertension (Soleimani, 2015). The milieu of increased ROS and vascular endothelial dysfunction in insulin resistance also amplify the factors associated with hypertension including arterial stiffness and increased sympathetic nervous stimulation via the renin-angiotensin aldosterone system (Sowers, 2004; Jia, DeMarco and Sowers, 2016).

1.2.4 Dyslipidaemia

IR results in increased FFA release to the liver. Therefore, enhanced hepatic very low-density lipoprotein (VLDL) production occurs due to increased substrate availability, decreased apolipoprotein B-100 (ApoB) degradation and increased lipogenesis (Cannon, 2008; Sorrentino et al., 2010). In T2DM and the metabolic syndrome, these changes lead to a lipid profile characterized by high triglycerides (TGs), low high-density lipoprotein cholesterol (HDL-C), increased remnant lipoproteins, apolipoprotein B (ApoB) synthesis and small, dense LDL particles. This has been labelled as ‘mixed dyslipidaemia’ and is associated with both T2DM and the metabolic syndrome. What is more, it
appears that frank hyperglycaemia and T2DM enhances this phenotype of lipid abnormalities. This LDL subtype plays an important role in atherogenesis as it is much more prone to oxidation. In patients with T2DM, atherogenic dyslipidaemia is an independent predictor of cardiovascular risk, stronger than isolated high TG or a low HDL cholesterol (Sorrentino et al., 2010).

1.2.5 Platelet Function and Coagulation

Increased platelet reactivity is an important contributor to increased atherothrombotic risk in T2DM. A number of mechanisms contribute to platelet dysfunction, affecting the adhesion and activation, as well as aggregation, phases of platelet mediated thrombosis. Hyperglycaemia alters platelet calcium ion homeostasis, leading to cytoskeleton abnormalities and increased secretion of pro-aggregant factors (Ferreiro and Angiolillo, 2011). Moreover, hyperglycaemia-induced upregulation of glycoproteins (IIb and IIb/IIIa), P-selectin and enhanced P2Y12 signalling are key events underlying atherothrombotic risk in T1DM and T2DM. In T2DM patients, IR and hyperglycaemia participate to the pathogenesis of a prothrombotic state characterized by increased plasminogen activator inhibitor-1 (PAI-1), factor VII and XII, fibrinogen and reduced tissue plasminogen activator (tPA) levels (Sarma et al., 2002; Grant, 2007).
1.2.6 Macrophage dysfunction and peroxisome proliferator-activated receptor gamma.

Another putative mechanism of atherothrombotic risk that is described in the literature, is the increased accumulation of macrophages that been observed in obese adipose tissue. In addition, the insulin-resistant macrophage increases expression of the oxidized LDL scavenger receptor B (SR-B), promoting foam cell formation and atherosclerosis. These findings are reversed by peroxisome proliferator-activated receptor gamma (PPARγ) activation, which enhances macrophage insulin signalling. In this sense, it seems that macrophage dysfunction may also contribute to atherogenesis, linking DM and CVD by both enhancing IR and by contributing to the development of fatty streaks and vascular damage (Kranendonk et al., 2015; Cannon, 2008).

1.2.7 T1DM and Cardiovascular risk: epidemiology from large studies

T1DM is defined as insulin deficiency due to T-cell mediated auto-immune destruction of the pancreatic beta cells (ADA 2004). It is associated with high cardiovascular risk. Perhaps the most well-known study which describes patients with T1DM and cardiovascular outcomes is the prospective randomised control trial Diabetes Control and Complications Trial (DCCT) and the prospective follow-up study Epidemiology of Diabetes Interventions and Complications (EDIC) study completed in 2005. In this study approximately 1400 patients with T1DM were followed up. Over a period of 17 years, the incidence of CV event as defined by Non-fatal MI, stroke, CVD death, confirmed angina, coronary
artery revascularization, subclinical/silent MI via annual electrocardiograph (ECG) was 0.38 in 100-person years in the intensive arm, versus 0.80 per 100 person-years in the conventional treatment arm (Nathan et al., 2005).

The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) looked at 996 individuals with T1DM over a period of 10 years and examined cardiovascular mortality and links with diabetic nephropathy (Klein et al., 2004). The 20-year age-adjusted cumulative incidences were 18.1% for angina, 14.8% for myocardial infarction, and 5.9% for stroke. Of 273 deaths, 176 involved heart diseases. Interestingly, the authors noted that some microvascular parameters in the diabetic retinopathy data such as arteriovenous ratio was associated with myocardial infarction, but that these associations were confounded by diabetic nephropathy and indeed this was more informative about the cardiovascular end points than were the blood vessel characteristics.

The Epidemiology and Prevention of Diabetes (EURODIAB) study was a prospective complications study examining approximately 2500 individuals with T1DM over a period of 7.4 years. Incident cardiovascular disease as defined by CV death, non-fatal MI, stroke and centrally coded ECG abnormality was 6.5%. The authors also found that pulse pressure, a measure of arterial stiffness, was associated with incident CVD (Schram et al., 2003).

There have been approximately a dozen other retrospective studies looking at the incidence of cardiovascular disease in people with T1DM. The largest to date is examined the United Kingdom General Practitioner Research Database (UKGPRD) in a case-control cohort study in 2006 (Soedamah-Muthu et al., 2006). The authors of this study compared 7 479 people with T1DM and 38 116 without, over a period of follow up over 4 years. Mean and standard deviation for age was 33 +/- 14.5 years, and duration of diabetes was 15 years. The data was described as absolute risk per 1000-person years. The absolute risk acute coronary heart disease (CHD) was 3.5 for men and 2.9 per 1000-person years.
for women, while the risk of coronary revascularization was 2.0 in men and 1.5 per 1000-person years in women. This study is typical of the UK population and is of particular interest for two reasons – one is that the population studied was in the era of statins and good BP control, and it was still showing a signal that people with T1DM were getting CV disease 10-15 years before their counterparts without diabetes.

Despite this high risk, many treatment approaches to the cardiovascular disease associated with T1DM is extrapolated from data from T2DM populations. The underlying pathophysiological mechanism in T1DM vs T2DM has only recently been explored in the literature and there are still many important unresolved issues. Similarities and contrasts are summarized subsequently.

1.2.8 T1DM duration, sub-clinical and early CVD

The Coronary Artery Calcification in T1DM (CACTI) study assessed 656 individuals with T1DM, and 764 control subjects aged 20-55 years. Coronary artery calcification was measured by electron-beam computed tomography (Dabelea et al., 2003). This study found that asymptomatic atherosclerosis was higher in the type 1 population than controls. Endothelial function is impaired even in children with T1DM (Singh, Groehn and Kazmers, 2003; Järvisalo et al., 2004). Adults in the Pittsburgh Epidemiology of Diabetes Complications study (EDC), showed an increased risk of CVD if they had endothelial dysfunction (Costacou et al., 2005).

Rates of cardiovascular disease tend to be lower in pre-menopausal women than in men. However, in the T1DM population, these trends are abolished. The Diabetes UK Cohort followed up 23 000 patients with T1DM and showed the same rates of CVD in women and men aged 40 years with T1DM (Laing et
In the long term follow up of the EDC study, CVD events occur earlier in T1DM population than controls, especially in individuals with duration of diabetes of more than 20 years (Secrest et al., 2010). The quoted rates were around 3% per year, in some individuals in the third decade of life.

The aforementioned CACTI study speculates that after adjustment for insulin resistance, and waist to hip ratio, and waist circumference, the excess atherosclerosis risk in people with T1DM were diminished. Therefore, the conclusion was that the excess atherosclerosis seen in women with T1DM is mediated by insulin resistance and adiposity distribution differences.

There are small angiographic studies examining the patterns of atherosclerosis in individuals with T1DM. These studies show that the T1DM population develop earlier, and up to fourfold atheroma burden and severity than controls. Again, women and men tend to be affected equally, in contrast to the pattern seen in non-T1DM populations (Valsania et al., 1991; Pajunen et al., 2000).

The characteristics of the vessel wall and atheroma in T1DM are different compared to the normal population but may be of a more ‘intermediate severity’ phenotype compared to people with T2DM. A multislice CT coronary angiography of 65 individuals with T1DM and 70 with T2DM showed similar coronary artery calcification scores, but with more severe coronary artery disease in the type 2 population. There was also a relatively lower proportion of non-calcified plaques in the T1DM cohort vs T2DM (Djaberi et al., 2009). Post-mortem studies of epicardial coronary arteries in people with T1DM shows a more fibrous composition of atheromatous disease (Mautner, Lin and Roberts, 1992).
1.2.10 T1DM and hypertension

Similar to albuminuria, hypertension is a strong independent risk factor for cardiovascular disease, and this is well documented. The CACTI study showed that hypertension occurred earlier in individuals with T1DM compared to age- and sex-matched controls (43% in people with diabetes vs 15% in controls, p<0.001) (Dabelea et al., 2003). A post-hoc analysis of the EUORDIAB study showed that hypertension was associated with increasing duration of diabetes, and with severity of complications, especially nephropathy (Collado-Mesa et al., 1999). In an analysis of the DCCT/EDIC cohort, higher glycated haemoglobin was associated with higher risk of hypertension. Interestingly, reduction in HbA1c also reduced the long term risk of hypertension by approximately 24% (de Boer et al., 2008). Further post-hoc studies by Orchard et al in the Pittsburgh EDC study shows that hypertension continued to be a strong predictor of CV risk over time. In contrast, the importance of glycaemic control diminished.

1.2.11 T1DM and dyslipidaemia

The DCCT research study group examined the lipid levels of the study cohort, and observed that individuals with T1DM in their study had similar lipid levels with the general population (DCCT Research Group,1992). As with the general population, increasing weight, worse glucose control, and insulin resistance as measured by the euglycaemic clamp method, were associated with higher lipid values (Maahs et al., 2011). A study of 334 adults with T1DM compared to 800 controls by Perez et al sought to characterise the phenotypes of dyslipidaemia in this individuals with T1DM (Pérez et al.,
In summary, levels of lipids and lipoproteins were similar in the two cohorts, but there was a significant trend of lower high-density lipoprotein, and there were more women with T1DM with hypercholesterolaemia than matched cohorts (15.6 % vs 8%, p = 0.04). Improvements in glycaemic control over 3-6 months were also associated with statistically significant improvements in lipid profile, especially LDL.

As in the general population, dyslipidaemia increases the risk for CV disease. Specific interest has focused on the specific subtypes of hepatic lipoprotein lipase enzymes that may contribute to the specific phenotype of low HDL-C in people with T1DM (Soedamah-Muthu et al., 2003). The ‘Golden Years cohort’, a study characterising long-term survivors of people with T1DM, found that this group had lower BMI, low insulin doses of less than 0.5 units of insulin per kilogram of body weight, and high HDL. In other words, features the reverse of what is typical for IR syndromes. At present there is no conclusive data that necessitates a difference in approach in people with T1DM with dyslipidaemia, versus the general population, but the ‘Golden years cohort’ among others have prompted a discussion around better phenotyping of people with diabetes rather than simply T1DM or T2DM.

1.2.12 ‘Double diabetes’

We have reviewed important large prospective studies of CV outcomes in people with T1DM. Soedamah-Muthu and colleagues modelled predictive risk factors in the EURODIAB, Pittsburgh EDC, FinnDiane and CACTI study. This work showed that age, waist to hip ratio, urinary albumin to creatinine ratio and HDL-C were independent prognostic markers for major CV outcomes (Soedamah-Muthu et al., 2014). Thus, features associated with the metabolic syndrome in people with T1DM predict (perhaps unsurprisingly) worse outcomes over and above glycaemic control. In the scientific
literature, this overlapping of risk factors has been labelled ‘double diabetes’ (Teupe and Bergis, 1991). A large cross-sectional study of people with T1DM in Germany and Austria looked at 31 000 people with ‘double diabetes’, defined as such if participants fulfilled two out of three NCEP criteria for metabolic syndrome. The prevalence of double diabetes in this cohort was approximately 25%; this group were older, had longer duration of diabetes, and had higher insulin requirements despite worse glycaemic control (Merger et al., 2016). Having double diabetes was associated with worse CV outcomes.

Both T1DM and T2DM are associated with decreased suppression of hepatic glucose production, reduced muscle glycogen, and a predisposition to fatty acid oxidation in peripheral mitochondria in favour of carbohydrate oxidation. (Bergman et al., 2012). This has led some experts to argue that T1DM is also associated with a predisposition for ectopic fat deposition (and its attendant risks) (Darabian et al., 2016; Perseghin et al., 2003), but at a lower BMI threshold compared to people with T2DM.

In contrast, people with T1DM have low levels of portal vein insulin due to beta-cell failure, whereas people with T2DM have high levels of portal insulin due to hepatic insulin resistance. Consequently, there is a reduced tendency for hepatic steatosis and improved hepatic lipid profiles and increased HDL. This has led some experts to hypothesise that portal insulinopaenia may mediate some of the cardioprotective effect in T1DM (Savage and Semple, 2010; Regnell et al., 2015). In patients with ‘double diabetes’ mellitus, this situation is reversed and atherothrombotic pathophysiology is putatively accelerated by the combination of chronic hyperglycaemia and abnormal lipids (Cleland, 2012).
Intensive glycaemic control is associated with weight gain and hypoglycaemia, and it has been argued this subset of patients with double diabetes may require a different therapeutic approach based on their phenotype (Cleland, 2012), which is in contrast to the ‘Golden years’ cohort described earlier.

1.2.13 Other Cardiac conditions associated with Diabetes Mellitus

1.2.14 Cardiac Autonomic Neuropathy

Estimating the true prevalence of cardiac autonomic neuropathy (CAN), and hence its impact, has been hampered by the lack of consensus on its definition and measurement. Several measures of autonomic neuropathy exist, including heart rate variability, the expiration to inspiration index, and the composite autonomic severity score. A study by Dimitropoulos and colleagues summarised the incidence of CAN in T1DM and T2DM as 6% and 2% per annum (Dimitropoulos, Tahrani and Stevens, 2014). CAN was examined in both the DCCT/EDIC and EURODIAB cohort, and in both studies were strongly associated with glycaemic control and duration of diabetes, in patients with T1DM (Witte et al., 2005; Pop-Busui et al., 2009). CAN however is more prevalent in T2DM, most likely reflecting the older population group with accumulated traditional CV risk factors (Low et al., 2004; Pfeifer and Schumer, 1994). CAN is associated with long term CVD events in the EURODIAB and DCCT/EDIC cohorts (Witte et al., 2005; Pop-Busui et al., 2017).

In the Detection of Ischaemia in Asymptomatic Diabetics (DIAD) study, 1123 people with T2DM were studied with myocardial perfusion studies, and CAN, as quantified by the Valsalva ratio, was a strong predictor for silent ischaemia (Wackers et al., 2004). What was also a very interesting finding in this
study was that the link between silent ischaemia and CAN appeared independent of traditional risk factors. The underlying mechanism of this link is still to be elucidated.

The DCCT study showed a 50% reduction in CAN in the intensive control arm at 6.5 years, and the benefit was sustained in the EDIC arm (DCCT Research Group 1998; Pop-Busui et al., 2009). In contrast, the effect of intensive glycaemic control in people with T2DM is mixed. Two Scandinavian studies appear to show benefit: firstly, the Steno type 2 study was a small study of 160 patients with T2DM randomised to intensive stepwise multifactorial treatment targeting hyperglycaemia, hypertension and hyperlipidaemia. The RR for progression of autonomic neuropathy was 0.32 (95% CI 0.12–0.78) (Gaede et al., 1999). Secondly, the Anglo Danish-Dutch Study of Intensive Treatment in People with Screen Detected Diabetes in Primary Care (ADDITION) was a pragmatic cluster-randomised parallel group trial of approximately 1500 people with T2DM. This showed no benefit of intensive multifactorial intervention, over a period of 6 years (Charles et al., 2013). The Veteran Affairs Cooperative Study in T2DM (VA-CSDM) was a randomised controlled-trial (RCT) of 153 men in the United States, with a 24-month duration of intensive therapy (HbA1c < 7.3% or 57 mmol/mol), with a separation of 2% average HbA1c in the intensive and standard arm. This study did not show benefit to cardiac autonomic neuropathy outcomes (Azad et al., 1999). Thus, in aggregate, clinical trials to date show better outcomes for CAN with intensive glycaemic control in the T1DM population than T2DM.
1.2.15 Diabetic Cardiomyopathy

‘Diabetic cardiomyopathy’ (DC) was first coined as a term in 1972 describing abnormal myocardial structure and function, in absence of coronary artery disease, hypertension or valvular heart disease (Rubler et al., 1972). Hyperglycaemia, systemic IR and impaired insulin signalling at cardiac myocyte level are risk factors for DC, causing a phenotype of left ventricular hypertrophy and fibrosis and subclinical diastolic dysfunction (Jia, Hill and Sowers, 2018). In T1DM, each 1% or 10mmol/mol increase in glycated haemoglobin is associated with 30% increased risk of heart failure (Lind et al., 2011), whereas in T2DM each 1% is associated with 8% increased risk of heart failure independent of obesity, smoking, CHD and hypertension (Stratton et al., 2000). Hyperglycaemia therefore seems to be a graded risk factor for heart failure even accounting for typical risk factors for CVD. There appears to be differences in the phenotype of DC in people with T1DM compared to T2DM, which has led experts to conclude that the differences are explained by insulin signalling at the myocyte (Abel, O'Shea and Ramasamy, 2012). Furthermore, clinical trial data in T2DM showing decreased heart failure rates in people treated with long acting glucagon-like peptide receptor agonists (Marso et al., 2016a) has opened up great interest in molecular pathways separate from the ‘classical’ hyperglycaemia and CVD mechanisms described earlier. CV effects of T2DM drugs are explored more in detail later in this chapter.
1.2.16 Summary: Contrasts of T1DM versus T2DM and pathophysiology of CV disease

A summary of overlap and contrasts between T1DM and T2DM are detailed in Table 2.

<table>
<thead>
<tr>
<th>Overlap</th>
<th>Reference</th>
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<tr>
<td>When double diabetes defined by NCEP criteria, it is associated with poorer CV outcomes despite higher today daily insulin requirements</td>
<td>(Merger 2016)</td>
</tr>
<tr>
<td>Ectopic fat deposition occurs in T1DM including in epicardial compartment and skeletal muscle, and poorer outcomes possibly at a lower BMI threshold</td>
<td>(Cleland 2012)</td>
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<td></td>
<td>(Bergman 2012)</td>
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<td>(Perseghin 2003)</td>
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<td>(Darabian 2016)</td>
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<tr>
<td><strong>Contrasts</strong></td>
<td></td>
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<tr>
<td>Low levels of insulin in portal circulation reduces hepatic steatosis, with a tendency toward low TGs and high HDL-C</td>
<td>(Savage 2010)</td>
</tr>
<tr>
<td>‘Golden Years’ cohort of survivors of more than 50 years with T1DM exhibit protective lipid profiles the ‘reverse’ of T2DM</td>
<td>(Regnell 2015)</td>
</tr>
<tr>
<td>CAN in DCCT/EDIC and EURODIAB cohort benefitted from intensive glycaemic control.</td>
<td>(Bain 2003)</td>
</tr>
<tr>
<td>Results for Intensive glycaemic control and CAN mixed for T2DM in Steno-2, ADDITION, VA-CSDM</td>
<td>(DCCT Research Group 1998)</td>
</tr>
<tr>
<td></td>
<td>(Pop-Busui 2009)</td>
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<td>(Gaede 1999)</td>
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<td>(Azad 1999)</td>
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<td></td>
<td>(Charles 2013)</td>
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<tr>
<td>Heart failure outcomes shows an increase in a graded fashion for every 10mmol/mol of HbA1c when corrected for classical risk factors.</td>
<td>(Lind 2011)</td>
</tr>
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<td></td>
<td>(Stratton 2000)</td>
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<tr>
<td>Probable differences at cardiac myocyte insulin signalling in people with T2DM vs T1DM and DC, more research needed</td>
<td>(Abel 2012)</td>
</tr>
<tr>
<td>‘Non-classical’ pathways affecting cardiac myocytes in DC with newer agents for treatment of T2DM</td>
<td>(Jia 2016)</td>
</tr>
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<td></td>
<td>(Marso 2016)</td>
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**Table 2: Overlaps and Contrasts of features of T1DM and T2DM and association with cardiovascular outcomes**
CVD in T1DM happens earlier than in non-diabetic populations, and in women with T1DM the sex-protective effect seen in healthy population is lost. Sub-clinical coronary artery disease occurs early and is different in characteristics compared to T2DM cohort: an ‘intermediate’ phenotype more severe than the non-diabetic population but less severe than T2DM counterparts. One of the biggest predictors of adverse events is diabetes kidney disease, which has a multiplier effect related to the common risk factors of hypertension and dyslipidaemia IR. This risk factor is partially ameliorated by treating hyperglycaemia, as evidenced by the long-term outcomes from DCCT/EDIC and Pittsburgh EDC study (discussed in the following chapters). Microvascular damage in the diabetic heart may lead to the myocardial injury, fibrosis, and hypertrophy found in diabetic cardiomyopathy, and hyperglycaemia is a ‘graded’ risk factor, when other classical risk factors are taken into account. However, other novel pathways modulated by newer agents to treat T2DM may also be involved. Cardiac autonomic neuropathy is important in both T1DM and T2DM but appears to be more responsive to intensive glucose therapy in people with T1DM. More research is certainly required in this area, as it appears that the ‘T1DM milieu’, out with classical risk factors, still confers excess cardiovascular risk compared to their age-matched counterparts without diabetes.

Thus, there is overlap between the molecular pathogenesis of cardiovascular disease in T2DM versus T1DM, but there are important contrasts. Further, what is not clear is extent to which blood glucose control in isolation improves cardiovascular outcomes. Long term follow-up data with the DCCT and EDIC study continue to firm up the assertion that tight glycaemic control is highly important in T1DM, but much more contentious in T2DM, although important overlaps between the groups exist.
What is becoming more evident is also the fact that many treatments of diabetes, including insulin and other oral agents, appear to modulate cardiovascular outcomes. Perhaps the most publicised finding in recent times was the observation of a three-fold increase in cardiovascular death in the intensive treatment arm of people with T2DM of the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial in 2008. This has prompted several lines of enquiry, including whether hypoglycaemia itself may be harmful to the cardiovascular system. The results of ACCORD also stand in stark contrast to the newer cardiovascular outcome trials (CVOTs) of type 2 diabetes, which has unexpectedly showed cardiovascular benefit. The next segment examines the cardiovascular outcomes of RCTs in the past 30 years.
1.3.1 Intensive glycaemic control in T1DM

The DCCT remains the landmark randomised clinical trial of the effects of intensive glycaemic control on vascular complications. In the study, 1441 individuals with diabetes aged 13 to 39 years were randomised to near normal glycaemic control (n = 711) versus conventional therapy (n = 730) for a follow up period on average of 6.5 years. It is worthwhile noting that the individuals were young and had a diabetes duration of 1 to 15 years. Furthermore, people with CVD or hypertension as defined by blood pressure of above 140/90 mmHg, or hypercholesterolaemia defined as lipids above 3 standard deviations of the age and gender specific means, were not eligible for recruitment (DCCT/EDIC Research Group, 2016b). There was a primary prevention cohort consisting of people with no retinopathy, and a secondary prevention cohort consisting of people with mild retinopathy. Following completion of DCCT, 1394 of the surviving cohort then joined the EDIC long term follow up cohort beginning 1994. At this point, the participants in the conventional treatment arm were instructed in intensive glycaemic control, and diabetes management was returned to their parent health care team.

Intensive therapy consisted of at least three insulin injections per day or the use of an insulin pump, and blood glucose values were targeted using four self-monitored blood glucose values per day. Pre-meals target was 70 -120 mg/dL (3.9 to 6.7 mmol/L), whilst post meal targets were 180 mg/dL (10.0 mmol/L). The glycated haemoglobin target was 6.05% or 42.6 mmol/mol. There were also monthly visits to a team comprising of a physician, nurse educator and behavioural psychologist. By contrast, the treatment target of the conventional glucose therapy was prevention of symptoms of hypo or hyperglycaemia using one or two injections of insulin per day.
During the DCCT, glycated haemoglobin was measured every 3 months, whilst all cardiovascular parameters described in the following paragraph were measured in a central laboratory. The primary outcome measure of the study was time to first CVD event comprising non-fatal myocardial infarction or stroke, death adjudicated to be due to CVD, subclinical CVD diagnosed via yearly electrocardiogram; angina diagnosed by exercise tolerance test or by clinically significant obstructive lesions on angiography; heart failure, or revascularisation via angioplasty or coronary artery bypass. Secondary outcome measures were microvascular complications of retinopathy, nephropathy and neuropathy (Nathan et al., 1993).

The headline outcomes of this study were a 76% relative risk reduction of retinopathy (95% CI 62-85%), neuropathy by 60% (95% CI 38-74%), and albuminuria to 39% (95% CI 19-74%). However, no cardiovascular outcome benefit was demonstrated, although duration of the study was short. In the EDIC follow up cohort however, follow up has been ongoing for the last 30 years. In the latest published analysis, 149 CVD events occurred in 82 former intensive treatment individuals, versus 217 in 102 individuals in the former conventional treatment group. Intensive glycaemic therapy in individuals with T1DM, with no prior hypertension or cardiovascular risk factors, is associated with a relative risk reduction of 30% of any cardiovascular events (95% CI 7-48, p = 0.016) after 30 years of follow up. Individuals in the prior intensive treatment arm also had a relative risk reduction of major cardiovascular events (non-fatal MI, stroke or CV death) by 32%, (95% CI of 3-56) and statistical significance of p = 0.07. A detailed analysis of the statistical analysis of this study is not included here; it is however noteworthy that the numbers are still small, and the stronger statistical signal seems to be for any cardiovascular events which will include heart failure complications also. The intensive glycaemic control cohort had threefold increase in incidence of severe hypoglycaemia, defined as low blood glucose requiring third party assistance.
At the end of the DCCT, the benefits of intensive glycaemic control in a subset of people with T1DM without hypertension and hypercholestrolaemia had formed the basis for the recommendation for glycaemic targets in people with type 2 diabetes. In the years following the report of the EDIC follow-up cohort, the CV benefits seen after more than ten years of follow up were postulated to be down to ‘metabolic memory’. That is to say, that early intensive control of glucose brought about changes to oxidative stress, advanced glycation end products and possible epigenetic changes, that may protect the vascular tree and produce beneficial effects later on (Chen et al., 2016; Schisano et al., 2011; Russell and Cooper, 2015). We now pivot toward the RCT data for people with T2DM designed in part to answer the clinical question as to whether or not intensive glycaemic control improves cardiovascular outcomes in people with T2DM.

1.3.2 Intensive glucose control in people with T2DM

The impact of intensive glucose control in T2DM remains controversial. The landmark study of intensive control in people with T2DM is the United Kingdom Prospective Diabetes Study (UKPDS). The UKPDS examined 5212 individuals with newly diagnosed T2DM over a period of median follow-up of 10 years. One cohort was randomised to intensive glycaemic control with a median glycated haemoglobin of 7% (53 mmol/mol) using metformin, chlorpropamide, glibenclamide or insulin after stratification for obesity (defined as 120% ideal body weight for the individual). The conventional treatment group had a median HbA1c of 7.9%, and was allocated dietary treatment aiming for a fasting plasma glucose of <15 mmol/L. There was no difference in cardiovascular outcomes or cardiovascular death in this cohort (UKPDS 33, 1998). It is useful to note that the HbA1c difference between the two cohorts were small, whereas there was a 2% difference in HbA1c in the DCCT trial. In the cohort of
individuals defined as obese, metformin use versus conventional dietary therapy was associated with a reduction of myocardial infarction, (relative reduction of 16%, $p= 0.052$). A 10-year follow up of the UKPDS, using questionnaires after the cessation of the trial at 5 years, was reported in 2008. Of the insulin and sulfonylureas group, the reported risk reduction for myocardial infarction was 15% ($p= 0.01$) and death from any cause was 13% ($p=0.007$). In the metformin group, it was 33% ($p=0.005$) and 27% ($p=0.007$) respectively (Holman et al., 2008). This was despite the abolishment of the differences in glycated haemoglobin in the two cohorts after one year of completion of the UKPDS. Once again this favoured the theory of ‘metabolic memory’ and the early effects of glucose control reaping delayed benefits after more than 5 years.

Circa 2008 following the report of the 10-year UKPDS follow up, several other large RCTs examining intensive glycaemic control in T2DM were reported, which significantly challenged the established thinking at the time. The ADVANCE (Action in Diabetes and Vascular disease: PreterAx and Diamicron MR Controlled Evaluation) RCT evaluated 11 140 individuals with T2DM. The intensive glycaemic control cohort was randomised to use gliclazide MR and other drugs to achieve a mean HA1c of 6.5% (48 mmol/mol) versus 7.3% (55 mmol/mol) in the conventional treatment arm. There was no difference in cardiovascular outcome after median follow up of 5 years, although there was a statistically significant reduction in combined endpoints mainly explained by a reduction in nephropathy (Patel et al., 2008). Rates of severe hypoglycaemia were 2.7% in the intensive control group versus 1.5% in the conventional treatment group. In the VADT (Veteran Affairs Diabetes Trial), 1190 veterans with a mean diabetes duration of 11.5 years with sub-optimal control of diabetes were assigned to intensive glucose control versus conventional treatment. Approximately 40% of these individuals had had one cardiovascular event, with a mean age of approximately 60 years. The aim for the intensive arm of the study was a 1.5% reduction in Hba1c, and after a median follow up of 5.6 years, HbA1c in the intensive group was 6.9% and the conventional group was 8.3% (Duckworth et al.,
Hypoglycaemia occurred in 24.1% in the intensive treatment arm, in contrast to 17.6% in the conventional treatment arm.

In the ACCORD clinical trial, 10 251 individuals with T2DM with a mean age of 62 years and median HbA1c of 8.1% (65 mmol/mol) were studied. At baseline, women comprised of 38% of participants, and 35% had had a previous cardiovascular event. The intensive control arm was randomised to HbA1c of < 6.0% (43 mmol/mol), versus 7-7.9% (53-60 mmol/mol) in the standard therapy arm. A finding of a higher mortality in the intensive arm led to a discontinuation of the study after 3.5 years. 257 participants died in the intensive group, versus 203 in the conventional group. Hypoglycaemia requiring assistance and weight gain of more than 10 kg occurred more frequently in the intensive treatment group (p<0.001). It is against this backdrop that intense discussion surrounding the role of hypoglycaemia and cardiovascular disease was generated. Many post-hoc statistical analysis has been subsequently generated from the ACCORD cohort which dispute hypoglycaemia as a causative factor in the deaths that occurred in this RCT (Bonds et al., 2010; Miller et al., 2010; Gerstein et al., 2011). Nonetheless, these post-hoc statistical analysis unfortunately are reliant on reported episodes of hypoglycaemia, and do not study directly cardiovascular parameters which may be affected during acute hypoglycaemia. This clinical question forms the basis of the next section and the original research that attempts to answer it.
1.4 Hypoglycaemia and Cardiovascular Disease

1.4.1 Definition of Hypoglycaemia

Low blood sugar is a common consequence of insulin treatment in people with type 1 diabetes, and in people with T2DM on sulfonylureas or insulin itself. As a phenomenon, hypoglycaemia has been recognised clinically since the advent of insulin therapy in 1922 (Fletcher, 1922), and the ‘Whipple’s triad’ coined in 1938 classified hypoglycaemia as symptoms consistent with low blood sugar, a low plasma glucose concentration, and resolution of symptoms on correction of low blood glucose (Whipple, 1938).

The symptoms of low blood glucose, ranging from hunger, palpitations and tremulousness, to neurological disturbances like blurred vision, light headedness and a change in mood, can be very frightening to people with diabetes and their carers. Hypoglycaemia is frequently cited as a limiting factor to good glycaemic control. However, there is no consensus on the definition of hypoglycaemia. Documenting the symptoms of hypoglycaemia has been proposed, but is limited by the change in symptom profile in some people over time, which can lead to impaired awareness (Pramming et al., 1991). Therefore, a practical definition using biochemical values of 4.0 mmol/L was proposed by a working group of the American Diabetes Association based on a blood glucose value that ‘exposes the individual to potential harm.’ A critique of this value is that this is above the threshold which would provoke autonomic and neuroglycopenic symptoms (described subsequently). Possibly a more clinically relevant value would be 3.5 mmol/L (Frier, 2009); there has however been a preponderance toward defining hypoglycaemia as blood glucose less than 4.0 mmol/L to provoke corrective action earlier and reduce risk of potential harm.
This particular definition of severe hypoglycaemia is more widely accepted and is defined as an episode of low blood sugar requiring third party attention. However, even this definition can be prone to errors in reporting; recall of severe episodes is accurate up to one year after the event (Pedersen-Bjergaard, Pramming and Thorsteinsson, 2003), whereas milder episodes only up to a week subsequently (Pramming et al., 1991). This makes recording hypoglycaemia in clinical trials challenging and hampers any efforts to analyse hypoglycaemia data across different studies. This problem is particularly problematic when dealing with studies in the hospitalised and critically unwell patient cohort. One RCT investigating tight glycaemic control in critically unwell patients have by convention adopted a standard definition of severe hypoglycaemia as a biochemical venous blood glucose level of less than 2.2mmol/L (van den Berghe et al., 2001). This convention was chosen arbitrarily but does have some basis on the provoked physiological responses to hypoglycaemia (explored in the following segments). This level has then facilitated direct comparison of the prevalence and incidence of hypoglycaemia in the critically unwell patient cohorts in several further clinical trials (Finfer et al., 2009; Krinsley et al., 2011).

1.4.2 The frequency and incidence of hypoglycaemia

In T1DM, as described in previous sections, intensive glycaemic control increases the risk of severe hypoglycaemia threefold. The DCCT reported the incidence of severe hypoglycaemia to be 0.62 episodes per patient per year in the intensive treatment group, versus an incidence of 0.19 episodes per patient per year (DCCT Research Group 1991). Prevalence of severe hypoglycaemia in DCCT and EURODIAB studies ranged between 30-40% (Gruden et al., 2012; DCCT Research Group, 1997).
As noted in the previous section, the inclusion criterion for the DCCT meant that the cohort studies had a maximum duration of 15 years and were a young cohort. The cohort did not include people with a history of severe hypoglycaemia. In a 30-year follow up study of the DCCT/EDIC study, rates of severe hypoglycaemia became similar after cessation of the DCCT study (0.37 vs 0.41 episodes per patient per year in intensive and conventional treatment groups respectively). The most powerful predictor of a subsequent episodes of severe hypoglycaemia, was a preceding episode. Insulin pump use were associated with a lower risk (Gubitosi-Klug et al., 2017). As such, the estimates of hypoglycaemia in the DCCT may be an under-representation of the scale in the wider population of people with T1DM.

The United Kingdom Hypoglycaemia Study (UKHGS) was a 9-12-month observational study in six secondary care diabetes centres in the UK (UKHGS Group, 2007). The incidence of severe hypoglycaemia was higher than those reported in the DCCT/EDIC; in people with T1DM less than 5 years duration the incidence was 1.1 episode per person per year, whereas it was 3.2 episodes per person per year in those with diabetes duration longer than 15 years. Prevalence of severe hypoglycaemia in this study was reported as 22% in the cohort of individuals with less than 5 years duration and 46% in individuals with T1DM of more than 15 years duration.

In T2DM, the incidence and prevalence of severe hypoglycaemia, and biochemical ‘mild’ hypoglycaemia, represents a more heterogenous group. A generalisable theme is that the rate of hypoglycaemia is lower overall in people with T2DM versus T1DM, but the number of people affected by hypoglycaemia in T2DM is higher. The population affected is also older and thus require more help from the emergency services. The UKPDS reported an annual prevalence of 1% of severe hypoglycaemia (UKPDS 33, 1998). This is likely to be an underestimate due to the inclusion criteria of this study; in contrast the UKHGS reports 7% annual prevalence of severe hypoglycaemia in those
using oral agents, and 25% in those who have T2DM of longer than 5 years duration. In the population studies in ACCORD and VADT, the rates of severe hypoglycaemia were higher overall than those reported in UKPDS and was significantly higher in the intensive control arm. The ACCORD study reported 16.1% vs 5.1% prevalence of severe hypoglycaemia in the intensive versus conventional arm, and the VADT study reported 21.2% vs 9.9% in the intensive and conventional therapy arms respectively. The ADVANCE trial reported a lower prevalence at 2.7% in the intensive control arm vs 1.5% in the conventional control arm. Again, however, it bears repeating that ADVANCE recruited a cohort with a shorter duration of diabetes. The incidence of severe hypoglycaemia episodes in the UK Hypoglycaemia Group study was 0.1 episode per patient per year in those on oral agents with diabetes of less than 5 years duration, and 0.7 per patient per year in those with type 2 diabetes. Despite the lower incidence, it is estimated that about one third of these episodes require emergency assistance (Donnelly et al., 2005). The estimated cost of severe hypoglycaemia in T2DM to the NHS in the UK is approximately 7.4 million pounds annually (Amiel et al., 2008).
1.4.3 The pathophysiology of hypoglycaemia

Hypoglycaemia provokes profound symphato-adrenal responses in order to preserve glucose supply to vital organs, especially the brain. These physiological counter-regulatory responses produce the symptoms of hypoglycaemia, which can prompt people with diabetes to take corrective action.

The blood glucose thresholds are well described in previous clinical studies. At under 5.0 mmol/L, all endogenous insulin secretion is inhibited. At 3.8 mmol/L, adrenaline and glucagon are secreted, which results glycogenolysis to counter the falling blood glucose. The adrenaline release provokes sympathetic symptoms including tremulousness, anxiety, hunger and diaphoresis (McAulay et al., 2001). This milieu of symptoms tends to be noticed at a level of 2.8 to 3.2 mmol/L. The low blood sugar also begins to have an effect on the brain, and ‘neuroglycopenia’ begins to develop, manifesting in a change in mood and concentration. Neurophysiological changes in the electroencephalogram also occurs at this threshold. Below 2.8 mmol/L, a reduction in the capacity of executing complex tasks, and some visual disturbance occurs. At under 1.5 mmol/L seizures and loss of consciousness can occur (McAulay, Deary and Frier, 2001).

Increasing duration of diabetes is associated with a decreased glucagon (Gerich et al., 1973) and adrenaline (Bolli et al., 1983) secretion and subsequently the sympathetic symptoms and counter-regulatory glycogenolysis. Thus, the opportunity to take corrective action may also diminish. Recurrent low blood glucose is associated with the syndrome termed ‘hypoglycaemia-associated autonomic failure’ (Cryer, 2005). This term describes the phenomenon where antecedent hypoglycaemia decreases effective glucose counter-regulation and hypoglycaemia unawareness,
there causing a vicious cycle of hypoglycaemia. The mechanism by which low blood glucose decreases the threshold for sympathoadrenal activation is not clearly known but hypothesised to be due to altered brain metabolism of glucose in the ventromedial hypothalamus and pre-frontal cortex. The aggregate effect is that antecedent hypoglycaemia decreases the very protective mechanisms against further hypoglycaemia, and can lead to impaired awareness of hypoglycaemia (Frier, 2007).

From the haemodynamic perspective, hypoglycaemia increases heart rate by 20 beats per minutes, with a 10% increase in mean systolic blood pressure and a 20 mm Hg decrease in mean diastolic blood pressure (Hilsted et al., 1984). There is also an increase in ejection fraction by an estimated 50% during insulin-induced acute hypoglycaemia, which continued about 90 minutes following correction of blood glucose (Fisher et al., 1990). In people who do not have diabetes, acute hypoglycaemia has been shown to increase arterial elasticity, due to a decline in arterial wall stiffness. In males with diabetes of more than 15 years duration, there is a reduction in arterial elasticity. Arterial elasticity is an important component of coronary arterial perfusion. During each myocardial contraction, arterial elasticity generates a reflected pressure wave generated during early diastole, hence enhancing coronary artery perfusion. Reduction in arterial elasticity therefore may reduce coronary perfusion (Sommerfield et al., 2007). Thus, there is a putative plausible biological mechanism for increased myocardial workload and oxygen demand during hypoglycaemia.

Due to the profound physiological responses provoked by hypoglycaemia, the concern regarding a link between hypoglycaemia and cardiovascular disease is very clinically significant and is explored in the next section.
1.4.5 Hypoglycaemia and Cardiovascular disease

The concern regarding the link between hypoglycaemia and cardiovascular disease has existed for some time but has gained a lot more traction in recent years. Following the publication of the DCCT and UPKDS studies in the 1990s, intensive glucose control became widely accepted practice. The increased mortality in the intensive control arm re-focused interest in hypoglycaemia as a risk factor for cardiovascular disease. Post hoc analyses of the ACCORD study disputing hypoglycaemia as a direct cause of cardiovascular death (Bonds et al., 2010; Miller et al., 2010) were nonetheless problematic due to the exclusion criteria of people with severe hypoglycaemia from the clinical trial. Out with the specialist diabetes sphere, a clinical trial studying the effects of intensive glucose control in critically unwell patients also highlighted the potential harms of hypoglycaemia on survival. The NICE-SUGAR (Normoglycaemia in Intensive Care Evaluation and Surviving Using Glucose Algorithm Regulation) trial recruited 6104 patients in intensive care units in North America, Europe and New Zealand. The participants were randomised to conventional glucose control (target less than 10mmol/L or 180 mg/dL) and intensive glucose control (4.5 to 6.0 mmol/L, or 80-108 mg/dL). This trial had an unexpected outcome of a higher 90-day mortality and more hypoglycaemic episodes (Finfer et al., 2009). In a subsequent post hoc analysis of the study in 2012, the increased mortality was attributed to moderate to severe hypoglycaemia (defined as blood glucose values between 2.2 to 3.9 mmol/L) (Finfer et al., 2012).

Several population studies in more recent years have reported results which raise the concern regarding risk of hypoglycaemia and all-cause mortality. In 2010, a study by Currie and colleagues examined the United Kingdom General Practice Research Database (Currie et al., 2010). This database examined a cohort of 27,965 individuals with T2DM from the year 1998 to 2008. Cox survival models
were built and adjusted for age, sex, smoking status and comorbidity. There was a finding of a U-shaped association between glycated haemoglobin and all-cause mortality, with the lowest mortality rates in those with a median glycated haemoglobin of 7.5% or 58 mmol/mol. The hazard ratios for all-cause mortality was increased in those treated with insulin versus oral agents (1.49, 95% CI 1.39 to 1.59).

Goto and colleagues produced a systematic review of meta-analysis of observational studies. This study which was published in 2013 specifically looked at severe hypoglycaemia and cardiovascular disease (Goto et al., 2013). There were six studies which included 903, 510 participants. Severe hypoglycaemia was associated with higher risk of cardiovascular disease (relative risk 2.05, 95% CI 1.74-2.42, p<0.001). There was moderate heterogeneity of the studies, which highlights how the definition of hypoglycaemia can impair compound analyses of large studies; nevertheless, the bias analysis suggests that severe co-morbid illness does not explain the residual link between hypoglycaemia and cardiovascular disease.

Khunti et al studied whether there was link between insulin treated individuals with T1DM and T2DM, hypoglycaemia, and cardiovascular and all-cause mortality (Khunti et al., 2015). This was a retrospective cohort database study of primary care patients in England between 1997 to 2007, with a last follow up date in 2010. The study participants included a total of 3260 individuals with T1DM and 10 422 people with T2DM. 573 (18%) of participants with T1DM experienced hypoglycaemia, versus 1423 (18%) of people with T2DM during follow up. The approximate duration of follow-up for both cohorts were approximately 5 years. The mean age in this cohort was 60 years, with a mean body mass index of 29 kg/m² and glycated haemoglobin on average of 8.6% to 8.9% (68-74mmol/mol). In both groups, cardiovascular events rate were approximately three times higher in the subset of the group with previous cardiovascular events, as compared to the subsets of the group with no prior CVD. Cox regression analysis was performed in both groups. In the T1DM cohort, hypoglycaemia was
associated with a twofold increase in all-cause mortality. In the T2DM cohort, hypoglycaemia was associated with a hazard ratio of 1.74 (95% CI 1.39-2.18) in those with no prior CVD, and 2.48 (95% CI 2.21-2.79) in those with previous CVD. The temporal relationship between hypoglycaemia and incident rate of cardiovascular disease and death were also examined. The median time to death in both cohorts ranged from 0.8 years for the T1DM cohort and 1.5 years in the T2DM cohort. The findings of this study reinforce the concerns brought up by ACCORD and NICE SUGAR and bears a big clinical relevance as the cohort studied was representative of the clinic population in the UK.

Plausible mechanisms pertaining to hypoglycaemia provoking myocardial ischaemia, arrhythmias, and thrombosis have been described in case reports and in clinical physiology studies. Anecdotal evidence of a temporal relationship between hypoglycaemia, chest pain and ischaemic changes on electrocardiography is numerous (Graveling, 2010; Strouse, 1932; Strouse and Nesto, 1989; Bansal, and LaBresh, 1983). De Souza et al conducted a small study where simultaneous continuous glucose monitoring was combined with continuous electrocardiography in 19 people with T2DM on insulin therapy (Desouza et al., 2003). They identified 10 episodes of hypoglycaemia associated with chest pain, 6 associated with ischaemic ECG changes. Although small in number, this study is important in showing a potential direct pathophysiological link between hypoglycaemia and ischaemia. Furthermore, Holter ECG monitoring is not a sensitive tool to detect myocardial ischaemia (Nair et al., 2001), and hence this study may be underestimating the effect size.

Hypoglycaemia also provokes changes to the ECG aside from markers of ischaemia. The surge of catecholamines during the counter-regulatory response to hypoglycaemia can lead to hypokalaemia, which affects electrophysiological function and may predispose to arrhythmias (Fisher et al., 1991;
Hypoglycaemia has also been shown to cause cardiac repolarisation prolongation (Robinson et al., 2003; Koivikko et al., 2008), as well as the QT interval. Prolongation of the QT interval is a strong risk factor for arrhythmias (Al-Khatib et al., 2003). The combination of potassium shifts and prolongation of cardiac repolarisation has been suggested as a plausible mechanism to explain sudden cardiac death in people with T1DM, the so-called ‘dead in bed syndrome’ (Tattersall and Gill, 1991; Gill et al., 2009). As explored previously, hypoglycaemia also tends to promote hypoglycaemia. Antecedent hypoglycaemia is a risk factor for attenuated cardiac baroreflex sensitivity (Adler et al., 2009), and this may also plausibly be arrhythmogenic.

Acute experimentally-induced hypoglycaemia has been shown to increase platelet monocyte aggregation (Wright et al., 2010), and coagulation factors such as fibrinogen and factor VIII (Dalsgaard-Nielsen and Hilsted, 1982). Pro-inflammatory cytokines are also released into the circulation (Gogitidze Joy et al., 2010; Dandona and Dhindsa, 2010). These effects potentially could be persistent for days following the initial acute hypoglycaemic episode (Hutton et al., 1979). These proinflammatory and prothrombotic milieu associated with hypoglycaemia is yet another plausible mechanistic factor in provoking cardiovascular disease.

These concerns have led to calls for more research to definitively answer the direct pathophysiological link between hypoglycaemia and cardiovascular risk. Many experts have also urged caution with pursuing intensive glycaemic control, especially in those individuals with T2DM with previous cardiovascular disease. Further, it has highlighted a need for therapeutic agents which can address both glucose control, cardiovascular risk, whilst avoiding weight gain and hypoglycaemia. This is discussed in the next section.
1.5 Cardiovascular effects of anti-diabetes medications

We have explored the concerns surrounding intensive glucose control and the risk of hypoglycaemia to cardiovascular events and all-cause mortality. Aside from risk of hypoglycaemia, there has been concerns raised regarding the cardiovascular safety profiles of some anti-diabetes medications themselves. Perhaps the most commonly referred to scenario was the finding of increased myocardial infarction with the drug rosiglitazone. A drug of the thiazolidinedione (TZD) class, rosiglitazone is an intracellular agonist of PPARγ, and has been shown to improve glycaemic control by increasing insulin sensitivity (Kahn et al., 2006). The TZD class of drugs received widespread attention in the late 1990s and early 2000s as an expansion in the armoury of drugs used to treat diabetes, especially T2DM. However, widespread concern regarding its cardiovascular safety profile culminated in a publication of a meta-analysis by Nissen and colleagues in 2007 (Nissen and Wolski, 2007), which eventually brought about a withdrawal of the use of the drug in the United States and Europe circa 2010. Perhaps a direct fallout of this controversy was also a more robust regulatory requirement of cardiovascular safety clinical trials for new anti-diabetes agents (Food and Drug Administration, 2008). More indirectly, it increased efforts to examine the cardiovascular safety profile of all diabetes treatments, explored here subsequently.
1.5.1 Metformin

Metformin is a drug of the biguanide class thought to improve glycaemic control predominantly by inhibiting gluconeogenesis. It also has an insulin sensitizing effect to the liver, skeletal muscle, adipose tissue and endothelium. The exact molecular mechanism of metformin is incompletely understood but is thought to be mainly exerted by its property of activating 5-Adenine Mono Phosphate-Activated Protein Kinase (AMP-K), an intracellular enzyme that regulates glucose and FFA uptake. Metformin is also thought to effect the gut microbiome, the effect of which is still a topic of great interest and research (Rena and Sakamoto, 2013). Metformin is the first line drug of choice for people diagnosed with T2DM, as recommended by the American Diabetes Association (ADA) and European Association of the Study of Diabetes (EASD) (Bennett et al., 2011; Burant CF, 2012). Metformin is weight neutral and comes with low hypoglycaemia risk (Bennett et al., 2011). Metformin reduces triglyceride levels, which is hypothesised to be related to its effect on hepatic lipoprotein secretion (Sirtori et al., 1977).

In the UKPDS study, the cohort of overweight patients on metformin had the biggest reduction of cardiovascular events (UKPDS 34, 1998). In observational studies of older individuals with T2DM in the US, metformin is associated with reduced hospitalisation for congestive heart failure and improved cardiovascular mortality (Masoudi et al., 2005; Roussel et al., 2010). A recently conducted meta-analysis included 13 RCTs looking at metformin (Griffin and Irving, 2017). The studies included had low heterogeneity but was heavily weighted towards the UKPDS. Metformin was associated with improvement of cardiovascular outcomes apart from stroke; however, did this not achieve statistical significance. Overall, metformin, when tolerated, is a safe drug that may exert a modest cardiovascular benefit. Factorial RCTs involving metformin, or CVOTs in people who do not have type 2 diabetes, is required to definitively answer this question.
1.5.2 Sulfonylureas

Sulfonylureas are a class of drug that work by stimulating pancreatic beta cells to produce insulin. The sulfonylurea class binds to a closes the potassium-sensitive adenine triphosphatase (ATP) receptor on the pancreatic beta cells, causing a cascade of intracellular events resulting in the secretion of insulin from the pancreatic beta cells (Proks et al., 2002). Sulfonylureas therefore, in cases of insulin to carbohydrate intake mismatch, cause hypoglycaemia. It is also associated with weight gain (Hermansen and Mortensen, 2007). The cardiovascular safety profile of sulfonylureas is predominantly neutral but some concern has been raised especially with first generation of sulfonylureas, and in elderly patients with T2DM with previous history of CVD. The University Group Diabetes Programme (UGDP) showed that tolbutamide caused excess cardiac deaths compared to insulin and placebo (UGDP, 1975). However, these concerns were not confirmed in the UKPDS (UKPDS 33, 1998), in which chlorpropamide, glyburide and glipizide were used, and similarly in ADVANCE where gliclazide modified release was used (Patel et al., 2008).

The concern for the cardiovascular effects of the sulfonylureas class come not only with the previously mentioned hypoglycaemia risk, but also potentially due to direct effects on the potassium sensitive ATP channels on ischaemic preconditioning of cardiac myocytes. However, this is not a ubiquitous property of the sulfonylurea drug class, happening in tolbutamide and glyburide but not in gliclazide or glipizide (Riddle, 2003). Meta-analyses of RCTs examining sulfonylureas and cardiovascular outcomes have been published, with conflicting results (Rados et al., 2016; Pladevall et al., 2016). 40 years after the UGDP, sulfonylureas are still being used due to its efficacy in improving HbA1c and its low cost.
Azoulay and colleagues reviewed 19 large observational studies on sulfonylureas and cardiovascular outcomes with conflicting outcomes. They analysed the comparators, the outcomes, and potential study-design related biases. They then conducted a meta-regression of analysis to evaluate heterogeneity (Azoulay and Suissa, 2017). Of these studies, sulfonylureas increased hazard ratios when metformin was the comparator and death was the outcome examined.
1.5.3 Thiazolidinediones

We have already reviewed the effect of one drug in the TZD class, rosiglitazone. It is however important to highlight pioglitazone, particularly because the cardiovascular outcomes compare favourably to rosiglitazone. This contrast in cardiovascular outcomes have been speculated in the scientific literature to be due to the differences in action at a molecular basis, and examining these differences may be instructive and provide vital clues for future research. Pioglitazone is a PPARγ agonist, and to a lesser extent a PPARα agonist. It is associated with weight gain and fluid retention, but no increase in hypoglycaemia risk unless used in combination with an insulin secretagogue (Smith, 2001). In the PROACTIV study (PRospective PiOglitAzone Clinical Trial in macroVascular events), treatment did not produce significant reduction in cardiovascular outcomes, although the investigators noted a trend toward reduction of coronary and peripheral vascular disease events (Dormandy et al., 2005). A meta-analysis of 19 RCT in 2017 showed that pioglitazone was associated with lower risk of composite outcomes of death, myocardial infarction and stroke (Hazard ratio 0.82, 95% CI 0.72 to 0.94, p = 0.05). However, there is an increase in heart failure (HR 1.41, 95% CI 1.14 – 1.76, p = 0.002) presumably related to fluid retention (Lincoff et al., 2007). This finding certainly gives pause to many physicians in recommending the use of pioglitazone for individuals with previous cardiovascular disease, as its modest cardiovascular benefits may come at a price of congestive heart failure.
1.5.4 Glucagon- Like Peptide 1 Receptor Agonists (GLP1-RA)

GLP-1 is secreted by L cells in the small intestine and has a paracrine insulinotropic effect (Drucker and Nauck, 2006). It also slows down gut transit, lowers circulating lipoproteins, and lowers blood pressure. GLP-1 receptor agonists, since entering the market circa 2004, have garnered massive attention due to its anti-hyperglycaemic and weight reducing potential. The GLP1-RA class was also subject to the FDA stipulation of non-inferiority in cardiovascular outcomes noted previously. The first large trial to be reported was the ELIXA (Evaluation of Lisexenatide in Acute Coronary Syndrome) (Pfeffer et al., 2015). This trial studied individuals with T2DM within 180 days of acute coronary syndrome. 6068 people were recruited and randomised to once daily lisexenatide versus standard care. Glycaemic control in the protocol aimed for a similar target in both groups. ELIXA was a neutral study, showing no significant difference between the study group for the primary composite end point of CV death, MI, stroke, or hospitalisation for unstable angina. It is worthwhile pausing to note that in this study the participants were started on GLP1-RA within 180 days of an acute coronary event, with median follow up of 25 months in each study group.

In 2016, the LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results) trial reported its results. A total of 9 340 participants were enrolled, and they had a mean age of 64 years with one cardiovascular risk factor. An acute coronary syndrome or cerebrovascular accident within 14 days was an exclusion criterion. The mean follow-up time was 3.8 years. The cohorts were randomised to liraglutide vs placebo and standard glycaemic control. The primary outcome, a composite of CV death, nonfatal myocardial infarction (MI) or stroke, showed benefit for liraglutide over placebo, was 13.0% vs. 14.9%, (HR 0.88 95%CI 0.78-0.97, p < 0.001 for noninferiority; p = 0.01 for superiority). There was no increase in chronic heart failure hospitalisations, and in the
liraglutide cohort the absolute number for hospitalisations were lower, although this did not reach statistical significance. Also of note is that the liraglutide arm used less insulin and insulin secretagogues to achieve a similar glycated haemoglobin (Marso et al., 2016b).

Evidence for cardiovascular benefit also is demonstrated in the SUSTAIN-6 (Trial to Evaluate Cardiovascular and Other Long-Term Outcomes with Semaglutide in Type 2 Diabetes). This RCT evaluated semaglutide, which is not yet approved by regulatory authorities. This trial is once again designed as a non-inferiority cardiovascular safety study, examining 3297 patients with T2DM with high cardiovascular risk. The follow up was over 108 weeks. There was no difference in cardiovascular deaths, but an improvement in primary outcome in the semaglutide group versus placebo was seen, driven by a reduction in MI (HR 0.74 95% CI 0.51-1.08) and stroke (HR 0.61, 95% CI 0.38-0.99) (Marso et al., 2016a).

### 1.5.5 Sodium Glucose Co-Transporter 2 Inhibitors or Gliflozins

SGLT-2 inhibitors are a class of drugs which promote glycosuria and weight loss by inhibiting sodium glucose transporter 2 in the proximal convoluted tubule of the kidney (Shubrook, Bokaie and Adkins, 2015). The three available drugs in this class currently are empagliflozin, dapagliflozin and canagliflozin. Dapagliflozin is the first drug in this class to market. However, the drug that has attracted the most attention is empagliflozin, due to the striking outcomes of the EMPA-REG OUTCOME trial (Zinman et al., 2015). The trial was designed to show non-inferiority to placebo. 7020 participants with
high cardiovascular risk were randomised to empagliflozin versus placebo. After a median follow up time of 3.1 years, there was a 38% relative risk reduction of death from any cardiovascular causes, and 35% risk reduction for hospitalisation for heart failure. There were no differences in hypoglycaemia event rates in the study cohorts.

The CANVAS (Canagliflozin cardiovascular Assessment study) was another RCT designed for non-inferiority in cardiovascular outcome. The RCT recruited 10 142 participants and compared canagliflozin versus standard care plus placebo. The mean age of the participants was 63.3 years, and 65% had a history of cardiovascular disease. The primary outcome was a composite of death from cardiovascular causes, non-fatal MI and non-fatal stroke. After mean follow up of 3.6 years, treatment with canagliflozin was associated with a reduction in the rates primary outcome in the canagliflozin group versus placebo (HR 0.86, 95% CI 0.75 to 0.97)
1.5.6 Lessons from recent CVOTs: Avoid weight gain and hypoglycaemia?

The focus of treatment in diabetes mellitus has in the past been intensive glycaemic control, which compared to addressing the traditional risk factors of blood pressure and cholesterol, have been calculated to confer a modest benefit in T2DM (Ray et al., 2009). Until recently, aside from metformin, cardiology colleagues would have been more attuned to the possible deleterious cardiovascular side effects of diabetes drugs, encapsulated by the example of rosiglitazone (Nissen and Wolski, 2007). The results of particularly the EMPA-REG (Zinman et al., 2015) and LEADER (Marso et al., 2016b) programme of RCTs have prompted a reassessment of diabetes care beyond glycaemic control and treatment of traditional risk factors.

Both RCT programmes aimed for conventional glucose targets of approximately 53-58 mmol/mol, and had glycaemic equipoise in the control arm of the studies. Another common theme is that the recent CVOTs had significant weight loss in the treatment cohorts, and low levels of hypoglycaemia. In this regard, the more recent RCTs are similar to the obese subset of the metformin treatment arm of UKPDS, in which a clearer signal toward cardiovascular benefit was seen (UKPDS 34, 1998). Additionally, these findings are in sharp contrast to the intensive treatment arm of ACCORD especially, where there was a weight gain of 3-7 kilograms (Fonseca et al., 2013), and the rates of severe hypoglycaemia were approximately three times that of the standard arm (Miller et al., 2014).

These observations make a compelling case for clinicians recommending glycaemic control strategies that avoid weight gain and hypoglycaemia. Further, it raises interesting research questions on how weight loss mediates improves cardiovascular outcomes, and whether avoidance of hypoglycaemia is a necessary and important part of that paradigm. We have already explored the numerous plausible mechanisms whereby hypoglycaemia may mediate cardiovascular harm. However, scarce data exist
regarding the direct effects of acute hypoglycaemia to the coronary circulation, and whether acute hypoglycaemia can cause small amounts of myocardial injury. Answering this particular research question forms one part of the research study described in this thesis.

As to the avoidance of weight gain aspect, there remains numerous areas of uncertainty and debate. Obesity is associated with excess ectopic fat, adipocyte dysfunction, inflammation, and insulin resistance (de Ferranti and Mozaffarian, 2008). In people with T1DM, raised BMI is associated with increased mortality (Edqvist et al., 2019). However, in the large look-AHEAD (Action for Health in Diabetes) RCT, weight loss of 5-10% body weight via dietary and lifestyle intervention alone in people with T2DM does not appear to improve cardiovascular outcome (Wing et al., 2013). This was despite significant reduction in classical cardiovascular risk parameters. There does appear, therefore, other mechanisms at play beyond reduction of blood pressure and lipids. It also appears that method of weight loss may be crucial.

In the attempt to explain how some methods of weight loss can mediate beneficial CV outcomes, the regulation of energy balance has been an area of interest. For example, the SGLT-2 class of drugs are estimated to cause a negative calorie balance of approximately 200 kilocalories per day through promoting glycosuria. However, the caloric deficit predicted causes less than expected actual weight loss seen in people taking SGLT-2 inhibitors (Ferrannini et al., 2015). This discrepancy suggests compensatory mechanisms and possible wider interactions in the regulation of total body energy. In this context, there has been a large body of work describing the molecular mechanisms that regulate energy homeostasis (Spiegelman and Flier, 2001). Many of the molecular pathways regulating energy intake, such as Adenine Monophosphate activated Protein Kinase (AMP-K), and energy expenditure, such as the uncoupling proteins (UCP2), share common upstream nuclear regulators. This area opens up a new avenue of drug targets, and have particularly been studied in the field of aging and caloric
restriction. The study of potential drug targets in this area forms another part of the original research in this thesis. We describe this subsequently.

1.6 Sirtuins, Aging, and the cardiovascular system

Aging is associated with increasing prevalence of hypertension, atherosclerosis, myocardial infarction and stroke. However, this relationship is not linear over time. For example, there are people who are prone to atherosclerosis in a relatively early chronological age group. Therefore, there are factors inherent within aging which may amplify and influence the expression of cardiovascular risk in certain individuals. Over time, aging cardiovascular tissue show an increase in left ventricular hypertrophy, diastolic function, endothelial function and arterial stiffness (Lakatta and Levy, 2003). These factors interact with each other and cause myocardial fibroblast proliferation, increasing myocardial hypertrophy and fibrosis (Lakatta, 2003). Heart rate variability and maximum heart rate also decrease with increasing age (Antelmi et al., 2004), thought to be due to sinoatrial node cell loss and senescence, as well as less efficient propagation of electrical conduction due to fibrosis. Cardiac myocyte apoptosis becomes more dysregulated with aging, causing impaired turnover, and an increase in myocyte size, causing hypertrophy (Goldspink and Tan, 2003).

Further, there is a decrease in repopulation of cardiomyocytes from cardiac stem cell reserves. Although cardiac myocytes are post-mitotic, they undergo division and regeneration. Work by Anversa and colleagues introduced the concept of cardiac stem cells which are distinct from haematopoietic stem cells, and that there exists a reserve in the myocardium. Cardiac myocyte regeneration is thought to be part of maintenance of myocardial health (Anversa et al., 2006). Aging increases cardiac cell senescence, which is defined as a decrease in the telomere length and increase in DNA markers of damage (Chimenti et al., 2003). This increase in senescence results in the reduced resilience of cardiac
myocytes to reactive oxygen species and inflammation, and can lead to increased vulnerability to ischaemia and heart failure (Camici et al., 2007; Chimenti et al., 2003).

With aging, the vascular tree exhibits infiltration and fibrosis of the smooth muscles, which causes luminal enlargement and stiffness. The endothelial cells themselves exhibit senescence, characterised by reduction in the ability to proliferate after injury, and a reduction in production of nitric oxide synthase (Brandes and Busse, 2005). The endothelial barriers become more porous, resulting in deposition of extracellular matrix in the sub-endothelial space, and causing intima media thickening (Vasa et al., 2000). This process of course bears a lot of resemblance to common pathways of vascular damage provoked by hyperglycaemia and hypertension. Senescent endothelial vascular cells appear to have diminished eNOS response to haemodynamic shear stress (Kang, and Muller-Delp, 2009), which in turn serves to protect against the effect of vascular aging. Therefore, endothelial cell senescence seems to promote a vicious cycle that accelerates endothelial dysfunction.

1.6.1 Caloric Restriction and Longevity

In the past, there has been a prevailing view that modern diets are harmful due to the rise in lipids accelerating atherosclerosis, and increasing inflammatory pathways and ROS, well described previously. However, another paradigm from the field of study of aging also exists, and that is the view that caloric restriction (CR) may promote the molecular and genetic mechanisms which protect against aging. One of the earliest examples of experiments in caloric restriction was by McCay in 1935. Rats subjected to a caloric intake of 20-40% of normal intake were smaller, but lived longer (McCay, 1935). In the past, the CR models were hypothesised as simply a passive slowing down of metabolic
processes, leading to reduced oxidative stress and prolonged lifespan. More recently, animal models of CR from yeast, worms, flies and some mammalian models have identified molecular and genetic pathways which suggest that protection against the effect of aging is a more active process, evolved to defend the organism during times of adversity (Weiss and Fontana, 2011).

Data from human studies of Okinawans, who eat a moderately restricted diet of 1785 kcal per day, show that they have lower rates of incidence of CVD. It is also observed that there is a higher prevalence of centenarians in this population group (Willcox et al., 2006). Small studies involving humans on approximately 6 months to 8 years of moderate CR have shown benefits to fat cell mass, insulin resistance and surrogate markers of atherosclerosis, which are consistent with the Okinawan Centenarian Study (Fontana et al., 2004; Larson-Meyer et al., 2006). Many of the molecular pathways which mediate the protective effects of caloric restriction are known. CR reduces inflammation by reducing the activity of vascular adhesion molecules, inflammatory cytokines and prostanoids in humans (Weiss et al., 2006). CR enhances endothelial function and attenuates progression of atherosclerosis and arterial stiffness in rodent models (Ahmet et al., 2005). Caloric restriction also increases mitochondrial function in the vascular tree, and simultaneously reduces oxidative inflammation (Nguyen and Pickett, 2009).

As aging underpins many of the processes that lead to cardiovascular risk factors, and molecular pathways that modulate aging are more understood, there has been a lot of research interest in potential therapeutic targets in this area. This class of drugs are termed ‘caloric restriction mimetics’, and influence the genetic and molecular signalling pathways summarised in figure 2. These molecular signalling pathways overlap and are interlinked. We pay particular attention to the sirtuin class in the next segment.
**FIGURE 2: HOW CALORIC RESTRICTION MEDIATES LONGEVITY**

Through the sirtuins, insulin and insulin like growth factor 1 pathway, target of rapamycin (TOR), liver kinase B1 (LKB1), and Adenine Monophosphate activated Protein Kinase (AMP-K), these pathways affect each other to modulate increased autophagy, genome resilience, mitochondrial generation and stress resistance to counter against cardiovascular and metabolic diseases.
1.6.2 Sirtuins, and interacting pathways

Work by Guarente and colleagues in researching longevity in the yeast species *Saccharomyces Cerevisiae* have focussed on a class of enzymatic proteins called sirtuins. The name sirtuins is derived from the yeast gene SIR2, which stands for ‘Silent mating-type Information Regulation 2’. This gene underpins many biological processes in yeast cells, including modulating aging by decreasing toxic rDNA circles (Sinclair and Guarente, 1997).

Over expression of SIR2 orthologs in the nematode *C. elegans* and in the *Drosophila* fly species also result in increasing lifespan (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). This highlighted the SIR2 gene as an important regulator of lifespan and is highly preserved across species. One of the human orthologs for SIR2, SIRT1, is noted to have a Nicotinamide Adenine Dinucleotide (NAD)+-dependent protein deacetylase activity (Landry *et al.*, 2000). This suggests a link between metabolism and lifespan, which is dictated by diet. It appears therefore that the sirtuins have an energy sensing effect that links metabolism and the downstream molecular signalling that protects against aging.

SIRT1, which is localised primarily in the nucleus, modulates AMP-K through deacetylation of liver kinase B1 (Lan *et al.*, 2008). Modulation of AMP-K has important potentially beneficial effects on metabolism, including inhibition of gluconeogenesis, increasing skeletal muscle uptake of glucose. It also increases cell autophagy directly and indirectly, as well as increasing mitochondrial genesis by regulating PPARG coactivator 1-alpha (PGC-1α) (Jeon, 2016). We have already seen that metformin
exerts some of its anti-hyperglycaemic effect through modulating AMP-K, and it appears that SIRT1 activation have overlap and similarities in the pathways. SIRT1, apart from having an indirect effect on AMPK and PGC-1α, also directly affects PGC-1α activity by increasing deacetylation (Rodgers et al., 2005). Increasing the activity of PGC-1α therefore might be predicted to increase insulin sensitivity.

As shown in Figure 2, SIRT1 affects the insulin and IGF-1 pathway. This effect is potentially via an increase in insulin secretion in the pancreatic beta-cells, by repressing the signalling of UCP2 (uncoupling protein 2) (Bordone et al., 2006). This is shown only in murine models currently. It is worthwhile noting that we might expect an insulin sensitising and therefore reduction in insulin secretion in the case of SIRT1 regulation of AMP and PGC-1α, whereas its effect on UCP2 would increase insulin secretion. The significance of this is as yet unclear.

Another target of SIRT1 is NF-κB, which as we have seen from previous sections regulates many genes involved in the inflammation cascade, which in turns impacts insulin resistance and endothelial dysfunction (Bordone and Guarente, 2005). Murine models have shown that SIRT1 knockout mice have increased susceptibility to ischaemic reperfusion injury, and the converse is shown in SIRT1 transgenic mice (Hsu et al., 2010). SIRT1, once again in murine models, has been observed to regulate endothelial angiogenesis, by acting as a negative modulator of the protein synthesis pathway ‘notch signalling’ (Siraj et al., 2015). SIRT1 could potentially also regulate arterial stiffness, via hyperphosphataemia-induced arterial calcification. SIRT1 activation in rat models reduced vascular smooth muscle calcification (Takemura et al., 2011). Over-expression of SIRT1 appears to protect against vascular smooth muscle hypertrophy mediated by angiotensin 2 (Takemura et al., 2011). However, massive over-expression of SIRT1 appears to cause reduce mitochondrial biogenesis in mice,
thereby increasing oxidative stress and apoptosis or cardiac myocytes (Kawashima et al., 2011). There appears therefore to be a dose dependent relationship between SIRT1 activation and its beneficial effects, which will be discussed later.

1.6.3 Mimicking the beneficial effects of CR: activation of sirtuins pathway by SRT2104.

Activation of SIRT1 therefore has a therapeutic potential to treat diseases associated with aging, including diabetes mellitus and cardiovascular diseases via modulating inflammation, glucose homeostasis, fatty acid metabolism and mitochondrial dysfunction. Over the last decades there has been numerous attempts to find a suitable way to activate the SIRT1 pathway. The most well described molecule in the literature that attempts this is resveratrol (Smoliga and Hausenblas, 2011), a small plant polyphenol found in red wine. The main limitation in resveratrol achieving its therapeutic potential has been a lack of bioavailability after first-pass metabolism in the liver, as well as its lack of specificity to SIRT1 and its lack of potency.

Nonetheless, unlike other drug development strategies focussing on kinases, membrane receptors and selective ion channels, SIRT1 activation retains its attraction due to the many putative beneficial upstream effects described earlier. Further research by many research groups then explored non-resveratrol compounds for the potential to activate SIRT1. High throughput screening is an automated method for drug discovery which tests a large number of compounds of a particular biological target (Broach and Thorner, 1996). Through this process, SRT2104 is one of the first synthetic SIRT1
activator compound that has more specific and potent direct activation of SIRT1 compared to resveratrol (Milne et al., 2007). SRT2104 is a molecule created and owned by Sirtris pharmaceuticals and GlaxoSmithKline (GSK). SRT2104 activates the deacetylase activity of SIRT1 to a maximum of 2-fold in vitro (Libri et al., 2012). The pre-clinical safety of SRT2104 has been investigated in the bacterial reverse mutation assay (AMES test), mouse lymphoma and mouse micronucleus genetic toxicology models, and in safety pharmacology studies in rats and dogs. SRT2104 was not genotoxic and was not associated with adverse central nervous system, cardiovascular system, or pulmonary effects in these preclinical safety and pharmacology studies. In vitro studies in human liver microsomes and cultured hepatocytes suggest that SRT2104 does not inhibit CYP1A, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, or significantly induce cytochrome P450 isoforms CYP1A and CYP3A4.

The effect of once daily oral administration of SRT2104 on fasting blood glucose and fed insulin levels, body weight, triglyceride, and plasma lipid levels were evaluated in a number of animal models of diabetes and obesity (DIO mice and ob/ob mice). SRT2104 lowered fasting blood glucose and fed insulin and enhanced the response to a glucose tolerance test (Libri et al., 2012).

SRT2104 was well tolerated and safe in young healthy volunteers for up to 7 days dosing of 0.03–3.0 g/day (Hoffmann et al., 2013). The elimination half-life (t1/2) was similar for doses up to 3.0 g/day, although increases in exposure were less than dose proportional at doses greater than 1.0 g/day.

No clinical studies have looked at the effects of SRT2104 in people with T2DM, and in another model of aging, that of young otherwise healthy smokers. Our group in the Centre for Cardiovascular Sciences at the University of Edinburgh have a track record of studies pertaining to surrogate markers of cardiovascular disease, including endothelial dysfunction, arterial stiffness and thrombosis. A collaboration between Sirtris pharmaceutical and our research group was set up in order to explore the safety, tolerability, and pharmacokinetics of SRT2104 in doses above 1.0 g/day, building on
previous data of first in man studies in other research groups. This led to the design of a short Phase I RCT.

Additionally, our group took the opportunity collect data on surrogate markers of cardiovascular outcomes, and cardiometabolic markers on this cohort of volunteers. This is to explore the putative benefits of SRT2104 in the aforementioned cohorts.

1.7 Aims of the Thesis

Insulin resistance, hypertension and dyslipidaemia interact in a complex way to produce endothelial dysfunction, and a pro-inflammatory and pro-thrombotic milieu in the vascular tree. Newer anti-diabetes agents like GLP-1RA and SGLT2 inhibitors may exert beneficial CV effects independent of glycaemic control, and in novel ways perhaps related to weight loss. The beneficial effects of the more recent CVOTs has renewed interest in sub-cellular signalling pathways underpinning caloric restriction, fatty acid and glucose metabolism, mitochondrial dysfunction and cardiovascular disease. SIRT1 is a NAD-dependent deacetylase which appears to be an important regulator of the aforementioned molecular pathways regulating metabolic disorders including diabetes mellitus and vascular disease. By mimicking caloric restriction, novel modulators of SIRT1 in humans therefore have the potential to treat both diabetes mellitus and cardiovascular diseases, which would be a welcome addition to the range of available treatments.

Whilst there are similarities in risk factors between T1DM and T2DM in multiplying risk of CVD, one of the contrasts is the effect of intensive glycaemic control. Hypoglycaemia, which is associated with
tight glucose control especially with insulin therapy, may exert important cardiovascular effects. Another common theme of the recent CVOTs is the low rate of hypoglycaemia, in contrast with ACCORD especially. This has raised speculation that avoidance of hypoglycaemia could contribute to CV benefit not observed in earlier clinical trials advocating tight glycaemic control. To date, there has been scarce evidence of low blood sugar itself causing direct harm to coronary microvasculature and to the myocardium. It has been difficult to study the coronary blood vessels non-invasively, and there is also the ethical concern of putting people with T2DM and known cardiovascular risk factors through experimental hypoglycaemia. In our research group, we have had a good track record of safely inducing experimental hypoglycaemia in young people with T1DM. Individuals with T1DM without any other cardiovascular risk factors can safely be put through experimental hypoglycaemia, and could represent a pre-pathological model of established cardiovascular disease, especially compared with their peers without T1DM. Collaboration with our cardiology colleagues have also highlighted novel methods to investigate the coronary vasculature non-invasively, and to use novel sensitive markers of myocardial injury not otherwise expected in younger people.

Summary

The research programme of this thesis therefore broadly investigates the cardiovascular effects of treatments for diabetes mellitus, which are highly clinically relevant. More specifically, our hypothesis is that mimicking caloric restriction and weight loss might benefit glycaemic control and cardiovascular outcomes. We also aim to investigate the possible adverse cardiovascular effects of hypoglycaemia directly in a people with and without T1DM. Our hypothesis is that avoidance of hypoglycaemia may also mediate potential benefit in cardiovascular outcomes.
This research programme to test the aforementioned hypotheses is mentioned below, with a brief description of the study designs and relevant chapters:

1) Is SRT2104 safe and tolerable in people with T2DM? How does SRT2104 affect markers of glycaemic control, lipids and surrogate markers of endothelial function and thrombosis in people with T2DM? We study this in a short Phase Ia RCT with crossover design (Chapter 3)

2) Is SRT2104 safe and tolerable in otherwise healthy smokers? Does SRT2104 have beneficial effects on cardiometabolic markers in this cohort? Separate cohort studies in Phase 1a RCT (Chapter 4)

3) How does SRT2104 affect arterial stiffness in people with T2DM and otherwise healthy smokers? We combine the two cohort models of aging in a post hoc analysis (Chapter 5)

4) Does hypoglycaemia, a common side-effect of insulin treatment, cause direct effects on myocardial perfusion in people with T1DM and people without T1DM? We study this in a prospective randomised open-end point study using non-invasive imaging of the coronary arteries, and novel sensitive biomarkers of myocardial injury (Chapter 6)?

The methods in answering the above questions in the original research studies are explored subsequently.
Chapter 2: Methods
2.1 Study Participants

Three original research studies are described in this body of work. Participants of all three studies were recruited by various methods. For people with T1DM and T2DM the methods were as follows:

- Direct approach in diabetes outpatient clinics in the Royal Infirmary of Edinburgh and the Western General Hospital for people with T1DM and T2DM
- Database search of the Scottish Diabetes Research Network at the Wellcome Clinical Research Facility at the Western General Hospital
- Radio advertisements on local radio stations.

Participants of the studies who were non-diabetics were recruited as follows:

- Poster recruitment around the University of Edinburgh Medical School, the Royal Infirmary of Edinburgh, and the Western General Hospital of Edinburgh.
- A database of pre-existing study participants available from the Department of Cardiovascular Sciences, University of Edinburgh
- Radio advertisements on local radio stations.

The first study recruited 15 individuals with T2DM aged between 18-70 years, with a HbA1c less than 9% (75 mmol/mol), a blood pressure of < 160/90 mm Hg. Exclusion criteria included current smokers, being on an ACE-inhibitor, the presence of major comorbidities, chronic illness, renal or liver impairment, history of gastrointestinal diseases or surgeries influencing drug absorption,
history of alcoholism, history of neoplastic disease within the last 5 years, a positive urinary test for recreational drugs, pregnancy, and participation in other clinical trials or blood donation within the last 3 months.

In addition to the 15 participants of the first study, a further 24 participants who were smokers who were otherwise fit and well were recruited. The inclusion criteria included age between 18-70 years of age, and participants had to be current smokers. The exclusion criteria were similar to the above. Study 2 was a phase 1a randomised cross-over clinical trial examining forearm vasodilatation studies and platelet monocyte studies, as well as lipids in otherwise healthy smokers. Study 3 combined the volunteers with T2DM cohort and the otherwise healthy smokers’ cohort. In all the above studies, participants who were females of childbearing potential had to undergo pregnancy tests at the initial screening visit and during the study visits, to ensure there was no exposure of the study drug to any potential unborn foetus.

Study 3 recruited 17 individuals with T1DM and 10 volunteers without T1DM. The inclusion criteria were: individuals with T1DM aged between 18-45 years with a HbA1c of 48 to 75 mmol/mol (6.5 to 9%) in the past 6 months prior to the study. Only male subjects are eligible to avoid the confounding effect of the documented variability of coronary flow reserve during the menstrual cycle (described later in the chapter). Exclusion criteria were as follows: co-existent systemic disease or malignancy, any history of cardiac conduction abnormality, impaired awareness of hypoglycaemia, past history of severe reaction to hypoglycaemia, past history of cerebral injury, seizure, chronic alcoholism or psychiatric disorder and any evidence of microvascular complications. The subjects without T1DM have the same exclusion criteria.
All study participants were recruited with written informed consent according to the principles of the Declaration of Helsinki.

2.2 Forearm Vasodilation Studies using Venous Occlusion Plethysmography

Venous occlusion plethysmography has been used extensively to study human vascular physiology in vivo, and is a well-validated surrogate marker of endothelial function when combined with intra-arterial administration of vasodilators, usually into the forearm vascular bed via the brachial artery (Wilkinson and Webb, 2001). This method is accurate and reproducible (Roberts and Breckenridge, 1986), is minimally invasive, and had the advantage of being a tried and tested method in our research group, with many colleagues having experience in the technique.

Forearm venous occlusion plethysmography measures the increase in forearm volume over time using a device called a strain-gauge plethysmograph. The study subjects lie supine in a temperature-controlled room, and blood pressure cuffs are applied at both upper arms and both wrists. Both forearms are positioned above the level of the heart, achieved by resting the elbows on foam pads and supporting the hands with pillows. The wrist and upper arm blood pressure cuffs are intermittently inflated; the pressure is 40 mm Hg so that it is above venous pressure but below diastolic blood pressure. The upper forearm cuffs are inflated for 10 seconds and deflated for 5 seconds. When both the wrist and upper forearm blood pressure cuffs are inflated, venous drainage is stopped, but arterial flow when venous drainage continues. Thus, for a short duration of time blood can enter the forearm but not escape, causing an increase in forearm blood flow.
and forearm volume. The increase in forearm blood flow can be measured using a mercury strain
gauge plethysmograph. The rate of increase in forearm blood volume (and hence forearm blood
flow) is linear over time. Under resting conditions, 70% of total forearm blood flow (FBF) is
through skeletal muscle, with skin blood flow accounting for most of the remainder. Hand blood
flow is excluded due to the fact that the hand contains a high proportion of arterio-venous shunts
and if the hand is not excluded then blood flow is nonlinear (Wilkinson and Webb, 2001). The
hands are excluded from the circulation during measurements by initial rapid inflation of a
smaller cuff, placed around the wrist, to well above systolic pressure (220 mmHg for
normotensive subjects). The wrist-cuffs are inflated at least 60 s before starting measurements
of flow in order to allow FBF to stabilize. This method can cause ischaemia to the hands, so the
plethysmography period is limited; however, measurements of up to 13 min have been
performed safely (Wilkinson and Webb, 2001). The strain gauges are be placed around the widest
part of the forearm, and act as resistors. Changes in forearm volume result in a corresponding
change in arm circumference and thus strain gauge length, which can be detected as an alteration
in electrical resistance of the gauge. This can then be computed to measure the forearm volume.
Venous occlusion plethysmography provides a measure of blood flow to that part of the forearm
enclosed by the two cuffs. This is usually expressed as ml per 100 ml of forearm volume per
minute. The process of inflating and deflating the cuff is automated and the plethysmography
values are run on a computer software.

Volunteers underwent brachial artery cannulation in the non-dominant forearm with a 27
standard wire gauge steel needle. After a 20 min baseline infusion with 0.9% saline, incremental
intra-arterial doses of bradykinin (American Peptide) at 100 pmol/min, 300 pmol/min and
1000 pmol/min (an endothelium-dependent vasodilator that induces tissue plasminogen
activator (t-PA) release), acetylcholine (Chem. Pharm Fabrik) at 5 µg/min, 10 µg/min and 20 µg/min (an endothelium-dependent vasodilator that does not induce t-PA release) and sodium nitroprusside (Hospira) at 2 µg/min, 4 µg/min and 8 µg/min (an endothelium-independent vasodilator that does not induce t-PA release) were infused for 6 min at each dose, with a 30 min 0.9% saline washout infusion between drugs. Absolute forearm blood flow is measured, with the control arm included to ensure there has been no systemic effect of the local vasodilators in the infused arm. In the control arm, we can make inferences as to whether the changes in blood flow are mediated by the endothelium, and whether tissue plasminogen is affected, by using all three of the above vasodilators.

The effect of vasodilators on forearm blood flow can change with systemic treatment, and this method is therefore an attractive method to investigate the effect of systemic drugs onto a model of endothelial function. There is a correlation between the forearm vasomotor response and changes in forearm blood flow, to that of the coronary arteries (Tagawa et al., 1997). Therefore, forearm blood flow studies provide an elegant non-invasive means of measuring surrogate markers of cardiovascular disease. This method, when combined with cannulation at the antecubital fossa, also allows platelet studies, described subsequently. In this particular center, there is the added advantage of building up a body of skilled operators in this method for troubleshooting any issues encountered, and building upon the work of Webb, quoted here.

It is important to note that in this occasion we excluded subjects using ACE-inhibitors to ensure no effect on the measurement of forearm blood flow associated with the bradykinin infusion. This is discussed further in the results and discussion segments.
2.3 Platelet Monocyte Aggregation Studies

Study 1 and 2 examined the effect of a novel SIRT1 activator to surrogate markers of thrombosis. The method of choice in this research programme was flow-cytometric measurements of platelet–monocyte aggregation (PMA) and platelet surface expression of P-selectin, soluble CD40 ligand (sCD40L) and monocyte CD11b expression (Mac-1/CD11b). The background to the utility of this method and the rationale are explained in the following.

Platelets are cells in the bloodstream generated from precursor megakaryocyte cells in the bone marrow. Platelets are involved in a key role in haemostasis by adhering to sites of endothelial damage; this process cascades changes to its cell structure which promote aggregation to other platelets, and to promote conversion of fibrinogen to fibrin. Fibrin formation triggers the coagulation cascade, causing a thrombus to form at the site of vascular injury (Ghoshal and Bhattacharyya, 2014). The cellular signalling events of platelet activation are triggered by multiple factors including thrombin, thromboxane A2 and Adenine Diphosphate (ADP). The final common pathway is platelet activation, resulting in upregulation of integrin cellular adhesion molecules. The most crucial is the cross-linking of von Willebrand factor and glycoprotein IIa/IIIb, leading to platelet aggregation.

Platelets also have a less completely understood role in host immune and inflammation responses. Platelets produce a number of inflammatory mediators that have no role in thrombosis and haemostasis. However, when activated, platelets adhere to neutrophils and monocytes (Thomas and Storey, 2015). Platelet activation is associated with low grade inflammation, and interaction with the endothelium that is thought to be important in the development of vascular disease (Freedman and Loscalzo, 2002). Binding between platelets and
other cell types is via the cell adhesion protein ‘P-selectin’, also known as CD 62p. Upon platelet activation P-selectin is rapidly expressed in in platelet cells and translocated to the cell membrane. P-selectin facilitates aggregation of platelets, leukocytes and the endothelium, triggering host inflammatory response and cell adhesion to site of cellular injury. Quantification of P-selectin expression on platelets therefore is a marker of platelet activation which is associated an increase in vascular inflammation. Activated platelets also expresses sCD40L, which can induce vascular cells to express IL-6. Both these markers are examined in this study programme. Expression of these markers have a short detectability in the peripheral bloodstream and platelets continue to exert its function. Therefore, in addition, quantification of platelet-monocyte aggregates (PMA) is also a useful marker of platelet activation, and is considered more sensitive (Michelson et al., 2001). PMA is associated with poorer outcomes in atherosclerosis (Gremmel et al., 2016). Platelet monocyte aggregates are stabilised by various mechanisms, including the binding of surface protein CD-11b and platelet glycoprotein 1bα (Simon et al., 2000).

In summary, this study programme quantified platelet activation by examining the expression of P-selectin (CD62p), PMA and CD-11b by flow cytometry, and soluble CD-40 ligand by ELISA.

The above quantification can be achieved by enzyme linked immunosorbent assay (ELISA) or Western Blotting. However, we used ‘flow cytometry’ in our research programme, because it is far more sensitive and is independent of platelet count and uses a smaller volume of blood. Flow cytometry is a method of sorting immunolabelled cells and quantifying them based on physical and chemical properties. A sample containing the cells is of interest is fixed and immunolabelled with fluorescence, then suspended in a fluid. The fluid is then injected into the flow cytometer machine, which creates a ‘flow’ of single cells, which in turn is then passed through a focused laser. The immunolabelled cells, with different fluorescence properties, emit a different spectrum of light as they pass through the laser. These different spectra of emission can be
measured using filters, and thus in this manner different population of cells can be measured based on the spectrum of fluorescent light refracted. These data are computed into a scatter histogram of different cell populations. The data is then analysed by computer software and expressed in terms of absolute percentage of cells. In this research programme we used the FACSCalibur machine (Becton Dickson) and used its paired software on the platform Cell Quest Pro to analyse generated data. A schematic diagram of the flow cytometry system is in Figure 3.

**Figure 3: A schematic diagram of the FACSCalibur flow cytometry system used in the experiments.**

'Sheath fluid' represents the fluid that runs in a flow cytometer. The fluid runs in a laminar flow in an outer channel, and the cells are injected at a slightly higher pressure in the central channel, thereby focussing single cells through the nozzle. This cell stream goes through a blue laser, creating a scatter of lights, which is detected by the computer system and integrated into a diagram in the following figure. Forward scatter correlates with cell size, and side scatter correlates with cell content. Fluorescence emissions from fluorochrome-immunolabelled cells provide information about cell surface expression.
The fluorochromes we used were: Phycoerythrin (PE) and Fluorescein Isothiocyanate (FITC). These fluorochromes can be paired with mouse anti-human monoclonal antibodies. In this specific example, CD14 is used as it is a cell surface receptor expressed predominantly by monocytes; mouse anti-human CD 42a is the cell surface receptor for platelets to bind to von Willebrand factor and therefore can be used to select platelets. Using appropriate antibody control isotypes, we can select out the appropriate cell type pairings; in this case we are interested in platelet monocyte aggregates. These pairings are described in terms of positive signals of fluorochrome-immunolabelled CD 62p (P-selectin) and CD-11b as described earlier, as markers of platelet activation. Directly conjugated monoclonal antibodies were obtained from DakoCytomation and Serotec. Samples were stained with the following conjugated monoclonal antibodies: phycoerythrin (PE)-conjugated CD14, PE-conjugated CD62p, PE-conjugated CD11b, fluorescein isothiocyanate (FITC)-conjugated 42a, and FITC-conjugated CD14 and appropriate control isotypes.
Figure 4: Histogram analysis of flow cytometric output.

Monocytes were labelled with CD 14 PE and Platelets with CD 42 FITC. In 1) Different forward and side scatter emissions are plotted on a graph. Monocytes are identified based on their known scatter properties. In 2) Isotype controls identify true positives and a histogram of these ‘gated events’ are created in 3) CD14 bright cells are identified. 4) CD14 fluorescent monocytes were plotted against CD42 platelets, identifying platelet monocyte aggregates, expressed as percentage. These images were adapted from own data printouts with identifiers removed.
There are many considerations in making these experiments as reproducible and accurate as possible. Work by colleagues in the Centre for Cardiovascular Sciences in Edinburgh is the one quoted here (Harding et al., 2007). In brief summary, the high sensitivity of the flow cytometric analysis leaves it open to artefactual error, including in vitro activation. Harding and colleagues looked at the effect of choice of anti-coagulant, the effect of a serial sampling via a large antecubital fossa venous cannula, time-delay to immunolabelling, erythrocyte lysis, and sample stability after fixation. There is a significant effect on using cannulation on platelet activation, so in our protocol venepuncture for platelet monocyte aggregation was done separately. Direct thrombin inhibitors or citrate could be used, with a possible minor advantage with citrate if there were significant time delays (more than several hours). There was no effect on platelet activation with erythrocyte lysis. Handling and processing should be standardised.

In our protocol, peripheral venous blood was drawn from a large antecubital vein and anticoagulated with the direct thrombin inhibitor D-phenylalanine-L-arginine chloromethyl ketone (75 mmol/L PPACK; Cambridge Biosciences). Tubes were gently inverted to ensure mixing of whole blood with anticoagulant. The blood samples were then immunolabeled within 5 minutes of phlebotomy for subsequent flow cytometric analysis. Once stained, samples were incubated for 20 minutes at room temperature before being fixed with FACS-Lyse (Becton-Dickinson).

Venous blood was collected in citrate at baseline and after each dosing period to assess plasma-soluble CD40 ligand (sCD40L) concentrations. Blood was centrifuged at 1500g for 15 minutes at
4°C, and plasma was decanted and stored at --80°C for further analysis by ELISA (Bender Medsystems).

### 2.4 Pulse Wave Analysis

Pulse wave analysis is a clinical research method which has been used extensively to quantify ‘arterial stiffness’. Some experts argue that this measure should be used in clinical practice due to its non-invasive nature and the powerful information it gives about the vascular tree, and its predictive accuracy in cardiovascular conditions (Segers et al., 2017; Laurent et al., 2006).

‘Arterial stiffness’ is now a widely used term in the scientific literature, and describes the mechanical properties of the arterial tree such as distensibility, compliance, and elasticity. These properties are complex and heterogenous along the arterial tree, as muscular and elastic vessels differ in mechanical properties centrally and peripherally. The arterial system also has homeostatic compensatory properties. There is therefore a variety of methods trying to quantify the physical properties of the arterial tree at different central and peripheral sites with varying degrees of reproducibility. The most widely accepted model of arterial stiffness is termed the propagative model; it is assumed that the arterial tree is a visco-elastic tube which allows propagation of a forward waveform in the arterial vessels. The arterial tree’s branch points and high resistance in the periphery create a reflected retrograde waveform, which can also be measured. Using this model, an increase in arterial stiffness causes a higher wave velocity forward, and the higher the speed of retrograde waves also.
Work by Safar, O’Rourke and colleagues building on work over decades has resulted in models of arterial stiffness which can compute generated waveforms into a validated integrative mathematical equation (Safar and London, 2000; O’Rourke et al., 2002). The of the equations based on inputs of blood pressure and waveforms from various methods method. ‘Applanation tonometry’ is the most commonly used and is described subsequently. It is useful to note however that other methods measuring systemic and local arterial stiffness exist, including doppler ultrasound devices measuring aortic pulse wave velocity, echotracking devices at the common carotid and common femoral and brachial arteries, and Magnetic Resonance Imaging (MRI) devices measuring local arterial stiffness at the aorta. These methods are extensively reviewed in the consensus statement by Laurent and colleagues (Laurent et al., 2006). Briefly, Carotid-Femoral Pulse Wave Velocity (PWV) is considered the gold standard in measurement of arterial stiffness. This is measured transcutaneously over the right common carotid or the right common femoral artery. This method is however limited in people who are obese, and pressure measurement over the carotid in this subset of people is not without risk (Van Bortel et al., 2002).

We concentrate on the applanation tonometry methodology in this segment. The term ‘applanation tonometry’ describes a method where a probe is placed on the skin overlying the artery of choice, and pressure is applied to ‘applanate’ or flatten the artery. This can be done over the carotid artery, but this can be technically challenging, as we have described. A more popular and well tolerated method is radial applanation tonometry, which is used in this particular research programme. A pressure transducer (Millar Instruments, Texas, USA) is applied to the radial artery, and this generates a radial pulse wave. The peak and trough of the radial pulse wave correspond, respectively, to systolic and diastolic blood pressure, with blood pressure is assumed to be identical in brachial and radial arteries. This can then be transformed using a mathematical transfer function (SyphgmoCor system, AtCor, Sydney Australia), generating aortic pressure
waveform from the radial waveform. Radial tonometry is technically simple to perform, as it is supported by the radial bone underneath, and is easy to compress effectively. The method is also well tolerated and is less risky than compressing the carotid artery. The Augmentation index is an independent predictor of cardiovascular events, which makes it a useful clinical research tool for intervention studies (Weber et al., 2005; Williams et al., 2006).

The aortic pressure waveform is an amalgamation of the pressure wave generated by left ventricular contraction, and the retrograde wave reflected from the peripheral vascular resistance. In elastic vessels, because the pressure wave velocity is low, the pressure wave is reflected and arrives back to the aortic root in diastole. In stiff arteries, the PWV is reflected back in systole, thereby adding to systolic pressure. A quantification of this phenomenon is termed the Augmentation Index (AIx), defined as the difference between the first and second systolic peak divided by the pulse pressure, shown in Figure 5.
Figure 5: A diagram of the aortic waveform, derived from radial applanation tonometry.

The augmentation pressure is the second systolic peak minus the first systolic peak. The augmentation index (aix) is the augmentation pressure divided by pulse pressure.
The pulse wave velocity can be modulated by many factors, including temperature, heart rate, physical activity, and recent ingestion of alcohol and caffeine. Therefore, in order to standardise the studies, volunteers in this study programme were requested to abstain from caffeine and alcohol 24 hours prior to the study. The experiments were conducted in a temperature-controlled room at 22 degrees Celsius, with the subject supine for at least 10 minutes beforehand. Blood pressure was measured at the brachial artery (Omron 705 IT, Omron Europe, The Netherlands). Augmented Index was obtained, and the Sphygmacor corrects this value for heart rate. Pulse wave velocity was calculated by measuring the time for pressure to be transmitted to the carotid and femoral arteries. A minimum of three waveforms were obtained and accepted based on the SphygmoCor quality control criteria. The operator performing analysis was kept constant for each participant of the study.
2.5 Coronary Flow Reserve Measurement by Transthoracic Colour Doppler Echocardiography

Quantification of myocardial ischaemia is a big area of clinical research, due to its significance in clinical practice. Quantifying myocardial ischaemia is complex and multifactorial. Briefly, myocardial ischaemia is the imbalance between the oxygen demand of the myocardium, and its oxygen supply at the cellular level. Stenosis of the coronary artery is but one of the components contributing to ischaemia. Other factors include endothelial function, coronary autoregulation, endothelial function, and metabolic factors such as myocardial free fatty acid uptake.

Modelling this complex interplay of factors is difficult and still an area of clinical practice that is only slowly gaining consensus. For example, an individual exhibiting the symptoms of ischaemic heart disease may undergo invasive coronary artery catheterisation. A coronary stenosis may be seen, but its significance may be in doubt. In this case, a ‘fractional flow reserve’ study may be performed. A guidewire is passed through the stenotic lesion, and the vasodilator adenosine is injected into the coronary artery, causing vasodilation. The fractional flow reserve is the ratio of blood flow distal to the stenosis, to the blood flow proximal to the stenosis. As this is a ratio it generates an absolute number. From previous studies, an FFR of less than 0.75 has prognostic significance and affects outcomes such as coronary revascularisation and myocardial infarction (Tonino et al., 2009; Pijls et al., 2007). This example illustrates the concept that modelling myocardial ischaemia requires an element of functional testing in response to stress.

The method described above however is invasive and coronary artery catheterisation is typically only done when there is a good index of clinical suspicion for coronary artery disease. We have
already explored in the introduction chapter how endothelial dysfunction, and low-grade atherosclerosis, may precede clinical presentation in people with T1DM and T2DM. Furthermore, there is a concern that low blood sugar may trigger myocardial ischaemia. There is of course the possibility of triggering low blood sugar experimentally during coronary arteriography. This approach however would be problematic for various reasons in people with diabetes, including the fact that it may cause harm.

Our group therefore looked at non-invasive methods of quantifying myocardial ischaemia, and a transthoracic colour doppler echocardiography method by Hozumi and colleagues was ideal on the basis of practicality, safety and reproducibility (Hozumi et al., 1998). In the first description of the method Hozumi and colleagues found a good correlation between this non-invasive method and significant coronary artery stenosis quantified by arterial catheterisation into the left anterior descending artery (Hozumi et al., 1998). Following on from this paper in 1998, Hirata and colleagues went on to use this method to correlate ‘coronary flow reserve measurement’ to endothelial dysfunction and subclinical coronary artery dysfunction (Hirata et al., 2004).

In this research study, our volunteers were positioned in a lateral decubitus position. Transthoracic Doppler echocardiography was performed using a 7.0 MHz transducer (Acuson Sequoia 512, Siemens Medical Solutions, Berkshire, UK). The echocardiograph acoustic window was between the fourth and fifth intercostal space in the midclavicular line. The left ventricle is imaged in the long axis, and the probe then tilted laterally to elicit the colour doppler flow of the distal left anterior descending (LAD) coronary artery. The study subjects underwent simultaneous electrocardiograph monitoring and the Doppler images were gated to the electrocardiograph. The
pattern of coronary doppler signals are biphasic, with predominance of flow during diastole and a small component in systole. However, due to cardiac motion it is difficult to measure the doppler signals during systole, so by convention only the signal during diastole is used. During diastole, the doppler signal of the LAD can be seen as a ‘flash’, using Doppler colour flow mapping. This Doppler signal can then be mathematically transformed into a spectral flow signal which can be measured as coronary flow velocity in centimetres per second. The Coronary flow velocities can be expressed as mean velocity, maximum velocity, or volume time integral of the spectral Doppler signals. By convention we use maximum velocity (Vmax) in our studies. Hozumi and colleagues show comparable sensitivity of 92% and specificity of 82% with Vmax and mean velocity, and subsequently the review literature has tended to select Vmax (Meimoun and Tribouilloy, 2008).

Coronary flow reserve is defined as coronary flow velocities during hyperaemia divided by baseline coronary flow. In order to induce hyperaemia, a standard protocol of 0.14 mcg/kg/minute Adenosine (Adenocor). Other agents can be used to induce hyperaemia and is reviewed extensively in the review by Meimoun (Meimoun and Tribouilloy, 2008). These include dobutamine and dipyridamole. In our protocol, due to repeat testing, the shorter half-life of adenosine allowed repeated testing under different experimental conditions without confounding. Hyperaemia induced by adenosine can be continued for up to four minutes to obtain Doppler signals, and measurements were averaged over three cardiac cycles. As the coronary flow reserve is a ratio of peak coronary velocity during hyperaemia over peak coronary velocity during baseline, the number is expressed as an absolute number without units. **Figure 6** shows the images of the spectral Doppler flow obtained in diastole, during both hyperaemia and baseline.
FIGURE 6: SPECTRAL DOPPLER IMAGES OF CORONARY FLOW WITH GATED ECG COMPLEXES

The top and bottom pictures are images from the same subject. The top picture was taken during baseline conditions, and the bottom pictures during hyperaemic conditions (‘pre-adenosine and post adenosine respectively). The green line in both pictures is the ECG complexes. The Echocardiographic window is indicated by the labels, as are the spectral doppler images. The coronary flow reserve is the coronary flow velocity in hyperaemia divided by coronary flow velocity during baseline.
The feasibility of measuring coronary flow reserve at the left anterior descending artery has been described in the review literature as high, around 90% in experienced hands and virtually 100% when supplemented with intravenous contrast agents (Hozumi et al., 1998; Caiati et al., 1999; Nohtomi et al., 2003). Inter and intra-observer variability has been quoted as around 5% for both. Inter-individual variability across different visits 3 weeks apart was examined by Meimoun et al using the Bland-Altman between measure interval of agreement method, ranging from -10 to +12%. Our own validation studies showed that in people without diabetes, our experiment was successful in obtaining images in 12 out of 13 volunteers, with no significant difference in observations using the Bland Altman method (Warraich 2011). However, we did encounter some challenges when combining the echocardiography study with experimental hypoglycaemia, and our success rate in obtaining the images were lower. We describe this further in the following chapters relevant to the experiment.
2.6 Hyperinsulinaemic Glucose Clamp Technique

The hyperinsulinaemic glucose method is a well-described technique which has been used extensively in our group (DeFronzo and Andres, 1979). After confirmation of avoidance of biochemical hypoglycaemia in the preceding 2 days, a retrograde IV cannula is inserted into the forearm for regular sampling of blood glucose. This hand is placed in a heated blanket in order to arterialise venous blood. A cannula in the antecubital fossa is also inserted for the infusion of 20% dextrose and soluble human insulin (Human Actrapid NovoNordisk pharmaceuticals, Crawley, UK). Insulin is infused at a rate of 1.5 mg/kg/min using a Gemini PCI pump (Alaris Medical Systems, San Diego CA). Dextrose is infused at a variable rate according to the blood glucose concentrations measured at least every five minutes using the glucose oxidase method which is highly accurate (2300 Stat, YSI, Yellow Springs OH). During a run-in period, blood glucose is maintained at 4.5-5 mmol/L for approximately 20 minutes. Blood glucose is then stabilised and maintained at this level during the euglycaemic clamp. Whereas in the hypoglycaemic clamp it is lowered to 2.0-2.5 mmol/L for approximately 20 minutes. Following this experimental period, euglycaemia is restored and maintained for around 20 mins. Following this, the clamp is discontinued and subjects are given a standardised meal. Subjects with diabetes are advised regarding the timing and dose of the next insulin administration. The experimental studies are performed during the initial run in, experimental condition, and recovery.

The subjects and operator are not informed as to which condition in which they are being studied, and hypoglycaemia and euglycaemia is randomised and counterbalanced.
2.7 Venous Blood Sampling

In the four experiments described in this thesis, various blood samples were taken including those already described in the platelet monocyte aggregation studies, which has some special considerations. Venous blood samples of full blood count, renal and liver function tests, lipids and coagulation profile were obtained to monitor safety in study 1, 2, and 3. Analyses of these samples were conducted by the regional NHS Lothian clinical haematology and biochemistry reference laboratories using an automated haematology analyser (XE2100, Sysmex Corporation (Japan) and ACL TOP, Instrumentation Laboratory), an automated chemistry analyser using colorimetric, kinetic and enzymatic ultraviolet and colour assays (AU2700/AU640 analysers, Beckman and Coulter), ion selective electrodes (sodium, potassium and chloride assays) and two point and multiple point rate assays (Ortho Clinical Vitros 250 analyser, USA). Samples of venous blood for pharmacokinetic assessment of plasma SRT2104 concentrations (Simbec Laboratories Limited) were taken serially into pre-labelled heparinised tubes. Plasma was separated by centrifugation of whole blood at 1500g at 4°C for 15 minutes, and decanted and stored at −80°C until analysed. Plasma concentrations of SRT2104 were measured using liquid chromatography with tandem mass spectrometry detection in positive ion mode. High-pressure liquid chromatography was performed using Betasil silica–100 columns using a Phenomenex C18 guard column. The safety analyses are used in routine clinical practice and are highly precise, with coefficient of variation (CV) of less than 5%.

During the previously described venous occlusion plethysmography study, we also obtained paired venous blood samples from each forearm before and during the infusion of intra-arterial bradykinin. Samples were collected into acidified buffered citrate (Stablyte; Trinity Biotech Plc) and citrate (BD Vacutainer; BD UK Ltd) for determination of t-PA and PAI-1 concentration, respectively. Samples were
placed on ice before centrifuging at 2000g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at −80°C before further analysis. Venous blood samples were collected into Ethylene Diamine Triacetic Acid (EDTA) tubes at the beginning and end of the vascular study to determine haematocrit. Plasma t-PA antigen and activity (t-PA Combi Actibind t-PA ELISA kit; Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed) concentrations were determined by ELISAs with Intra- and inter-assay coefficient of variation of under 5%.

High-sensitivity cardiac troponin I (hs-cTnI) concentrations were determined using the ARCHITECT STAT high-sensitive troponin I assay (Abbott Laboratories, Abbott Park, IL). This is the first clinically approved high-sensitivity troponin I assay. The limit of detection is 1.2 ng/L and precision profiling in our own laboratory in NHS Lothian demonstrates an inter-assay CV of <10% at 5 ng/L. The upper reference limit or 99th centile is 16 ng/L for women and 34 ng/L for men (Shah et al., 2015a; Shah et al., 2015b).
2.8 Statistical Analysis

Results were analysed using a combination of SPSS for Unix version 9.3 and higher (SPSS, Chicago, USA) and GraphPad Prism Version 6 and higher (GraphPad, San Diego California). Specific statistical tests including power calculations will be addressed in the relevant subsequent chapters. In general, parameters in study 1 and 2 are expressed as model adjusted least square means and 95% confidence intervals. Where appropriate, linear mixed-model repeated measures of analysis of covariance (ANCOVA) were used. Treatment differences were analysed using a model adjusted for period and treatment using SAS for Unix. Post-hoc analyses are dealt in the appropriate chapters. P-value of < 0.05 was adopted for statistical significance, and a p of <0.1 was deemed significant for period by treatment interactions. Coefficient of variation, where appropriate, was measured using the Student’s t-test, and between day reproducibility was measured using the Bland-Altman method.

Study 3 used analysis of covariance in SPSS. Mean values were model-adjusted for period, treatment, and treatment by period interaction.

Study 4 uses a generalised linear mixed model analysis using euglycaemia or hypoglycaemia experimental condition as within-subjects factor, and diabetes status as between-subjects factor. We chose a value of p<0.05 as statistically significant. When appropriate, student’s t-tests were used.
Chapter 3

The cardio-metabolic effects of SRT2104, in people with T2DM


3.1 Introduction

As explored in the introduction chapter, the effect of CR on energy metabolism has attracted intense interest over the past decade because it extends life span in yeast, nematodes and flies (Lin and Guarente, 2000; Tissenbaum and Guarente, 2001; Sinclair and Guarente, 1997). CR also lowers the incidence of age-related disorders such as diabetes and cancer in mammals. These beneficial effects may be mediated by sirtuins (Bordone and Guarente, 2005). The sirtuin gene regulates many cellular pathways including inflammation, apoptosis and mitochondrial biogenesis. The activity of sirtuins is dependent on nicotinamide adenine dinucleotide, thereby linking energy metabolism and CR to these important cellular pathways, many of which mediate processes of aging when upregulated.

The mammalian homologue of the sirtuins is SIRT1 (Bordone and Guarente, 2005). In murine models, activation of SIRT1 has been shown to regulate PGC-1α, NFκB, FOXO box protein and UCP proteins (Picard et al., 2004; Bordone et al., 2006; Motta et al., 2004). These pathways are implicated in skeletal muscle energy metabolism and adipocyte differentiation, and play a role in glucose regulation in skeletal muscle and pancreatic β cells, as well as modulating insulin sensitivity (Rodgers et al., 2005). Inhibition of SIRT1 in murine models upregulates tissue factor expression in endothelial cells and promotes intra-arterial thrombosis (Breitenstein et al., 2011), as well promoting atherogenesis by increasing foam-cell formation (Stein et al., 2010). In contrast, upregulation of SIRT1 increases nitric oxide in rodent models (Mattagajasingh et al., 2007). The logical conclusion of these studies is that SIRT1 activation is a novel drug target which has the potential mitigate the adverse effects of atherosclerotic disease and therefore improve cardiovascular health.
The major cause of mortality in patients with T2DM is cardiovascular disease. One of the putative mechanisms of the link between cardiovascular disorders and T2DM is endothelial dysfunction (van Sloten et al., 2014). Thus, SIRT1 activation is a potentially promising strategy for the treatment of both diabetes and its well documented association with cardiovascular disease.

To date, studies have primarily investigated the ex-vivo effect of SIRT1 activation. We hypothesised that therapeutic SIRT1 activation would have beneficial effects on cardiometabolic health in people with diabetes. Specifically, we examined the effect of a novel SIRT1 activator, SRT2104, on vascular vasomotor and fibrinolytic function, platelet activation, lipid profile and markers of glycaemic control in people with T2DM.

3.2 Methods

The study was approved by the research ethics committee, was given clinical trial authorization by the Medicines and Healthcare products Regulatory Agency (MHRA), and carried out at the MHRA phase I accredited Wellcome Trust Clinical Research Facility at the Western General Hospital, UK. Written informed consent was obtained from each participant, and the study was carried out in accordance with the Declaration of Helsinki.
3.2.1 Study participants

As described previously, fifteen individuals with T2DM were recruited using various methods, including outpatient clinics at the Royal Infirmary of Edinburgh, radio advertisement in Edinburgh, and database search. Inclusion criteria included age between 18 years and 70 years, glycated haemoglobin (HbA1c) <9.0% (75 mmol/mol) and resting blood pressure of <160/90 mm Hg. Exclusion criteria included current smokers, the use of ACE inhibition therapy (as the potentiation of bradykinin and effects on endothelial function would confound the vascular studies), the presence of major comorbidities, chronic illness, renal or liver impairment, history of gastrointestinal diseases or surgeries influencing drug absorption, history of alcoholism, history of neoplastic disease within the last 5 years, a positive urinary test for recreational drugs, pregnancy, and participation in other clinical trials or blood donation within the last 3 months. After obtaining informed consent, the eligibility of participants including absence of relevant medical history was confirmed through a standardized form completed by the volunteers own general practitioner. Tests for pregnancy (serum human chorionic gonadotropin (HCG) concentrations at screening and urinary HCG concentrations at study visits) were conducted on all female participants of childbearing potential.

3.2.2 Study design

This was a prospective, double-blind, randomized, placebo-controlled cross-over study. Subjects were randomized 1:1 to receive 2.0 g daily of oral SRT2104 or matched placebo (Sirtris
Pharmaceuticals) for a 28-day period, followed by cross-over to the alternate study arm for another 28 days, giving a total dosing duration of 56 days. A safety visit was conducted on day 70, with a follow-up by telephone on day 86. Assessment of drug safety, tolerability and efficacy on vascular function was carried out at baseline, and during and at the end of each treatment period. The overall study design included otherwise healthy smokers in addition to people with T2DM, and volunteers were stratified by these two categories. This chapter focuses on the T2DM group, with observations in the otherwise healthy smokers group explored in later chapters.

**Figure 7: A schematic diagram of the study in Chapter 3**

various study points beginning screening (denoted SCR), two 28-day periods with physiological studies at Day 1, 28 and 56, and follow-up period via telephone, 30 day after study termination. There were bi-weekly safety visits to ensure tolerability of the drug, as well as venous sampling for safety parameters, and pharmacokinetic assessments (labelled PK in the diagram)
3.2.3 Vascular studies

Vascular studies were undertaken before and at the end of each 28-day trial period. All studies were performed with the patient lying supine in a quiet temperature-controlled (22°C to 25°C) room. Participants were fasted for 10 hours, and avoided caffeine and alcohol for 24 hours, before the study. Venous cannulae (17G) were inserted into large subcutaneous veins in the antecubital fossae of both arms to facilitate periodic venous sampling. Platelet monocyte aggregate study sampling however were done separately. Supine heart rate and blood pressure were monitored at intervals throughout the study using a semiautomated, non-invasive oscillometric sphygmomanometer (Omron 705 IT).

3.2.4 Forearm venous occlusion plethysmography

FBF was measured in the infused and non-infused forearms using forearm venous occlusion plethysmography as described in previous chapters. Volunteers underwent brachial artery cannulation in the non-dominant forearm with a 27 standard wire gauge steel needle. After a 20 min baseline infusion with 0.9% saline, incremental intra-arterial doses of bradykinin (American Peptide) at 100 pmol/min, 300 pmol/min and 1000 pmol/min (an endothelium-dependent vasodilator that induces t-PA release), acetylcholine (Chem. Pharm Fabrik) at 5 µg/min, 10 µg/min and 20 µg/min (an endothelium-dependent vasodilator that does not induce t-PA release) and sodium nitroprusside (Hospira) at 2 µg/min, 4 µg/min and 8 µg/min (an endothelium-independent vasodilator that does not induce t-PA release) were infused for 6 min.
at each dose, with a 30 min 0.9% saline washout infusion between drugs. The order of drugs was randomized between subjects but kept constant for each participant across the three visits.

### 3.2.5 Blood sampling

Paired venous blood samples were obtained from each forearm before and during the infusion of intra-arterial bradykinin. Samples were collected into acidified buffered citrate (Stabilyte; Trinity Biotech) and citrate (BD Vacutainer; BD UK) for determination of t-PA and PAI-1 concentrations, respectively. Samples were placed on ice before centrifuging at 2000 g for 30 min at 4°C. Platelet-free plasma was decanted and stored at −80°C before further analysis. Venous blood samples were collected into EDTA at the beginning and end of the vascular study to determine haematocrit.

Plasma t-PA antigen and activity (t-PA Combi Actibind t-PA ELISA kit; Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed) concentrations were determined by ELISAs.

### 3.2.6 Platelet and monocyte activation

Flow-cytometric measurements of platelet–monocyte aggregation and platelet surface expression of P-selectin and monocyte CD11b expression (Mac-1/CD11b) were performed at baseline and at the end of each treatment period as described in previous chapters. Peripheral venous blood was drawn from a large antecubital vein separate to the venous cannulae to
prevent artefactual platelet activation. The samples were anticoagulated with the direct thrombin inhibitor D-phenylalanine-L-arginine chloromethyl ketone (Cambridge Biosciences) and immunolabelled within 5 min of phlebotomy for subsequent flow cytometric analysis. Directly conjugated monoclonal antibodies were obtained from DakoCytomation and Serotec. Samples were stained with the following conjugated monoclonal antibodies: PE–conjugated CD14, PE-conjugated CD62p, PE-conjugated CD11b, FITC–conjugated 42a and FITC-conjugated CD14 and appropriate control isotypes. Once stained, samples were incubated for 20 min at room temperature before being fixed with Fluorescence-activated cell sorting (FACS) Lyse (Becton-Dickinson). All samples were analyzed using a FACS Calibur flow cytometer using CellQuest Pro software (Becton-Dickinson). Venous blood was collected in citrate at baseline and after each dosing period to assess plasma-soluble CD40 ligand concentrations. Blood was centrifuged at 1500 g for 15 min at 4°C, and plasma was decanted and stored at −80°C for further analysis by ELISA (Bender Medsystems).

3.2.7 Safety and pharmacokinetic analyses

Venous blood samples were collected twice weekly to measure haematological and biochemical parameters including full blood count, coagulation profile, liver and renal function, creatine phosphokinase, lactate dehydrogenase, lipid profile and free fatty acids. Analyses were conducted by the regional clinical haematology and biochemistry reference laboratories using an automated haematology analyser (XE2100, Sysmex Corporation and ACL TOP, Instrumentation Laboratory), an automated chemistry analyser using colorimetric, kinetic and enzymatic ultraviolet and colour assays (AU2700/AU640 analysers, Beckman Coulter), ion-selective
electrodes (sodium, potassium and chloride assays) and two-point and multiple-point rate assays (Ortho Clinical Vitros 250 analyser). Venous blood samples were taken into prelabelled heparinised sodium tubes for pharmacokinetic assessment of plasma SRT2104 concentrations (Simbec Laboratories Limited). Serial blood samples were collected on days 1, 28 and 56 immediately before (0 min) and 15 min, 30 min, 60 min, 120 min, 180 min, 240 min, 480 min, 720 min and 1440 min following study medication. Plasma was separated by centrifugation of whole blood at 1500 g at 4°C for 15 min, and decanted and stored at −80°C until analysed.

Plasma concentrations of SRT2104 were measured using liquid chromatography with tandem mass spectrometry detection in positive ion mode. High-pressure liquid chromatography was performed using Betasil silica–100 columns using a Phenomenex C18 guard column.

3.2.8 Data analysis and statistics

Plethysmographic data were analysed as described in the methods section. Estimated net release of t-PA and PAI-1 antigen and activity was defined as the product of forearm plasma flow (based on blood flow and haematocrit) and the difference in plasma antigen (or activity) concentrations between the two forearms. Fibrinolysis and forearm blood flow data were analysed using a linear mixed-model repeated-measures analysis of covariance. Treatment differences were investigated in a model adjusting for period, treatment by period, vasodilator dose, treatment by vasodilator dose, and vasodilator dose by period using SAS for UNIX (V.9.1.3 or higher; SAS Institute). Treatment differences in weight, HbA1c, fructosamine and lipid variables were analysed using linear mixed-model repeated-measures analyses of covariance adjusting for baseline, period and treatment by period interaction. Values for these parameters are expressed
as model adjusted (least square) means and 95% CIs. Between-day reproducibility of forearm venous occlusion plethysmography data were assessed using the Bland-Altman method, and coefficient of reproducibility was determined for 95% CIs using the Student's t-distribution. All other values are expressed as mean±SD. Statistical significance for treatment differences was concluded if the two-sided p was <0.05. Interactions and period effects were tested using a significance level of 0.10.

3.3 Results

The Consolidated Standards of Reporting Trials (CONSORT) flow diagram summarises the participant screening, exclusion and recruitment process. The participants were predominantly middle-aged men (58±7.8 years, 13 male), who were obese (body mass index 30±3.5 kg/m²) and who had reasonable glycaemic control (mean HbA1c 54±8.0 mmol/mol (7.4%±0.80%).)
**FIGURE 8: CONSORT TEMPLATE FOR STUDY IN CHAPTER 3**

Diagram of randomised control trial numbers from recruiting programme to analysis
3.4 Safety of SRT2104 in people with T2DM

All of the participants tolerated the study medication. Commonly reported side effects occurring in two or more volunteers included headache (33%), diarrhoea (27%), nausea (13%) and hypoglycaemia (13%) (Table 4). Apart from one subject describing diarrhoea as ‘severe’, the remaining reported adverse events were mild to moderate in intensity. All side effects resolved without any sequelae. There were no meaningful differences in the frequency of treatment-emergent adverse events between the active treatment and placebo groups. One individual underwent part of the first period of the study but was withdrawn because an adverse event criterion was met with the concentration of alanine aminotransferase being recorded at five times the upper limit of normal. After unblinding, this patient was found to have been taking placebo at the time of the event.
### Table 3 Summary of Treatment Emergent Adverse Events

Multiple adverse events for each subject is counted once within each treatment and unique category.

<table>
<thead>
<tr>
<th>System</th>
<th>Adverse Event</th>
<th>Number of Subjects with Events (percentage)</th>
<th>Placebo (n=14)</th>
<th>SRT2104 (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Any Event</strong></td>
<td>-</td>
<td>11 (79%)</td>
<td></td>
<td>14 (93%)</td>
</tr>
<tr>
<td><strong>Injury, Poisoning and Procedural Complications</strong></td>
<td>Any Event</td>
<td>3 (21%)</td>
<td>1 (7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Contusion</td>
<td>1 (7%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Muscle strain</td>
<td>1 (7%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nail injury</td>
<td>0</td>
<td>1 (7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[post-procedure discomfort]</td>
<td>1 (7%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Neurological</strong></td>
<td>Any Event</td>
<td>1 (7%)</td>
<td>7 (47%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Headache</td>
<td>1 (7%)</td>
<td>5 (33%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Paraesthesia</td>
<td>0</td>
<td>1 (7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lethargy</td>
<td>0</td>
<td>1 (7%)</td>
<td></td>
</tr>
<tr>
<td><strong>Gastrointestinal</strong></td>
<td>Any Event</td>
<td>4 (29%)</td>
<td>8 (53%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diarrhoea</td>
<td>2 (14%)</td>
<td>4 (27%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nausea</td>
<td>1 (7%)</td>
<td>2 (13%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Upper Abdominal Pain</td>
<td>0</td>
<td>1 (7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dyspepsia</td>
<td>0</td>
<td>2 (13%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abdominal Discomfort</td>
<td>0</td>
<td>1 (7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Constipation</td>
<td>1 (7%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Frequent Bowel Motions</td>
<td>0</td>
<td>1 (7%)</td>
<td></td>
</tr>
<tr>
<td>System</td>
<td>Adverse Event</td>
<td>Number of Subjects with Events (percentage)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------</td>
<td>--------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Placebo (n=14)</td>
<td>SRT-2104 (n = 15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolism and Nutrition</td>
<td>Any Event</td>
<td>2 (14%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypoglycaemia</td>
<td>1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anorexia</td>
<td>1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Investigations</td>
<td>Any Event</td>
<td>1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alanine Aminotransferase Increased</td>
<td>1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver Function Tests Abnormal</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory, Thoracic or Mediastinal</td>
<td>Any Event</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rhinorrhoea</td>
<td>1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cough</td>
<td>1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epistaxis</td>
<td>1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hiccups</td>
<td>1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>Any Event</td>
<td>1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pruritis</td>
<td>2 (13%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dry Skin</td>
<td>1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alopecia</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Musculoskeletal</td>
<td>Any Event</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Muscle Spasm</td>
<td>1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Muscular Weakness</td>
<td>1 (7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>System</td>
<td>Adverse Event</td>
<td>Number of Subjects with Events (percentage)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------------</td>
<td>--------------------------------------------</td>
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<td></td>
</tr>
<tr>
<td>Placebo (n=14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRT-2104 (n = 15)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Immune System</td>
<td>Any Event</td>
<td>1(7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Seasonal Allergy</td>
<td>1(7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular</td>
<td>Any Event</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flushing</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>General</td>
<td>Any Event</td>
<td>2(14%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fatigue</td>
<td>1(7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flu-like Illness</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asthenia</td>
<td>1(7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malaise</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infections</td>
<td>Any Event</td>
<td>3(21%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nasopharyngitis</td>
<td>3(21%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Upper Respiratory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tract infection</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Renal and Urinary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dysuria</td>
<td>1(7%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


3.5 Pharmacokinetics of SRT2014 in people with T2DM

After 28 days of active treatment, the mean maximum plasma concentration ($C_{max}$) of SRT2104 was 517±355 ng/mL and 716±359 ng/mL in the first and second treatment periods, respectively. The mean time at which the maximum plasma concentration was observed ($T_{max}$) on day 28 of dosing was 2.6±1.2 hours and 2.9±1.5 hours in the first and second treatment periods, respectively. The mean area under the curve was 5300±3473 h.ng/mL in the first treatment period and 7312±3708 h.ng/mL in the second treatment period. The pharmacokinetics in this group was consistent with levels seen in previous studies by Sirtris pharmaceuticals (Hoffmann et al., 2013).

3.6 Cardiovascular effects of SRT2104 in people with T2DM

There were no changes in heart rate and blood pressure during the study. No effects were observed on cardiac rhythm or the 12-lead ECG, and specifically the corrected and uncorrected QT intervals were unaffected. As expected, there was a dose-dependent increase in the infused forearm blood flow was observed with all three agonists (acetylcholine, bradykinin and sodium nitroprusside) in the presence of either SRT2104 or placebo (p<0.0001 for all three agonists; figure 9). There were no differences in response to acetylcholine (p=0.318) and sodium nitroprusside (p=0.083) in the presence of SRT2104 compared with placebo. There was a reduction in bradykinin-induced vasodilatation with SRT2104 (7.753 vs 9.044, SRT2104 vs placebo, mean difference=−1.291, (95% CI −2.296 to −0.285, p=0.012)) with a trend for a period-by-treatment effect (p=0.092).
**FIGURE 9: FOREARM BLOOD FLOW RESPONSE SRT2104 VS PLACEBO IN PEOPLE WITH T2DM**

Effect of bradykinin (100, 300, 1000 pmol/min), acetylcholine (5, 10, 20 µg/min), and sodium nitroprusside (2, 4, 8 µg/min) on absolute forearm blood flow. Blue, placebo; red, SRT2104; closed circle and square, infused forearm blood flow; open circle and square, non-infused forearm blood flow. Data presented as mean±95% confidence interval.
3.7 Endogenous fibrinolysis and monocyte and platelet activation in people with T2DM

Post hoc analysis showed that dose-dependent increments were recorded in bradykinin-induced net t-PA antigen and activity release (p<0.0001 for both) in the infused arm, which is expected and well described. Estimated net PAI antigen release was reduced with SRT2104 compared with placebo (mean difference=−38.89 ng/100 mL tissue/min, (95% CI −75.47 to −2.305, p=0.038)) with a non-significant period effect for the plasma PAI-1 antigen concentrations (p=0.138). There were no differences in net PAI-1 activity release, or t-PA antigen and activity release (p>0.05 respectively). SRT2104 had no effect on markers of in vivo platelet or monocyte activation (Figure 10).
FIGURE 10: PMA RESPONSE, SRT2104 VS PLACEBO IN PEOPLE WITH T2DM

Data presented as mean±SD. PMA indicates platelet–monocyte aggregate; CD 11b- CD11b/macrophage-1 antigen; sCD40L, soluble CD40 ligand.
3.8 Metabolic effects in people with T2DM

During SRT2104 administration, body weight decreased by 0.93 kg (95% CI −1.72 to −0.15, 
p=0.0236) with a treatment-by-period effect (p=0.080). HbA1c rose by 0.48% (95% CI 0.26% to 
0.70%, p=0.004) after 28 days of SRT2104, as did plasma fructosamine, which rose by 33.41 
µmol/L (95% CI 20.24 to 46.58 µmol/L, p<0.001).

Post hoc analyses showed that the lipid profile did not change (table 5) although trends were 
noted towards lower concentrations of total cholesterol (−0.36 mmol/L, 95% CI −0.87 to 
0.16 mmol/L, p=0.158) and triglycerides (−0.22 mmol/L, 95% CI −0.53 to 0.09 mmol/L, 
p=0.150; table 5) with SRT2104. Non-esterified fatty acids (NEFAs or FFAs) rose by 0.09 mmol/L 
(95% CI 0.04 to 0.15 mmol/L, p=0.003) with a treatment-by-period effect (p=0.0087).
<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=14)</th>
<th>SRT2104 (n=15)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cholesterol [mean (SD)], mmol/L</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4.09 (0.743)</td>
<td>4.24 (0.714)</td>
<td></td>
</tr>
<tr>
<td>Day 28/56</td>
<td>3.91 (0.843)</td>
<td>3.65 (0.710)</td>
<td></td>
</tr>
<tr>
<td>Change from baseline</td>
<td>-0.18 (0.410)</td>
<td>-0.59 (0.766)</td>
<td></td>
</tr>
<tr>
<td>Mean difference (95% CI)</td>
<td>-0.36 (-0.87, 0.16)</td>
<td></td>
<td>0.158</td>
</tr>
<tr>
<td><strong>HDL cholesterol, [mean (SD)], mmol/L</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.07 (0.212)</td>
<td>1.08 (0.207)</td>
<td></td>
</tr>
<tr>
<td>Day 28/56</td>
<td>1.09 (0.210)</td>
<td>1.07 (0.249)</td>
<td></td>
</tr>
<tr>
<td>Change from baseline</td>
<td>0.03 (0.080)</td>
<td>0.00 (0.167)</td>
<td></td>
</tr>
<tr>
<td>Mean difference (95% CI)</td>
<td>-0.03 (-0.13, 0.07)</td>
<td></td>
<td>0.542</td>
</tr>
<tr>
<td><strong>LDL cholesterol, [mean (SD)], mmol/L</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.21 (0.585)</td>
<td>2.29 (0.552)</td>
<td></td>
</tr>
<tr>
<td>Day 28/56</td>
<td>2.05 (0.692)</td>
<td>1.90 (0.656)</td>
<td></td>
</tr>
<tr>
<td>Change from baseline</td>
<td>-0.15 (0.467)</td>
<td>-0.39 (0.656)</td>
<td></td>
</tr>
<tr>
<td>Mean difference (95% CI)</td>
<td>-0.23 (-0.65, 0.19)</td>
<td></td>
<td>0.259</td>
</tr>
<tr>
<td><strong>Triglycerides, [mean (SD)], mmol/L</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.81 (0.800)</td>
<td>1.93 (0.767)</td>
<td></td>
</tr>
<tr>
<td>Day 28/56</td>
<td>1.67 (0.497)</td>
<td>1.46 (0.445)</td>
<td></td>
</tr>
<tr>
<td>Change from baseline</td>
<td>-0.14 (0.568)</td>
<td>-0.46 (0.638)</td>
<td></td>
</tr>
<tr>
<td>Mean difference (95% CI)</td>
<td>-0.22 (-0.53, 0.09)</td>
<td></td>
<td>0.150</td>
</tr>
</tbody>
</table>

**Table 4: Effect of SRT 2104 on Serum Lipid Concentrations in People with T2DM**
3.9 Discussion and Conclusion

The cardiometabolic effects of SRT2104 have not been studied previously in people with T2DM. In this chapter safety data, venous occlusion plethysmography studies, and platelet monocyte studies are presented. This is also repeated in a different smokers cohort, with comparable results (see next chapter)

In the current T2DM cohort we found modest reductions in bradykinin-induced vasodilatation and net PAI-1 antigen release with SRT2014. This finding is unexpected, as murine and rat models of SIRT1 activation seem to promote improved endothelial function, whilst inhibition induced atherogenesis and thrombosis. We did observe some treatment-by-period interactions suggesting an inadequate washout period. Moreover, our sample size was modest and this could represent a chance finding. An alternative explanation is that SRT2104 affects a hitherto undescribed pathway which works via bradykinin alone and not acetylcholine or sodium nitroprusside. This study cannot answer that question presently but generates a hypothesis for further examination in the future.

We found a statistically significant decrease in PAI-antigen release. There was no significant treatment-by-period interaction in this case (p>0.1) which would suggest a potential fibrinolytic benefit in the treatment arm, although this was not confirmed by other measures of t-PA and PAI-1 activity, which were unaltered. Taken in aggregate, the effect of SRT2104 on cardiovascular measures are predominantly neutral although we acknowledge some inconsistent changes in isolated measures of vasomotor and fibrinolytic function.

Obesity is a major factor in the development of T2DM through the promotion of insulin resistance. Weight reduction is difficult to achieve in many patients and remains a major focus of therapeutic and lifestyle intervention. In the present study a striking reduction in weight over a
The loss of weight was associated with an apparent short-term deterioration in measures of glycaemic control. The elevations of HbA1c and fructosamine were puzzling. These observations contradict the findings in models of mice and other higher mammals. It is possible that the acute administration of SRT2104 mimics the early changes of fasting states (decreased insulin secretion), and that the subsequent effects of decreased insulin sensitivity take longer to develop. This may explain the weight loss that was observed and to a certain extent the lack of benefit to glycaemic control. Supporting this theory, a trend towards decreased peak insulin secretion to a glucose challenge was observed in a separate Sirtris pharmaceutical study (2-hour post glucose challenge insulin concentrations 143 mmol/L vs 117 mmol/L, placebo vs SRT2104, p=0.046) where a similar degree of weight loss (approximately 1 kg in 28 days) was observed (Baksi et al., 2014). It may be that in people with T2DM with established relative or absolute insulin deficiency, attenuation of insulin secretion, without a concomitant reduction in insulin resistance, is sufficient to permit a net rise in blood glucose.

We also observed an increase in non-esterified fatty acid values. There is some evidence in the scientific literature, linking SIRT1 modulation to an acute rise in insulin resistance. This is via the
inhibition of PPAR-γ and the mobilisation of fat from white adipose tissue (Picard et al., 2004). This may be responsible for the modest rise in non-esterified fatty acids seen after SRT2104 administration that could contribute to the change in glycaemic control, contributing to insulin resistance via intracellular competition with glucose metabolism (Karpe, Dickmann and Frayn, 2011).

People with T2DM have complex changes to other important glucoregulatory hormones such as glucagon and cortisol (Sims and Horton, 1968). During acute starvation, important metabolic adaptations occur such as gluconeogenesis and an increase in glucocorticoids. These metabolic adaptations maintain the supply of blood glucose to the brain during periods of prolonged starvation, but result acutely in a constellation of effects similar to insulin resistance. However, in people with established T2DM, these protective glucoregulatory mechanisms described above may exacerbate hyperglycaemia in the short term. Speculatively, these effects may be worsened by a caloric restriction mimetic.

Without further study, it is unclear whether long-term exposure to SRT2104 will ultimately be metabolically beneficial in patients with T2DM. The promotion of hyperglycaemia, which is undesirable, conflicts with the beneficial effects of weight loss, and it is unknown whether these effects are sustained over time. The finding of weight loss certainly raises the question as to whether the drug might be beneficial in individuals with impaired fasting glucose who are treatment-naive, and whether an exposure to the drug beyond 28 days would improve glycaemia once the downstream effects of increased mitochondrial activity and decreased adiposity are further established.

The principal aim of the present study was to establish whether SRT2014 could improve a range of markers of cardiovascular health in patients with T2DM. Ultimately, we did not demonstrate
any improvements in vasomotor or fibrinolytic vascular function or measures of platelet and monocyte activation.

An important question was whether the dose of SRT2104 was sufficient to have an effect. As yet, the pharmacokinetics and pharmacodynamics of SRT2104 are not fully elucidated. However, an effect on metabolic measures was demonstrated with substantial exposure to SRT2104, achieving high plasma SRT2104 concentrations in the current study. Even if adequate SIRT1 activation is assumed, its downstream effects may vary in different tissues in different disease states. It may be that in advanced states of disease associated with ageing, such as T2DM, the beneficial effects are abolished by higher caloric consumption, or that the benefits require treatment for longer than 28 days to become apparent. It might therefore be anticipated that a more prolonged exposure is required before any meaningful effect is apparent.

In conclusion, SRT2104 appears to be well tolerated in patients with T2DM but has no demonstrable beneficial effects on a range of measures of cardiovascular health. It is possible that while short-term exposure to SRT2104 is effective in mediating weight loss, it appears to be associated with an inadequate effect on diminishing insulin resistance, thereby causing deterioration in glycaemic control. Further larger-scale studies are required to confirm or refute these preliminary findings. Future perspectives, and further in-depth dissection of the methodology described here will be summarised in the final chapter.
Chapter 4

The cardiometabolic effects of SRT2104 in otherwise healthy smokers
4.1 Introduction

Smoking tobacco is one of the most important and modifiable risk factors for coronary vascular disease. Smoking is associated with an up to 7-fold increased risk of nonfatal myocardial infarction (Teo et al., 2006). It is associated with both accelerated atherosclerosis and a propensity for acute coronary thrombosis (Zieske et al., 2005). This is mediated through a variety of mechanisms including alterations in vascular, endothelial, fibrinolytic, and platelet function (Newby et al., 1999). The precise cellular mechanism for these effects is as yet unknown, but cigarette smoke is associated with oxidative stress, endothelial nitric oxide synthase acetylation, and increased endothelial cell senescence that has been associated with SIRT1 modulation (Arunachalam et al., 2010).

To date, there have been few clinical studies to assess the effect of SIRT1 activation in vivo in humans. The potential beneficial effects of SIRT1 activation have been described previously. Therefore, the aim of the present study was to examine the in vivo effects of a novel oral SIRT1 activator, SRT2104, on the lipid profile and vascular, endothelial, and platelet function in otherwise healthy cigarette smokers. We hypothesized that SIRT1 activation could improve the cardiovascular risk profile and reverse or improve the vascular and endothelial dysfunction associated with cigarette smoking.
4.2 Methods

The study was approved by the Research Ethics Committee, was given Clinical Trial Authorization by the MHRA, and carried out at the MHRA Phase 1 accredited Wellcome Trust Clinical Research Facility at the Royal Infirmary of Edinburgh, United Kingdom, between June 2010 and September 2011. Written informed consent was obtained from each volunteer, and the study was carried out in accordance with the Declaration of Helsinki. The study design was a Phase Ia, double-blind, randomised control trial of 28 days duration followed by a crossover. Pharmacokinetic studies, safety analyses, venous occlusion plethysmography and platelet monocyte studies were analogous to methods described in Chapters 2 and 3. In addition to all requirements asked of the people with T2DM cohort, the smoker cohorts were asked to avoid from smoking 10 hour prior to the vascular studies.

Twenty-four otherwise healthy male and female volunteers aged between 18 and 70 years who smoked $\geq$10 cigarettes daily for at least 1 year were eligible for the study. Exclusion criteria included the presence of significant comorbidities, chronic illness, renal or liver impairment, history of gastrointestinal diseases or surgeries influencing drug absorption, history of alcoholism, history of neoplastic disease within the last 5 years, a positive urinary test for recreational drugs, pregnancy, and participation in other clinical trials or blood donation within the last 3 months. Eligibility of participants including absence of relevant medical history was confirmed through a standardized form completed by the registered general practitioners after informed consent. Tests for pregnancy (serum HCG concentrations at screening and urinary HCG concentrations at study visits) were conducted on all female participants of childbearing age.
4.3 Results

Volunteers had a mean age of 38±13 years (median, 37 years) and relatively equal sex distribution (58% male) and were normotensive without any significant coexisting medical conditions. Volunteers had a body mass index of 25±4 kg/m^2 and a mean cigarette consumption of 17±6 cigarettes per day over 21±14 years. All 24 volunteers completed all study visits. Before drug administration, 1 subject was withdrawn from the study because of problems with venous access and was replaced.

4.4 Safety and Tolerability of SRT2104 in otherwise healthy smokers

Three hours post dose, mean plasma SRT2104 concentration was 1328±748 ng/mL after 28 days of active treatment. The median plasma SRT2104 concentration after 28 days of treatment was 366 ng/mL (Interquartile range (IQR) 940 ng/mL). The median time at which the maximum plasma concentration was observed (T_{max}) on day 28 of dosing was 3.05 hours, which coincided well with study measurements performed on those days (2 to 4 hours post dose). All subjects tolerated study medication well. Commonly reported side effects included headache (25%) and rhinitis, nasopharyngitis, and respiratory tract symptoms (17%). The reported adverse events were mild in intensity and resolved without any intervention or sequelae. There were no meaningful differences in the number of events between active treatment and placebo. There was only 1 reported serious adverse event in the study (SRT2104 arm): a traumatic facial bone fracture that was considered unrelated to SRT2104. Blood pressure and heart rate remained unchanged throughout the study. There were no effects on cardiac rhythm or the 12-lead electrocardiogram, and specifically there were
no effects on the corrected or uncorrected QT intervals. There were no clinically significant adverse effects involving any of the clinical haematological or biochemical analytes.

4.5 The effect of SRT2104 on Lipid Profile in Otherwise healthy smokers

The changes in lipid profile after treatment with SRT2104 is summarised in Table 6. There was a reduction in total and LDL cholesterol as well as triglyceride concentrations. There was no effect on HDL concentrations. Post-hoc analyses of the lipid date revealed that a statistically significant period effect was observed in the analysis of total and LDL cholesterol concentrations. Baseline values were higher in subjects receiving placebo in the first period. Regardless of treatment arm, the level of change from baseline was greater in period 2 for total and LDL cholesterol and less in period 2 for triglycerides. and the 7% fall in total cholesterol was attributable to the 11% fall in LDL cholesterol concentrations.
**Table 5: Effect of SRT2104 on Serum Lipid Concentrations in Otherwise Healthy Smokers**

* indicates p<0.05. HDL: high-density lipoprotein, LDL: low-density lipoprotein
4.6 Vasomotor Function

Non-infused forearm blood flow remained unchanged throughout all assessment periods, as were the predose measurements of blood flow in the infused arm between visits \((P>0.05)\). There was a dose-dependent increase in the infused forearm blood flow with all 3 agonists (acetylcholine, bradykinin, and sodium nitroprusside) in the presence of either SRT2104 or placebo \((P<0.0001\) for all 3 agonists; Figure 11). There were no significant differences in response to either endothelium-dependent or -independent vasodilators in the presence of SRT2104 compared with placebo (bradykinin, \(P=0.1169\); acetylcholine, \(P=0.1683\); sodium nitroprusside, \(P=0.9039\): placebo versus SRT2104).
FIGURE 11: FBF RESPONSE, SRT2104 VS PLACEBO IN OTHERWISE HEALTHY SMOKERS

Effect of bradykinin (100, 300, 1000 pmol/min), acetylcholine (5, 10, 20 μg/min), and sodium nitroprusside (2, 4, 8 μg/min) on absolute forearm blood flow. Blue, placebo; red, SRT2104; closed circle, infused forearm blood flow; open circle, noninfused forearm blood flow. Data presented as mean±95% confidence interval.
4.7 Endogenous Fibrinolysis and Platelet-Monocyte Aggregation studies

There was a dose-dependent increase in bradykinin-evoked net t-PA antigen and activity release ($P<0.0001$ for both) in the infused arm that was unaffected by SRT2104 ($P=0.3691$ and $P=0.1377$, placebo versus SRT2104, for net t-PA antigen and activity, respectively). Plasma PAI-1 antigen and activity concentrations were similar in both treatment arms ($P=0.8877$ and $P=0.6635$, placebo versus SRT2104, for plasma PAI antigen and activity, respectively). There were no changes in markers of platelet activation after treatment with SRT2104 (figure 12)
**Figure 12:** PMA Response SRT2104 vs Placebo in Otherwise Healthy Smokers

Data presented as mean±SD. PMA indicates platelet–monocyte aggregate; Mac-1, macrophage-1 antigen; sCD40L, soluble CD40 ligand.
4.9 Discussion and conclusion

In this randomized, double-blind, placebo-controlled crossover trial of otherwise healthy cigarette smokers, oral SRT2104 is safe and well tolerated at a dose of 2.0 g daily. Treatment with SRT2104 was associated with an 11% mean reduction in serum LDL cholesterol concentrations, but without demonstrable differences in vasomotor function, endothelial function, or platelet activation assessments compared with placebo. The favourable effects on lipid profile suggest that SIRT1 activation may have a beneficial role in patients at risk of developing or with established cardiovascular disease.

Elevated serum cholesterol is an established risk factor for atherosclerosis and coronary heart disease. In general, coronary heart disease risk is reduced by 2% to 3% for each 1% decrease in total cholesterol concentrations (Gotto, 1999). The mechanism of this lipid-lowering effect is yet to be elucidated but consistent with observations associated with SIRT1 activation in animals (Yu and Wang, 2012). One mechanism whereby SIRT1 activators could improve lipid profiles may involve a positive effect on liver X receptor proteins (LXRs), nuclear receptors involved in cholesterol and lipid homeostasis. Nuclear receptor LXR is a substrate for SIRT1 (Li et al., 2007). These initial findings raise the possibility that SIRT1 activation could be a useful addition to therapy to current lipid-lowering strategies, leading to improvements in cardiovascular disease pathophysiology and therefore possibly to clinical outcomes. However, the findings in otherwise healthy smokers were not seen in people with T2DM, although a similar trend was observed. As has already been described in the previous chapter, SIRT1 activation can manifest in inhibition of PPARγ and this may explain the increase in non-esterified fatty acids seen in people with type 2 diabetes. If this finding is a true signal, it may reflect the fact that SIRT1 activation...
at different receptors can produce inconsistent results, or that people with T2DM have a more severe phenotype of aging and caloric excess than our current cohort of otherwise healthy smokers. Alternatively, our inconsistent findings may be related to the fact that these studies are underpowered, and that there was an inadequate washout period. These may attenuate any small signals from the experimental studies. Though a post hoc statistical correction for this is attempted by correcting for a period and treatment effect, this is not a substitute for a full washout period.

There are currently no published data directly examining the effects of SIRT1 activation on vasomotor function or endogenous fibrinolysis in vivo in humans. Despite the several beneficial effects of SIRT1 activation on endothelial function observed in preclinical in vitro studies we were unable to demonstrate improvements in vascular, endothelial, or platelet function in these otherwise healthy smokers.

There are various potential explanations why we did not observe an improvement with SRT2104 in these parameters in the current study. One possibility is that the SRT2104 exposure achieved in this study did not lead to adequately consistent SIRT1 activation, which would be required to reverse the vascular and endothelial dysfunction in these smokers. At present, there is no current biomarker for SIRT1 activation in humans. Therefore, we do not have a good understanding of the pharmacokinetic-pharmacodynamic relationship between SRT2104 drug exposure and SIRT1 activation. Previous studies have shown inter-subject variability of SRT2104 levels, and these two factors alone may be a significant confounding factor in this study (Hoffmann et al., 2013).
Although we were able to demonstrate improved lipid profiles in the smokers cohort, it is unclear whether the same exposure levels would also lead to improved vascular and endothelial function. There are at least 70 known substrates for SIRT1. We speculate that SRT2104 may differentially modulate certain pathways than others, depending on the precise interaction between SRT2104 and the substrates as well as the concentration level and activity of the substrates in a particular disease state. It is also possible that certain abnormalities may be reversed more readily than others through SIRT1 activation. Further, although a 28-day exposure may be adequate for observing improvement in lipid metabolism, longer treatment may be required to reverse some of the vascular and endothelial abnormalities. The study design and power cannot answer the above questions.

In conclusion, SRT2104 has been demonstrated to be safe and well tolerated in otherwise healthy cigarette smokers and provides potential positive effects on lipid profiles. There were no demonstrable effects on forearm vasodilation studies or platelet function compared with placebo.
Chapter 5

The effect of SRT2104 on arterial stiffness in otherwise healthy smokers and in people with T2DM
5.1 Introduction

As described in previous chapters, arterial stiffness increases with age and is recognised to be an independent predictor of cardiovascular risk. Specifically, raised pulse pressure and aortic stiffness are associated with increased risk of coronary vascular events and overall mortality (Vlachopoulos and Stefanadis, 2010). Markers of central aortic stiffness is correlated with coronary atherosclerosis plaque load (McLeod et al., 2004).

It is well established that cigarette smoking and diabetes mellitus are significant risk factors for the development of cardiovascular disease. There are strong associations between diabetes and cigarette smoke exposure with increased aortic stiffness, endothelial dysfunction and cardiovascular disease (Stehouwer and Ferreira, 2008; Roos et al., 2011; Rehill et al., 2006). Ageing is associated with structural changes to arteries, mediated by fraying of elastin structures within the intima media, and a compensatory increase in collagen content and fibrosis. This process of biological ageing appears to be accelerated or have a more severe expression in the presence of diabetes mellitus. Vascular change induced by cigarette smoke is considered to be a model of accelerated vascular ageing (Díez, 2007).

CR has been shown to improve age-related arterial stiffness in animal models, probably related to improvement in endothelial NO and a reduction in ROS (Weiss and Fontana, 2011). SIRT1 activation is thought to mediate many of the beneficial effects of caloric restriction (Bordone and Guarente, 2005). The current hypothesis, therefore, was that activation of SIRT1 may inhibit this process of vascular ageing and improve arterial stiffness.

The aim of this study was to assess the effect of the oral SIRT1 activator, SRT2104, on measures of arterial compliance in otherwise healthy cigarette smokers and patients with T2DM.
5.2 Methods

Twenty-four otherwise healthy cigarette smokers and 15 participants with stable T2DM, aged between 18 and 70 years, were eligible for the study. Healthy cigarette smokers were required to have smoked ≥10 cigarettes daily for at least 1 year. Participants with T2DM were non-smokers and were selected on the basis of having a diagnosis of T2DM for at least 6 months prior to inclusion in the study, with no change in medications having been made for at least the preceding 3 months, and a HbA1c<9% (75 mmol/mol) on screening. Exclusion criteria included the presence of significant comorbidities, chronic illness, renal or liver impairment, history of gastrointestinal diseases or previous surgical procedures that would influence drug absorption, history of alcoholism, history of neoplastic disease within the last 5 years, a positive urinary test for recreational drugs, pregnancy and participation in other clinical trials or blood donation within the last 3 months. People with T2DM on ACE inhibitors, antiplatelet or anticoagulant therapies were excluded from the study. Tests for pregnancy (serum HCG concentrations at screening and urinary HCG concentrations at study visits) were conducted on all female participants of childbearing potential.

This was a prospective double-blind randomised placebo-controlled cross-over study. Following ethical approval, and informed consent as per the Helsinki declaration, participants were randomised to receive 2.0 g daily of oral SRT2104 or matched placebo (Sirtris, a GSK company, Massachusetts, USA) for a 28-day period, followed by cross-over to the alternate study arm for a further 28 days, giving a total dosing duration of 56 days. An end of study visit was conducted at day 70 with a telephone call follow-up on day 86. Measures of arterial stiffness were undertaken prior to and at the end of each 28-day trial period. All studies were performed in a quiet temperature controlled (22–
25°C) room. Participants were fasted and asked to refrain from smoking for 10 h, and abstain from caffeine and alcohol for 24 h prior to assessment. Participants remained supine for at least 30 min before any recordings were started. Systolic and diastolic blood pressures were recorded using a non-invasive oscillatory sphygmomanometer (Omron705 IT, Omron Healthcare Europe, the Netherlands).

Pulse wave analysis of the radial artery was performed at the wrist using micromanometer applanation tonometry (Millar Instruments, Texas, USA) and the SphygmoCor system (AtCor Medical, Sydney, Australia) in accordance with the manufacturer's recommendations. To briefly re-iterate the description in the previous section, pulse wave analysis derives an aortic pulse pressure waveform from the radial artery wave via a mathematical transfer function. The arterial pressure waveform is a composite of the forward pressure wave created by ventricular contraction and a reflected wave generated by peripheral vascular resistance. The augmentation pressure is the pressure difference between the second and first systolic peaks. The augmentation index, which is augmentation pressure as a percentage of the pulse pressure, is a measure of systemic arterial stiffness and wave reflection. Corrected augmentation index represents the augmentation index corrected for heart rate, which is more accurate. The time to wave reflection declines with increasing arterial stiffness, and provides a surrogate measure of aortic pulse wave velocity.

In the current methodology, at least three independent waveform analyses were obtained from each participant, with measurements only accepted on meeting SphygmoCor quality control criteria. Pulse wave velocity was calculated by measuring the time for the pulse wave to travel between the carotid and femoral arteries. The operator performing the analysis was kept constant for each participant throughout the study.

Data were analysed, where appropriate, using repeated measure analysis of covariance on the change from baseline for all parameters. Initially, analyses were conducted separately on cohorts. As a result
of the small sample size and similar trends for the two cohorts, these data were pooled *post hoc*. Treatment differences were investigated in a model adjusting for baseline, period, treatment by period and treatment by cohort using SAS for UNIX (V.9.1.3 or higher) (SAS Institute, Cary, North Carolina, USA). Unless stated otherwise, values are expressed as mean±SD. Tests for treatment effect were two-sided with a significance level of 0.05.

### 5.3 Results

#### 5.3.1 Baseline Characteristics

Participants in the study had a mean age of 45±15 years and were predominantly male (68%). Participants in the T2DM cohort were older (mean age 58±8 years) when compared with the participants in the otherwise healthy smokers group (mean age 38±13 years). All participants were normotensive with comparable systolic and diastolic blood pressures at baseline (Table 7).
<table>
<thead>
<tr>
<th></th>
<th>Otherwise Healthy Smokers n= 24</th>
<th>People with Type 2 Diabetes n= 15</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean age (years)</strong></td>
<td>38±13</td>
<td>58±8</td>
</tr>
<tr>
<td><strong>Sex (numbers, (percentage))</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14 (58)</td>
<td>13 (87)</td>
</tr>
<tr>
<td>Female</td>
<td>10 (42)</td>
<td>2 (13)</td>
</tr>
<tr>
<td><strong>Baseline Blood Pressure (mm Hg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>129±6</td>
<td>133±7</td>
</tr>
<tr>
<td>Diastolic</td>
<td>77±2</td>
<td>80±3</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>68±1</td>
<td>77±5</td>
</tr>
<tr>
<td>Body Mass Index (kg/M²)</td>
<td>25±4</td>
<td>30±4</td>
</tr>
<tr>
<td><strong>Smoking History</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cigarettes per day</td>
<td>17±6</td>
<td>Not applicable</td>
</tr>
<tr>
<td>No. of Pack years</td>
<td>16</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Urinary Cotinine concentration (ng/mL)</td>
<td>1352±950</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Baseline Glycated haemoglobin (%/ mmol/mol)</td>
<td>Not tested</td>
<td>7.4±0.8 % or 57 ± 8 mmol/mol</td>
</tr>
<tr>
<td></td>
<td>Otherwise Healthy Smokers n= 24</td>
<td>People with Type 2 Diabetes n= 15</td>
</tr>
<tr>
<td><strong>Concomitant Medications (numbers (%))</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antiplatelets</td>
<td>Not applicable</td>
<td>5 (33%)</td>
</tr>
<tr>
<td>Angiotensin-II Receptor Blockers</td>
<td>Not applicable</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>Statins</td>
<td>Not applicable</td>
<td>13 (86%)</td>
</tr>
<tr>
<td>Metformin</td>
<td>Not applicable</td>
<td>9 (60%)</td>
</tr>
<tr>
<td>Sulfonylureas</td>
<td>Not applicable</td>
<td>5 (33%)</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>Not applicable</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>Exenatide</td>
<td>Not applicable</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>Insulin</td>
<td>Not applicable</td>
<td>2 (13%)</td>
</tr>
</tbody>
</table>

**TABLE 6: A COMPARISON OF BASELINE CHARACTERISTICS OF THE T2DM AND OTHERWISE HEALTHY SMOKERS COHORTS**
5.4 Blood pressure

Resting systolic and diastolic blood pressures remained unchanged throughout the study with no significant differences between treatment and placebo treatment periods.

5.5 Pulse wave analysis and velocity

In a combined analysis of otherwise healthy cigarette smokers and participants with type 2 diabetes, a reduction in the augmentation pressure was observed in participants receiving SRT2104 compared with placebo (mean change from baseline: SRT2104−1.60 (5.304) vs placebo−0.06 (4.205); p=0.0273) and a trend towards improvement in the augmentation index (mean change from baseline in AIx: placebo−0.64 (8.361) vs SRT2104−3.47 (9.728); p=0.0813) and the corrected augmentation index (mean change from baseline AIx75: placebo−2.2−(7.453) vs SRT2104−4.84 (9.299); p=0.0747) (figure 13 (A)). Pulse wave velocity and time to wave reflection remained unchanged between placebo and treatment arms (p>0.05 for both parameters; figure 13 (B)). The effects of SRT2104 administration on measures of arterial compliance were consistent across the two cohorts. For example, in the SRT2104 arm, mean augmentation index at 75 bpm was reduced for healthy smokers and participants with T2DM (−4.97 vs −4.63, respectively). Measures of arterial compliance and stiffness for the individual cohorts are summarised in Table 8. A statistical interaction between cohort and treatment was not observed (p>0.05 for all variables tested).
**FIGURE 13: EFFECT OF TREATMENT WITH SRT2104 ON MEASURES OF ARTERIAL COMPLIANCE IN OTHERWISE HEALTHY CIGARETTE SMOKERS AND PARTICIPANTS WITH T2DM—CHANGE FROM BASELINE.**

(A) Pulse wave analysis—augmentation index, corrected augmentation index, augmentation pressure and time to wave reflection. (B) pulse wave velocity. Solid column: placebo; checked column: SRT2104. (C) baseline parameters of measures of arterial compliance—combined data.
**TABLE 7: All parameters of arterial compliance in the otherwise healthy smokers cohort and the T2DM cohort**

<table>
<thead>
<tr>
<th></th>
<th>Augmentation Index (%)</th>
<th>Corrected Augmentation Index (%)</th>
<th>Augmentation Pressure (mmHg)</th>
<th>Time to Wave Reflection (ms)</th>
<th>Pulse Wave Velocity (m/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Mean (SD)</strong></td>
<td><strong>Baseline</strong></td>
<td><strong>Day 28/56</strong></td>
<td><strong>Baseline</strong></td>
<td><strong>Day 28/56</strong></td>
</tr>
<tr>
<td><strong>Otherwise Healthy Smokers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>16.73 (18.91)</td>
<td>12.58 (18.09)</td>
<td>11.19 (15.35)</td>
<td>6.39 (17.60)</td>
<td>5.79 (5.46)</td>
</tr>
<tr>
<td>SRT2104</td>
<td>11.30 (18.02)</td>
<td>9.73 (21.51)</td>
<td>5.64 (14.19)</td>
<td>3.68 (19.97)</td>
<td>4.89 (7.03)</td>
</tr>
<tr>
<td><strong>Subjects with type 2 diabetes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>21.34 (10.04)</td>
<td>18.35 (11.80)</td>
<td>22.28 (5.15)</td>
<td>15.77 (10.84)</td>
<td>9.97 (8.25)</td>
</tr>
<tr>
<td>SRT2104</td>
<td>12.96 (12.36)</td>
<td>15.63 (11.81)</td>
<td>12.46 (7.91)</td>
<td>13.07 (10.45)</td>
<td>5.89 (5.91)</td>
</tr>
</tbody>
</table>
5.6 Discussion and conclusions

This study demonstrates that SRT2104, may improve markers of arterial stiffness in otherwise healthy cigarette smokers and in people with T2DM, without affecting resting measures of blood pressure.

In the present study, a 28-day period of treatment with the oral SIRT1 activator SRT2104 was associated with a reduction in augmentation pressure and trends towards improvement in augmentation index and corrected augmentation index. Augmentation pressure and index are measures of arterial compliance and wave reflection from small to medium sized arteries. As such, they can be influenced by endothelial function and a number of other dynamic and functional factors, such as heart rate and peripheral vascular tone (Laurent et al., 2006). This finding of improvement in dynamic measures of arterial stiffness following short-term administration of SRT2104 is potentially predicted by preclinical studies which have demonstrated improved endothelial function with SIRT1 activation (Donato et al., 2011; Csiszar et al., 2008). Pulse wave velocity, on the other hand, is a more direct measure of arterial stiffness that is determined by the structural composition of the arterial wall, such as decreasing elastin and increasing fibrosis. Changes in pulse wave velocity are therefore expected to happen more gradually. In the present study, a change in pulse wave velocity was not observed with SRT2104 administration. This is perhaps not surprising given the short-time period of exposure to SRT2104 (28 days) and the brief period of observation. If the benefits of SRT2104 are indeed sustained over time, we speculate that an improvement in pulse wave velocity might be anticipated with a longer period of treatment, to allow the attenuation of senescence processes, and therefore reduce the maladaptive structural changes in the larger arterial tree.

Although these favourable findings have potentially exciting therapeutic implications, some limitations should be noted. Some outcomes did not achieve statistical significance, and this is likely
attributable to the trial being designed specifically to examine the acute effects of treatment with SRT2104. A longer period of treatment may be required for benefits to emerge on variables such as pulse wave velocity that involve structural changes in the arterial wall.

Additionally, the sample sizes of the two groups examined were small, and a post-hoc pooled analysis was performed to improve the power of the study. Two heterogenous populations were studied in this trial, and the mechanisms of vascular dysfunction may be very different. Having noted these limitations, the direction of beneficial effects on treatment with SRT2104 was similar between the two groups, suggesting a consistency of effect.

In conclusion, the present study is suggestive that treatment in SRT2104, may lead to an improvement in measures of arterial compliance in otherwise healthy cigarette smokers and people with type 2 diabetes. Given that arterial stiffness and endothelial function are key factors in predicting cardiovascular outcomes, identification of novel pharmacological means of improving these predictive parameters is tantalising. More adequately powered studies with more clearly defined primary outcomes, and potentially longer treatment of SRT2104, would help in trying to confirm these exciting preliminary findings.
Chapter 6

The effect of hypoglycaemia on myocardial blood flow and injury in adults with and without T1DM
Cardiovascular disease is the main cause of death in people with T1DM and T2DM (Rawshani and Gudbjörnsdottir, 2017). Tight glycaemic control may be beneficial in people with T1DM, but is more controversial in people with T2DM. Tight glycaemic control is associated with an increased risk of hypoglycaemia.

The physiological counter-regulatory response to hypoglycaemia is well described, and provokes a profound autonomic response in humans. The release of catecholamines causes an increase in heart rate, and regional blood flow changes occur to maintain blood glucose to the brain and vital organs. Experimentally induced hypoglycaemia causes an increase in cardiac output, stroke volume and contractility (Fisher et al., 1990). T1DM is associated with increased arterial stiffness, which may interfere with coronary perfusion (Sommerfield et al., 2007). The cardiac responses in hypoglycaemia may be tolerated in individuals without diabetes, but may precipitate ischaemia in people with type 1 diabetes. Endothelial dysfunction has been measured in peripheral arteries and used as a surrogate indicator of coronary endothelial function. However, differences exist between peripheral and coronary arterial endothelium, such as the presence of shunt vessels (Baumgart et al., 1998; Khan et al., 2008). Direct measurement of coronary vasomotor function may therefore provide a more accurate estimate of potential cardiovascular impairment resulting from limited vascular responsiveness. Measurement of coronary microvascular dysfunction by calculation of the coronary flow reserve (CFR) is the preferred investigative technique (Hirata et al., 2007b). CFR is a measure of the capacity of the coronary circulation to increase flow during maximal resistance vessel vasodilatation. Maximal hyperaemia is achieved by the intravenous infusion of adenosine (Baumgart et al., 1998) and coronary flow velocity can be measured non-invasively by transthoracic Doppler
echocardiography (Hozumi et al., 1998; Okayama et al., 2002). CFR is reduced in patients with T1DM during euglycaemia (Pitkanen et al., 1998) and in individuals with T1DM with retinopathy (Akasaka et al., 1997). It is unclear how hypoglycaemia affects CFR.

Cardiac troponins are a biomarker of all myocardial injury. In recent years, the introduction of a highly sensitive cardiac troponin I assay (hs-cTnI) and lowering of the diagnostic threshold has improved outcomes for people with type 1 myocardial infarction (Mills et al., 2011). It has also provided prognostic information for risk stratification of long term outcomes in cohorts of people without myocardial infarction (Chapman et al., 2018). Due to its increased diagnostic sensitivity, the use of hs-cTnI in an experimental setting has the potential to detect sub-acute myocardial injury.

People with T2DM often have multiple cardiovascular risk factors that are confounders in attempts to investigate hypoglycaemia as a causative mechanism for cardiovascular morbidity and mortality. People with T1DM are less likely to have these additional risk factors, especially at a younger age and if recently diagnosed. We hypothesise that hypoglycaemia will adversely affect myocardial blood flow and cause myocardial injury, and that these effects will be more pronounced in people with T1DM.
6.2 Methods

6.2.1 Study Participants

Young healthy male adults with T1DM with no microvascular complications or cardiovascular risk factors were recruited from out-patient clinics in NHS Lothian. Healthy non-diabetic males, matched for age, were recruited for the study via responses to poster advertisements and a database of healthy volunteers. Only male subjects were studied to avoid the confounding effect of the variability of coronary flow reserve that occurs during the menstrual cycle (Hirata et al., 2001). Exclusion criteria included co-existent systemic disease or malignancy, chronic alcoholism or psychiatric disorder, any history of cardiac conduction abnormality, impaired awareness of hypoglycaemia (as assessed by the method of Gold et al (Gold, MacLeod and Frier, 1994)), past history of severe hypoglycaemia, and any evidence of overt microvascular complications including retinopathy and neuropathy and none had microalbuminuria.

A total of 17 male subjects with T1DM and 10 healthy non-diabetic individuals were studied (Table 9). Participants with T1DM had reasonable glycaemic control typical of the Scottish population with T1DM (average HbA1c 8±1% or 64 ± 10 mmol/mol), with a median duration of diabetes of 15 years, range 2 to 35 years. The study was conducted with the written informed consent of all subjects, the approval of the Lothian Medical Research Ethics committee, and in accordance with the Declaration of Helsinki.
<table>
<thead>
<tr>
<th></th>
<th>Type 1 diabetes participants</th>
<th>Non-diabetic participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=17</td>
<td></td>
<td>n= 10</td>
</tr>
<tr>
<td>Average age (years) (median, range)</td>
<td>30 (20-35)</td>
<td>24.5 (21-33)</td>
</tr>
<tr>
<td>(p=0.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (%M)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²) (mean ±SD)</td>
<td>25.9±2.1</td>
<td>24.2±2.7</td>
</tr>
<tr>
<td>(p= 0.11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (%/mmol/mol) (mean ± SD)</td>
<td>8±1.1</td>
<td>n/a</td>
</tr>
<tr>
<td>(64 ± 11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of Diabetes (years) (median, range)</td>
<td>15 (2-35)</td>
<td>n/a</td>
</tr>
</tbody>
</table>

**Table 8: Clinical characteristics of participants with and without T1DM**
6.2.2 Study Design

Participants attended for two study visits, performed on separate days at least two weeks apart to avoid any potential carry-over effects. Two experimental conditions, hypoglycaemia (blood glucose 2.5 mmol/L) and euglycaemia (4.5 mmol/L), were studied in a prospective randomised open-label blinded endpoint (PROBE) cross-over study.

Participants attended in a fasting state, having been asked to abstain from consumption of caffeine-containing food and beverages for 24 hours. Venous cannulae were inserted for intravenous infusion of dextrose and insulin, and blood sampling. The latter was obtained within a heated box (50 °C) to arterialise venous blood. A modified version of the hyperinsulinaemic glucose clamp was employed (DeFronzo and Andres, 1979). During a run-in period, arterialised blood glucose was maintained at 4.5 mmol/L for 20 min. A YSI 2300 blood glucose analyser was used to analyse blood samples taken at 5-min intervals. Blood glucose was either maintained at 4.5 mmol/L throughout (the euglycaemia condition), or lowered over 20 min to 2.5 mmol/L (the hypoglycaemia condition), and maintained at this level for 30 min before restoration of euglycaemia. During the glucose clamp, the participants underwent blood sampling and an ultrasound examination by a trained ultrasound operator, using a well described technique (Okayama et al., 2002; Hirata et al., 2001). The timepoints were labelled as baseline, experimental (either euglycaemia or hypoglycaemia - blinded to the sonographer), and recovery (Figure 14). Continuous electrocardiographic monitoring and regular blood pressure monitoring were performed during the study.
**FIGURE 14: SUMMARY OF STUDY, CHAPTER 6**

Diagram of the two sessions of the study, with euglycaemia at the top, and hypoglycaemia glucose clamp
6.2.3 Coronary Flow Velocity Measurements

During each study condition, the left anterior descending coronary artery was visualised by transthoracic echocardiography. Transthoracic Doppler echocardiography was used for a non-invasive estimation of coronary flow velocity (CFV), and maximal coronary vasodilatation was induced with an adenosine infusion to allow calculation of CFR. Imaging of the LAD artery and measurement of coronary blood flow velocity was performed using a 7.0 MHz transducer (Acuson Sequoia 512, Siemens Medical Solutions, Berkshire, UK). Baseline spectral Doppler signals were recorded initially in the distal portion of the LAD coronary artery over five cardiac cycles at end-expiration. To measure CFR, intravenous adenosine was administered (0.14 mg/kg/min; Adenocor) for up to 4 min (Baumgart et al., 1998) to record spectral Doppler signals during hyperaemic conditions. Coronary velocities were measured at baseline and at peak hyperaemic conditions from the Doppler signal recordings. Measurements were averaged over three cardiac cycles. CFR was defined as the ratio of hyperaemic to basal velocities (chapter 2). Blood pressure was recorded at baseline, during adenosine infusion and at recovery. CFR was calculated at baseline, during the experimental phase (0-20 min) and in the recovery phase.
6.2.4 High-sensitivity Cardiac Troponin I Concentration

Blood samples were taken prior to assessment of CFR, during the experimental hyperinsulinaemic clamp, and during the recovery period. (Figure 14). High-sensitivity cardiac troponin I concentrations were determined using the ARCHITECT STAT high-sensitive troponin I assay (Abbott Laboratories, Abbott Park, IL). This is the first clinically approved high-sensitivity troponin I assay with excellent precision at very low concentrations. The limit of detection is 1.2 ng/L and precision profiling in our own laboratory demonstrates an inter-assay coefficient of variation (CV) of <10% at 5 ng/L. The upper reference limit or 99th centile is 16 ng/L for women and 34 ng/L for men (Shah et al., 2015b).

6.2.5 Statistical Methods

The effects of hypoglycaemia on coronary artery blood flow were assessed by generalised liner mixed model analysis, with the experimental condition (hypoglycaemia and euglycaemia) as a within-subjects factor, and diabetes status as a between-subjects factor. Using results from a previous study using a similar technique (Hirata et al., 2007a); accepting 95% as statistically significant, with a SD of 0.5, a sample size of 12 allows an 80% chance of detecting a 0.57 difference in CFR, which is considered clinically relevant. Statistical significance was taken as a two-sided p<0.05. Unless specifically stated, results are mean ± standard deviation. The hs-cTnI data were log-transformed due to the skewed distribution. Analysis of the results was performed using GraphPad Prism (Version 7 for Windows, GraphPad Software, La Jolla California)
6.3 Results

Table 9 summarises the clinical characteristics of the volunteers. The study participants were young males with normal BMI. During hypoglycaemia, heart rate and systolic blood pressure rose in both groups, and diastolic blood pressure fell (Table 10) During the hypoglycaemic clamp experimental session, the mean glucose nadir was 2.34 ± 0.21 mmol/L (p<0.001 compared to baseline), indicating adequate hypoglycaemia to stimulate a symphto-adrenal counter-regulatory response. Heart rate, systolic and mean blood pressure during euglycaemia did not change in either group during euglycaemic clamping. During hypoglycaemia, there was an apparent trend toward increased heart rate and systolic BP in both groups, and a decrease in diastolic BP (Table 10). In the T1DM group, the increments in heart rate and systolic blood pressure did not reach statistical significance. In the non-diabetic group, the heart rate increased from 71 ± 9 beats per minute (bpm) to 78 ± 8 bpm. (p=0.02) whilst the systolic BP increased from 116 ± 11 mm Hg to 124 ± 12 mm Hg (p=0.001). We examine the possible reason for the differences in the two cohorts shortly.
<table>
<thead>
<tr>
<th></th>
<th>Euglycaemic Clamp</th>
<th>Hypoglycaemic clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Experimental</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.95±0.65</td>
<td>4.70±0.42</td>
</tr>
<tr>
<td>Subjects with type 1 diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>73±14</td>
<td>73±8</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>127±14</td>
<td>131±13</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>74±6</td>
<td>71±6</td>
</tr>
<tr>
<td>Non-diabetic subjects</td>
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<td></td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>74±13</td>
<td>76±10</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>124±15</td>
<td>126±13</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>69±6</td>
<td>69±6</td>
</tr>
</tbody>
</table>

**Table 9:** Plasma glucose, heart rate, systolic, and diastolic blood pressure values during hypoglycaemia and euglycaemia in the groups of participants, with and without T1DM
6.3.1 Coronary Flow Velocities, Reserve and cardiac troponin I

No differences were observed between the coronary flow velocities of the groups with T1D and the non-diabetic controls at baseline (Figure 15). During hyperaemia after adenosine infusion, a trend for lower coronary flow velocities was observed in people with T1D, but this did not reach statistical significance (Figure 15).

During euglycaemia, there was a non-significant trend for lower coronary flow reserve in young adults with T1D, compared to people without diabetes (3.66 ± 0.47 versus 3.92 ± 0.85). During hypoglycaemia, coronary flow reserve trended non-significantly lower in those with T1D than in the non-diabetic participants (3.54 ± 0.47 versus 3.89 ± 0.89) (Figure 16). From the generalised linear mixed model analysis, no statistical significance was reached for euglycaemia or hypoglycaemia affecting CFR (p=0.31). There was also no statistically significant trend for diabetes status affecting CFR (p=0.23).
FIGURE 15: CORONARY FLOW VELOCITY, REST AND ADENOSINE IN SUBJECTS WITH AND WITHOUT T1DM

Summary of coronary flow velocities, during rest and hyperaemia adenosine, and between euglycaemia and hypoglycaemia, in T1DM group (blue) and subjects without diabetes (red). Data shown as mean and SD.
**Figure 16** Summary of CFR during experimental conditions in subjects with and without T1DM

CFR (mean±SD) in participants with T1DM and participants without diabetes under euglycaemic and hypoglycaemic conditions. Blue T1DM subjects, Red – subjects without T1DM
Log transformation of highly sensitive troponin during baseline, experimental condition and recovery. T1DM group in blue and subjects without diabetes in red.
6.4 Discussion

The present study used a well validated non-invasive method to examine the effect of acute hypoglycaemia on real-time coronary arterial flow in adults with and without type 1 diabetes, with the coronary flow ratio (CFR) being measured using transthoracic Doppler echocardiography. During acute hypoglycaemia, young male adults with T1D had a lower coronary flow reserve compared with an age-matched group of non-diabetic males. A modest decline in CFR was well tolerated in young men with T1D who were otherwise healthy with no evidence either of microvascular complications or of coronary heart disease; they retained normal coronary reserve.

The direct effects of hypoglycaemia per se on cardiac function have been difficult to elucidate as insulin itself exerts direct effects on the heart. Fisher et al (Fisher et al., 1987) showed that administration of insulin caused an immediate increase in left ventricular ejection fraction and provoked sympathetic activation, both of which occurred before any decline in blood glucose. As blood glucose fell progressively, these responses became more pronounced, with the maximal changes coinciding with the glucose nadir. One strength of the present study is the ability to use a non-invasive real-time assessment of coronary flow reserve during acute hypoglycaemia. By using a hyperinsulinaemic glucose clamp the present study was able to compare CFR during euglycaemia and hypoglycaemia, and between T1DM and the non-diabetic state. The changes observed are primarily related to a low blood glucose and to counterregulatory mechanisms, rather than to the intravenous infusion of insulin per se. Furthermore, this study provides a direct assessment of coronary vasomotor function which excludes other potential confounders such as hypertension or microvascular disease.
The main limitation is a small sample size. This is related to the robust exclusion criteria, but also partially with study design, specifically the use of adenosine to induce maximal hyperaemia of the coronary blood vessels, and also to the demands of hypoglycaemia and euglycaemia glucose clamps, which required two procedures at least a fortnight apart. The rationale for using adenosine is its short half-life, which meant that the CFR values from one measurement to the next was not confounded by residual effects of adenosine. The disadvantage of this approach was that adenosine may be poorly tolerated because of unpleasant side-effects of chest tightness and facial flushing, which reduces the willingness of volunteers to participate. The study was designed to have the two experimental conditions separated by at least a two-week interval. Because each procedure was onerous, recruitment was affected by this study design.

It is of interest to note that the present study did not reproduce the findings of a previous investigation by Rana and colleagues, which to our knowledge is the only other published research that has explored the direct effect of acute hypoglycaemia on the myocardial circulation. Rana et al measured myocardial blood flow reserve during euglycaemia and acute hypoglycaemia in adults of both sexes, 28 with, and 19 without T1D, using sequential hyperinsulinaemic glucose clamps and dipyridamole-induced stress echocardiography (Rana et al., 2011). Their participants had a wider age range, and included people with microvascular disease. Hypoglycaemia was reported to induce a significant fall in myocardial blood flow reserve in both groups, with lower values being observed in the group with T1D at all times of measurement. A statistically significant association was observed with microvascular complications.
The interpretation of the results of the present study merits comparison with the study methodology used by Rana et al. In contrast to the present study, no time interval was allowed between the initial euglycaemia and the subsequent induction of hypoglycaemia, so that myocardial blood flow reserve rose during the period of protracted euglycaemia, which may have influenced the effect on the subsequent hypoglycaemia (Rana et al., 2011). Furthermore, the long half-life of dipyridamole may potentially have affected any positive findings. In the study by Rana et al, randomisation of the order of the euglycaemic and hypoglycaemic clamps was not applied and may therefore have introduced observer bias. It can therefore be argued that the present study provides a more direct model of coronary flow reserve with fewer confounding factors such as the presence of microvascular disease and a possible residual effect of dipyridamole.

**Analysis and Interpretation of Results**

In the present study no change in CFR was observed during hypoglycaemia. Although no change in CFR was also observed during euglycaemia, a trend towards a decline in CFR was observed in the participants, consistent with previous reports (Pitkanen et al., 1998). The lowest CFR values were observed in people with T1D during hypoglycaemia. Unexpectedly, the increments in heart rate and systolic blood pressure in the T1D group did not achieve statistical significance (Table 9). This may be because the study sample size was too small and insufficiently sensitive to detect small variations in blood pulse and blood pressure. An alternative interpretation is that some individuals with T1DM had
some degree of autonomic dysfunction, which could be contributing to the lower CFR values observed in the group with T1DM. Formal assessment of autonomic function was not measured in the participants so this possibility could not be answered definitively.

With respect to the absence of any change in the highly sensitive troponin values, this could be related to the close temporal proximity to the hypoglycaemia and it is possible that measurement in the immediate ‘recovery’ stage is too early to detect a rise in plasma troponin. In retrospect, a later measurement of troponin may have been of greater value in determining whether hypoglycaemia causes any myocardial insult.

**Clinical implications of the results**

The results of the present study imply that the putative harm of hypoglycaemia is not mediated solely through coronary vasomotor dysfunction. Other additive harmful factors associated with hypoglycaemia may be required, such as the promotion of pro-thrombotic mechanisms (Wright et al., 2010; Razavi Nematollahi et al., 2009), or pro-dysrhythmic factors, as have been demonstrated in previous studies (Marques et al., 1997; Koivikko et al., 2008). These abnormalities may be more likely to occur in people with type 2 diabetes. Recent randomised controlled trials (RCTs) that did not target strict glycaemic control but used drugs with low risk of hypoglycaemia have shown beneficial cardiovascular outcomes (Zinman et al., 2015; Marso et al., 2016b). This is in direct contrast to previous RCTs, which pursued very strict glycaemic control, where the incidence of severe
hypoglycaemia was high. These findings are intriguing and suggest that avoidance of hypoglycaemia is important to achieve cardiovascular benefit.

**Conclusion**

Although hypoglycaemia had no effect on markers of ischaemia in the participants of the present study, there was a small apparent reduction in CFR during hypoglycaemia, with the lowest numerical value occurring in young adults with T1D during hypoglycaemia. Further studies that include female participants and use larger sample sizes are necessary to confirm or refute this observation. If the present finding of lower CFR is confirmed, this would raise concern that hypoglycaemia may promote myocardial ischaemia in older people with diabetes with established coronary heart disease. Ideally, coronary blood flow should be studied during hypoglycaemia in people with type 2 diabetes, who have a higher risk of cardiovascular disease, but this was not undertaken for ethical reasons because of the potential risk to such participants. A possible approach would be to repeat this study using larger numbers using a more tolerable form of investigation, and using a real time biomarker of cardiac injury. As population studies have shown an association between severe hypoglycaemia and cardiovascular risk (Khunti et al., 2015; Goto et al., 2013), there is a pressing need for mechanistic studies to elucidate this association.
Chapter 7

Summary of studies, conclusion, and future perspectives
7.1 The effect of SRT2104 in people with type 2 diabetes

7.1.1 Summary of study in Chapter 3

The incidence of T2DM is increasing worldwide and is projected to affect 300 million people by 2030 (Wild et al., 2004). Cardiovascular diseases are the number one cause of mortality and morbidity in people with diabetes (Rawshani and Gudbjörnsdottir, 2017). Although advancements in treatment of CVD may have improved some outcomes, especially in the western world, there is an increase in obesity in younger age groups which in the future abolish some of the improvements made in cardiovascular outcomes. Indeed, the WHO considers tackling the burden of obesity and non-communicable diseases such as diabetes one of its main priorities.

Treatment of T2DM is multimodal, which includes lifestyle modifications and indeed, drugs. Tight glucose control in people with T2DM may be harmful, as evidenced by RCTs in people with diabetes and cardiovascular risk factors such as ACCORD and VADT (Duckworth et al., 2009; Gerstein et al., 2008). The agents used to treat diabetes themselves have important cardiovascular effects, and the Food and Drug Administration (FDA) in the United States have mandated a requirement that new diabetes medications are cardiovascular safe. As such, there is a strong ongoing need for drugs that can treat diabetes, and also be of benefit to the cardiovascular system.

Research in yeast species, roundworm, and fruit flies have revealed beneficial effects of caloric restriction in the lifespan of these organisms. Many of the beneficial effects of caloric restriction has been shown to be modulated by the sirtuins. Modulation of sirtuins in various animal models have
been involved in various processes that underpin the pathophysiology of metabolism, inflammation and vascular disease. These include for example molecular pathways that influence production of reactive oxygen species and endothelial dysfunction such as NFκB, and pathways that modulate oxidative metabolism such as AMP-kinase. In short, sirtuins link energy metabolism and protects against the cellular pathways involved in aging. SIRT1 is one of the sirtuin homolog in humans. Activation of SIRT1 in animal models have highlighted potential benefits in diseases of aging such as diabetes and vascular disease, and therefore provide an exciting novel therapeutic target.

Chapter 3 describes the study in which the safety and tolerability of a novel SIRT1 activator, SRT2104, was examined in a randomised controlled trial of a period of 28 days duration. The crossover design of the study meant that the participants consumed either placebo or SRT2104 for 28 days and then crossed-over to the contralateral arm, for a further 28 days. The effect of SRT2104 on vasomotor function, thrombosis, platelet activation, and cardiometabolic markers were also examined at day 1, day 28 and day 56. For vasomotor function, we selected venous occlusion plethysmography, and for platelet activation we selected flow cytometry of platelet monocyte aggregates. Venous samples of haematology, renal and liver function as well as markers of lipids and glycated haemoglobin were obtained during the study. A total of 15 volunteers with T2DM were recruited and underwent this phase 1 RCT at the Wellcome Trust Clinical Research Facility in the Western General Hospital Edinburgh. The participants were predominantly middle aged- males with reasonably good control of type 2 diabetes.
The study found that SRT2104 was well tolerated. There was predominantly neutral effects of markers of vasomotor function, thrombosis, and platelet monocyte activation. Bradykinin-induced vasodilatation was less during treatment with SRT2104 versus placebo (7.753 vs 9.044, respectively, mean difference=−1.291 (95% CI −2.296 to −0.285, p=0.012)). Estimated net PAI-type 1 antigen release was reduced in the SRT2104 arm versus placebo (mean difference=−38.89 ng/100mL tissue/ min, (95%CI −75.47, to −2.305, p=0.038)). There were no differences in other plasma fibrinolytic factors (p>0.05 for all). After 28 days, SRT2104 exposure was associated with weight reduction (−0.93 kg (95% CI −1.72 to −0.15), p=0.0236), and a rise in glycated haemoglobin (5 mmol/ mol or 0.48% (0.26 to 0.70), p=0.004) (Noh et al., 2017). There were no statistically significant changes in lipid values, although there was a trend to numerically lower lipid values with SRT2104 treatment compared with placebo.

The interpretation of these results is limited by the methodological considerations of the study. Firstly, the study is small in size. One the reasons for this is the use of ACE inhibitors as an exclusion criterion for the study. This criterion was due to the potential confounding effect of ACE inhibitors to bradykinin in the vasomotor studies. Many potential participants were deemed not suitable as a result of this criterion, and recruitment fell short of the even modest 24 participants modelled in the power calculations. Another possible limiting factor is the tolerability and duration of the study itself. Venous occlusion plethysmography is time consuming, taking 3-4 hours in duration where the subject is lying flat, with the infusion arm undergoing brachial artery cannulation. This particular aspect of the study was cited as a factor in potential candidates declining participation.
Secondly, the crossover design of the study did not include a washout period. The findings therefore are interpreted in the context of statistical modelling of treatment by period interactions, and carryover bias becomes more likely. A potential solution to this would be to use only the data the first treatment period; however, this would halve the power of the study. On the other hand, the study was placebo-controlled with a 4-week treatment period, so a carry-over effect is unlikely. On balance, the findings of chapter 3 have been presented with a treatment by period interaction analysis alongside it, which provides an estimate of likelihood of the carryover effect.

With the above limitations in mind, it appears that short duration treatment with SRT2104 in people with T2DM has neutral effects on platelet activation as measured by flow cytometric analysis of platelet monocyte aggregates. SRT2104 has predominantly neutral effects on vasomotor function as measured by venous occlusion plethysmography, but has a modest effect in reducing bradykinin-induced forearm blood flow, as well as PAI-antigen release. These two findings may be explained by a carryover effect, but we would have to also have consider that SRT2104 may cause a small reduction in vasomotor effect, by a mechanism mediated by bradykinin alone.

The finding of worsening glycaemic control by 5 mmol/mol, is problematic in a drug developed to modulate the cellular pathways that contribute to the development of type 2 diabetes, and vascular dysfunction. There was also a striking association with weight loss with SRT2104 exposure (−0.93 kg (95% CI −1.72 to −0.15), p=0.0236) and a modest increase in FFAs. Again, interpretation of this finding is tempered by the period by treatment bias analysis; we may simply be observing a carryover effect here. Having noted this, it is important to consider that this finding could be consistent with a reduction in insulin. Indeed, a reduction in insulin levels and a rise in FFAs are predicted in the early
stages of CR and activation of SIRT1 (Milne et al., 2007). Moreover, a separate Phase II RCT by Baksi and colleagues in people with T2DM with stable doses of metformin showed a similar magnitude of reduction in weight of around 1 to 1.5 kilograms (Baksi et al., 2014). In this particular study, the effect of various doses of SRT2104, from 0.5 grammes, 1.0 grammes and 2.0 grammes, onto fasting glucose and post-prandial insulin levels were examined. The authors found no consistent dose-dependent effect on glucose and insulin levels. For our purposes however, it is interesting to note that there was a statistically significant reduction in 2-hour post prandial insulin with 28 days of exposure of 2.0 g SRT2104 versus placebo. This potentially could explain weight loss and hyperglycaemia seen in the study and the one described in chapter 3 of this thesis.

Additionally, despite inconsistent pharmacokinetics findings in the study by Baksi et al, there is a consistent statistically significant beneficial on lipids, consistent with findings in the study described in Chapter 4 in this thesis. This is explored in next segment, and an overall conclusion from Chapters 3,4 and 5 which integrates all the SRT2104 related-studies follows on from the next section.
7.2 The effect of SRT2104 in otherwise healthy smokers

7.2.1 Summary of study in Chapter 4

Smoking is a model for aging and chronic inflammation of the vascular tree. Smoking accelerates endothelial cell senescence and alters endothelial function, fibrinolysis and platelet function. Activation of SIRT1 has been shown in animal models to improve endothelial function and improve markers of vascular inflammation. To date there are limited numbers of studies of SIRT1 activation in humans, and its potential putative beneficial effects on models of vascular aging. The study of a novel SIRT1 activator, SRT2104, in a group with a strong risk factor for vascular disease, is therefore highly clinically relevant.

Chapter 4 describes a phase Ia randomised clinical trial of 2.0g of SRT2104 versus placebo, for 28 days, with a crossover for another 28 days. Pharmacokinetics and safety data were obtained throughout the study. Additionally, data for vasomotor function via venous occlusion plethysmography, markers of platelet activation via flow cytometry analysis of platelet monocyte aggregates, and markers of endogenous fibrinolysis were obtained for Day 1, Day 28 and Day 56 of the study. The protocol used was similar to the study in Chapter 3.
7.2.2 Analysis of Results, Chapter 4

SRT2104 was well tolerated in otherwise healthy smokers. Significant inter-subject variability in the pharmacokinetics data was seen, consistent with previous human studies (Hoffmann et al., 2013). No change was observed in markers of vasomotor function, platelet-monocyte aggregation, and endogenous fibrinolysis (p>0.05 for all) with SRT2104 treatment, versus placebo.

There were statistically significant changes to lipid values in the study in Chapter 4. Compared with placebo, serum lipid profile improved during SRT2104 administration, with reductions in serum total cholesterol (−11.6±20 versus 6±21 mg/dL), low-density lipoprotein cholesterol (−10±17 versus 3±21 mg/dL), and triglyceride (−39.8±77 versus 13.3±57 mg/dL) concentrations (P<0.05 for all) (Venkatasubramanian et al., 2013).

The study in chapter 3 observed a similar trend, with lower concentrations of total cholesterol (−0.36 mmol/L, 95% CI −0.87 to 0.16 mmol/L, p=0.158) and triglycerides (−0.22 mmol/L, 95% CI −0.53 to 0.09 mmol/L, p=0.150) with SRT2104, compared with placebo. As observed previously, the signal of beneficial trend in lipids were also reported in a separate Phase II RCT of SRT2104, where total cholesterol and triglycerides decreased, even in lower doses of SRT2104 than used in Chapter 3 and 4. Post hoc statistical modelling for a dose dependent effect of SRT2104 on lipids showed an association between higher exposure to SRT2104 and lower total cholesterol and triglycerides (Baksi et al., 2014).
The interpretation of the results in study in chapter 4 is limited by the lack of a washout period similar to that described in chapter 3. However, the study in chapter 4 recruited more volunteers, possibly due to a younger cohort of participants who were better able to tolerate the physiological studies. This study therefore benefitted from being better powered than study 3. Despite the caveat of a carryover effect, a benefit to total cholesterol and triglyceride was found. Speculatively, had study 3 involved more participants and did not include people on statins, a stronger signal in the total cholesterol and triglycerides could have been found and may have achieved statistical significance. Ultimately, the primary outcome of the study was to show improvement in vasomotor function after treatment with SRT2104, and this hypothesis was not supported. The change in lipids is interesting and is consistent with other human studies out with our research group. Given that hyperlipidaemia is a strong risk factor for cardiovascular disease this signal merits further assessment. This is explored the conclusion segment integrating Chapter 3, 4 and 5.
7.3 The effect of SRT2104 on arterial stiffness in otherwise healthy smokers and in people with T2DM.

7.3.1 Summary of study in chapter 5

Arterial stiffness increases with age and is an independent risk factor for cardiovascular disease. It is well established that smoking and T2DM are associated with increased arterial stiffness. SIRT1 activation has been shown to improve markers of vascular function in animal studies. SRT2104 is a novel SIRT1 activator in humans. The study in chapter 5 hypothesises that treatment with SRT2104 can improve markers of arterial stiffness.

24 participants who were otherwise healthy smokers and 15 participants with T2DM were studied in a prospective randomised controlled trial. The participants took 2.0 grammes of SRT2104 for 28 days and crossed-over to a placebo arm. Arterial stiffness was measured by pulse wave analysis of the radial artery, using applanation tonometry. The studies were performed on Day 1, Day 28 and Day 56. The study described in chapter 5 uses the same protocol as chapter 3 and 4, and the pulse wave analysis data is pooled and analysed post-hoc.
7.3.2 Analysis of results, chapter 5

Resting systolic and diastolic blood pressures remained unchanged throughout the study with no significant differences between treatment and placebo treatment periods. In a combined analysis of otherwise healthy cigarette smokers and participants with T2DM, a reduction in the augmentation pressure was observed in participants receiving SRT2104 compared with placebo (mean change from baseline: SRT2104−1.60 (5.304) vs placebo−0.06 (4.205); p=0.0273) and a trend towards improvement in the augmentation index (mean change from baseline in Alx: placebo−0.64 (8.361) vs SRT2104−3.47 (9.728); p=0.0813) and the corrected augmentation index (mean change from baseline Alx75: placebo−2.2−(7.453) vs SRT2104−4.84 (9.299); p=0.0747). Pulse wave velocity and time to wave reflection remained unchanged between placebo and treatment arms (p>0.05 for both parameters). The effects of SRT2104 administration on measures of arterial compliance were consistent across the two cohorts. For example, in the SRT2104 arm, mean augmentation index at 75 bpm was reduced for healthy smokers and participants with T2DM (−4.97 vs −4.63, respectively). A statistical interaction between cohort and treatment was not observed (p>0.05 for all variables tested).

The interpretation of these results is limited by the post-hoc nature of the analysis. The potential for a carryover bias remains due to the lack of a washout period. The heterogenous groups may also have different putative mechanisms for vascular injury. Keeping these in mind, the pooled analysis increases the power of the study, and in all measures of arterial compliance the trends were consistent across both cohorts. We may therefore be observing a true signal of benefit here, of small magnitude.
Chapters 3, 4 and 5 examined the cardio-metabolic effects of a novel SIRT1 activator, SRT2104, in a series of first in human studies. These studies showed that SRT2104 was safe and tolerable in humans.

Consistent with other first-in-human studies of SRT2104 outside of our research group, there was inter-individual variability in SRT2104 exposure. Despite this variability in plasma SRT2104 levels, a signal toward improvement in lipids was observed. No benefit in markers of vasomotor function, platelet activation and endogenous fibrinolysis was seen. A trend toward improved augmentation index was observed in both the T2DM cohort and the otherwise healthy smokers cohort. In a pooled post-hoc analysis, this trend became statistically significant.

These findings are tempered by the lack of washout period in the crossover design of the study. In the context of people with type 2 diabetes, the finding of weight loss and increase in markers of average glucose is highly important, and may limit further development of this particular molecule as a therapeutic agent, especially if the deleterious effect is sustained over the long term.

The primary outcomes of the above studies were that of vasomotor function and platelet activation. The studies observed the null hypotheses for these primary outcomes. The positive findings in terms of the metabolic parameters of lipids and glycaemia, are of limited validity and generalisability until further more robust studies are conducted. With that in mind, there are still nevertheless some important lessons to extract in the design of future studies examining SIRT1 activation in general. Firstly, there are the methodological considerations. The neutral findings raise the question as to whether we selected methods sensitive enough to find differences in vasomotor function and platelet activation. This particular concern is heightened by the finding of inter-individual variability of plasma SRT2104 levels after exposure. The counter-argument to this point is that the venous occlusion
plethysmography method has been used extensively in our group with very reproducible results, and the same can be said of the platelet activation studies. Furthermore, despite the variation in exposure of SRT2104, there was a signal in lipid improvement and deterioration in glycaemic control that was observed in other first-in human studies. This suggests that at the very least there is enough SIRT1 modulation to achieve an effect in pathways that control lipid and glucose metabolism.

Secondly, we would have to consider that SIRT1 activation itself may have a complex effect based on a narrow therapeutic window, and/or have downstream effects that are abolished by the hyper-caloric state in advanced diabetes mellitus, the treatment of diabetes mellitus, or smoking. Indeed, human studies of the first known naturally occurring SIRT1 activator, resveratrol, has shown that this class of drugs suffers from problems with bioavailability despite good absorption (Walle et al., 2004). SRT2104 was developed by Sirtris pharmaceuticals to as a more selective SIRT1 activator with better bioavailability. However, the first in human pharmacokinetic studies of this compound still demonstrated significant variability in absorption (Hoffmann et al., 2013). Although the dose of 2.0 grammes of SRT2104 administered during the fed state emerged as the most acceptable dosing regimen from this particular pharmacokinetic study, there were still concerns noted regarding the inter-subject variability of exposure. This would be a concern if higher-doses were required to exert a beneficial effect in humans. SIRT1 activation is a tightly regulated pathway with over 70 known downstream effects (Bordone and Guarente, 2005). In studies examining the effect of resveratrol, low levels of activation is associated with beneficial antioxidant and anti-inflammatory effects, but higher levels can induce potentially deleterious effects of downregulating Akt/mTor, which regulates apoptosis of cancerous cells (Jiang et al., 2009). In the first in human pharmacokinetics paper by Hoffmann and colleagues, there was an acknowledgement that in vitro studies of selectivity of
SRT2104 are from unpublished data. The paper also acknowledges that to date, there was no biomarker available to ascertain SRT2104 activation of the SIRT1 pathway (Hoffmann et al., 2013).

Without access to these data that Hoffman et al alludes to, it is difficult to be certain which pathways may have been affected more than others during administration of 2.0 g SRT2104. We can speculate that PPARγ could have been selectively more upregulated, given that this controls lipolysis and fatty acid oxidation, and the beneficial signal to cholesterol concentrations were seen in the work by Baksi, and in study 2 of otherwise healthy smokers from our own research group. In the T2DM cohort of study 1, a beneficial trend was observed, and speculatively the magnitude of benefit could have been attenuated by concomitant statin therapy. Additionally, inhibition of NF-κB, but not enough upregulation of eNOS pathways, may potentially explain the discrepancy in study 4 (benefit in augmentation index but no overall change in forearm blood flow). Lastly, we can speculate that there is a dosing-related effect on PGC-1α, causing paradoxical hyperglycaemia. This potentially could have been further exacerbated by the effect of metformin therapy in study 1. Metformin increases PGC-1α in hepatocytes, but this effect is abolished by sirtuin 1 in mechanistic studies (Aatsinki et al., 2014).

**7.5 Future perspectives for SRT 2104 studies**

We have gleaned valuable lessons from the predominantly neutral outcomes of the studies on SRT2104. Specifically, its effects on glucose and lipid metabolism appear to be complex, and can be confounded in models of advanced vascular aging such as T2DM and smoking. Weight loss was observed in the T2DM cohort, but not measured specifically in the smoking cohort. Glycaemic markers were also omitted in the smoking cohort. Additionally, the study design was hampered by the crossover component with a lack of washout period, and suffered from the lack of control subjects
without vascular risk factors. Further, the study design straddled a Phase 1 first-in-man and pharmacokinetics study with some measures of outcomes expected in a Phase II RCT.

Potentially, a repeat randomised clinical trial with 2.0 grammes of SRT2104 in obese people with pre-diabetes could be performed, alongside the smoking cohort and a control cohort of non-smokers without dysglycaemia. The RCT could be enhanced by a factorial design. This future study should include periodic measurement of fasting insulin levels, and post prandial glycaemic excursions. In the past, markers of insulin resistance such as the homeostatic model assessment-insulin resistance (HOMA-IR) has been used, but this method is limited by inaccuracy in relatively leaner individuals (Kang et al., 2005). Therefore, direct glucose excursion measurements would be preferable. The latter could now feasibly be performed using flash or continuous glucose monitoring devices.

This future study period should be longer than 28 days. Ideally, the study should be at least over 6 months, in order to determine whether initial decrease in the postulated insulin secretion is then counteracted by improvement in insulin sensitivity later. We have already covered that SRT2104 activation does not as yet have a suitable biomarker. To test the hypothesis that the weight and glycaemic changes brought on by SRT2104 is mediated by AMP-K and PPARγ, several biomarkers could be suitable including AMPK Thr(172) phosphorylation via Western blotting (Lim et al., 2012) and PPAR response element (PPRE) luciferase assay (Chan and Cipolla, 2012). Other secondary outcomes of this RCT could include biometric indices to investigate whether there are associated changes in adipose tissue compartments and adipose inflammation. This could be achieved by quantitative volumetry of visceral and total adipose tissue via magnetic resonance imaging (Poonawalla et al., 2013), to ascertain whether there are any changes to visceral fat especially. Monitoring of caloric intake and physical
activity could enhance understanding of energy balance in this scenario, alongside periodic tracking of IL6, TNF-α, adiponectin, resistin and CRP (Hajer, van Haeften and Visseren, 2008). Theoretically, a study design such as the one described would be able to give a more rounded understanding of how SRT2104 mediates weight loss, and whether it remains a viable drug to control diabetes.

As regards to vascular outcomes, this programme of research has highlighted the underpowered nature of the studies, and technical challenges, especially with regards to venous occlusion plethysmography. Whilst plethysmography remains a highly reproducible method in which our research group specializes, it nevertheless is time intensive, which in turn effects recruitment. In the post hoc analysis, it is clear that more than 48 patients would be required to ascertain meaningful improvements in markers of arterial compliance and endothelial function. In the future therefore, a Phase II RCT examining the effect of SRT2104 on markers of endothelial function would need a completely separate study from that of the one examining weight and metabolic markers. A more tolerable, preferably non-invasive technique would be required to ensure good recruitment in cohorts that would include obese individuals with dysglycaemia, smokers, and a control cohort. In this regard, ultrasound measurement of brachial artery reactivity would be a good candidate. This technique is non-invasive, well validated and has good correlation with hard outcomes such as acute coronary syndrome (Corretti et al., 2002). Combined with pulse wave analysis, this theoretically study could examine endothelial dysfunction mediated by small artery constriction (brachial artery ultrasound) and large artery remodelling (pulse wave analysis).

In summary, our research group showed that SRT2104 was a well-tolerated agent. There is a signal toward benefit in terms of lipids and markers of arterial stiffness. In people with type 2 diabetes, SRT2104 may induce weight loss at a cost of short-term loss of glycaemic control. Although the use of
SRT2104 as an anti-diabetes agent may be problematic, its effect of weight and lipids merit further assessment and may provide vital clues to important molecular pathways underpinning lipid metabolism, weight, and cardiovascular disease.

Beyond the studies described by our group and the work by Hoffmann and Baksi, there has been two other studies in humans examining the effects of SRT2104 in separate to cardiometabolic areas. One study examined the effect of SRT2104 on plaque psoriasis. This study showed some histological improvement of psoriasis, although the results were inconsistent with the clinical severity index. Again, inter-subject of variability of exposure to SRT2104 was cited a problem (Krueger et al., 2015). SRT2104 was also examined as a potential for treatment of ulcerative colitis (Sands et al., 2016). The authors concluded that SRT2104 did not improve outcomes and further evaluation as a therapeutic strategy in this regard is not warranted. No further in vivo studies of SRT2104 in people with T2DM have been described in the scientific literature.

The sparsity of any further human studies despite massive investment in the research of SIRT1 activation suggests that there are difficult challenges to overcome in ensuring consistent exposure to the drug, and making sure that downstream pathways are activated in a predictable beneficial manner. It is perhaps pertinent to consider that Sirtris pharmaceuticals itself has been shut down and absorbed by GlaxoSmithKline, despite initial purchase of the company estimated at 720 million US dollars (Timmerman, 2013). This would suggest that the scope for future development of this drug could be limited.

Arguably, more effort should be focussed on understanding the effect of CR itself to obesity, type 2 diabetes, and the underlying molecular mechanisms. This is a complex challenge, likely requiring cross disciplinary collaboration, and this aspect is explored in the broader future perspectives section.
7.5 The Effect of Hypoglycaemia on coronary flow reserve and markers of myocardial injury in people with and without type 1 diabetes.

7.5.1 Summary

Tight glycaemic control is thought to be beneficial to microvascular and macrovascular complications in people from type 1 diabetes. This conclusion is evidenced mostly from the seminal clinical DCCT (Nathan et al., 1993), and its follow-on study the EDIC (Nathan et al., 2005). The findings of the DCCT drove clinical trials in T2DM to aim for a tight glycaemic target of glycated haemoglobin of approximately 6.5% or 48 mmol/mol, or less than 6% or 42 mmol/mol in some cases. The cardiovascular benefits of this glycaemic target are marginal in the UKPDS trial (Holman et al., 2008), and found to be harmful in ACCORD and VADT trials, with findings of increased mortality in people with high cardiovascular risk in the intensive treatment arm of the trials (Gerstein et al., 2011). Additionally, a randomised controlled trial in the critical care setting, NICE-SUGAR, also found evidence of harm with tight glycaemic control (Finfer et al., 2009).

Tight glycaemic control is associated with a threefold increase in hypoglycaemia in the DCCT (DCCT 1997). Following the finding of possible harm in randomised clinical trials, several population-based studies have found a similar trend of increased cardiovascular mortality with hypoglycaemia (Goto et al., 2013; Khunti et al., 2015). The study by Goto and colleagues found that the association between severe hypoglycaemia and cardiovascular mortality was not fully explained by co-morbidity. This finding is of course highly concerning, given that randomised clinical trials likely under-estimate risk of hypoglycaemia due to strict exclusion criteria during recruitment. Furthermore, the RCTs tend to
have protocols entailing close follow-up of participants at regular intervals, which is not replicated in clinical practice.

Against this background, mechanistic studies explaining hypoglycaemia and cardiovascular disease are highly clinically relevant. Hypoglycaemia provokes profound counter-regulatory response, which entail an increase in myocardial work load (Fisher et al., 1990), and markers of thrombosis (Wright et al., 2010). There are therefore putative mechanisms to explain the link between hypoglycaemia and cardiovascular disease. To date, a study by Rana and colleagues has shown the effect of hypoglycaemia on myocardial blood flow (Rana et al., 2011). This particular study does not examine the direct effect of hypoglycaemia directly on the coronary circulation, or on markers of myocardial injury. Study 4 examines the effect of insulin-induced hypoglycaemia on coronary flow reserve and on highly sensitive cardiac troponin I. In a prospective, randomised, open-label, blinded, endpoint, cross-over study, 17 young adults with T1DM and no cardiovascular risk factors and 10 healthy non-diabetic volunteers underwent hyperinsulinaemic euglycaemic (blood glucose 4.5-5.5 mmol/L) and hypoglycaemic (2.2-2.5 mmol/L) clamps. Coronary flow reserve coronary flow reserve was measured by transthoracic doppler echocardiography, with adenosine administration to induce coronary hyperaemia. Myocardial injury as measured by hs-cTnl was also measured during euglycaemia and hypoglycaemia.
7.5.2 Analysis of Results in Chapter 6, Discussion and Conclusion

During euglycaemia, CFR was numerically lower in adults with T1DM than in participants without T1DM. During hypoglycaemia, mean CFR declined and was lower in the T1DM group than in the group without T1DM ([3.66 ± 0.47 versus 3.92 ± 0.85]). A generalised linear mixed model analysis was performed, with diabetes status and euglycaemia or hypoglycaemia as factors affecting CFR. No significant change was associated with euglycaemia or hypoglycaemia condition (p = 0.31), or with diabetes status (p = 0.23). No changes in hs-cTnI occurred during hypoglycaemia and subsequent glucose recovery.

The interpretation of this study’s findings should take into consideration some methodological considerations. Firstly, this study is likely underpowered due to difficulty recruiting into the study. This was influenced by lack tolerability of the adenosine administration, which is of course known to cause chest tightness, discomfort and flushing. Secondly, there was difficulty obtaining echocardiographic images with some of the participants, likely related to sub-optimal positioning during echocardiography. Ideally, the subjects should adopt a lateral decubitus position for obtaining the spectral doppler flow of the coronary artery. The concomitant hyper-insulinaemic clamp, with intravenous administration of glucose and insulin, sometimes rendered this challenging. As such, our group obtained 70% of images, which of course decreases the power of the study. This is contrasted with our own validation study, in which images were obtained in 12 out of 13 volunteers, with low inter-visit and inter-subject variability (Warraich, 2011). In this study, the subjects only had one adenosine administration per visit, and did not have to undergo the hyperinsulinaemic glucose clamp, which may have aided the positioning during echocardiography. The scientific literature describes a
90% success rate in obtaining images using this echocardiographic method, and above 90% if intravenous contrast is used (Hirata et al., 2004).

### 7.5.3 Future perspectives on the hypoglycaemia and myocardial flow and markers of injury studies

To enhance recruitment in any future studies in this area, it is crucial to improve the tolerability profile of the study. To date, no well validated and safe method in inducing experimental hypoglycaemia exist, apart from the hyperinsulinaemic glucose clamp technique. Therefore, at present, any studies examining experimental acute hypoglycaemia must work around the necessity of the insulin glucose clamp, and its logistical implication. We have touched upon the optimal positioning of the volunteer in obtaining echocardiographic images above. In future studies, extra consideration must be given with regards to the placement of glucose and insulin infusion on the volunteers’ arm, so that it facilitates the volunteer’s positioning and maximises the echocardiographer’s chances in obtaining the relevant. Additionally, the use of intravenous contrast could be used to enhance Doppler flow signals of the LAD coronary artery. In the current study protocol, adenosine was administered at least three times per visit in the baseline, experimental and recovery phase. Similar to our pilot study, any future studies should minimise adenosine delivery to perhaps only the experimental euglycaemia or hypoglycaemia phase. The advantage of adenosine is mainly its short half-life, which reduces confounding factors during repeated CFR measurements. However, by moving to a study where one CFR measurement is done per visit, dipyridamole could confidently be used without concerns regarding confounding factors, so long as the two experimental studies are at least several weeks apart as per our protocol.
Alternatively, a cross-sectional imaging method with a higher resolution of images, such as CFR by cardiac magnetic resonance may be more appropriate, whilst retaining the advantage of being non-invasive. Recently, Kato and colleagues used phase contrast imaging via cardiac magnetic resonance to quantify blood flow in the coronary sinus at peak stress, using adenosine. This method was shown to quantify coronary flow reserve well, and strongly correlated with major adverse cardiac events after a median follow-up of 2.3 years (Kato et al., 2017). This is a potential candidate for future non-invasive coronary imaging that can be combined with an insulin-clamp technique. Apart from being non-invasive, this technique does not use ionising radiation, unlike the other cross-sectional imaging technique in this area, which is Positron Emission Tomography-Computer Tomography CFR. In our current study, female participants were excluded on the basis of the effect of menstrual cycle to CFR. Additionally, there was the pragmatic modesty factor of undergoing transthoracic echocardiography. A cardiac MRI could potentially overcome this problem, whilst larger sample numbers might be expected to overcome any effects of the menstrual cycle on CFR.

Additionally, our research group used hs-cTnI, which has a peak of up to 6 to 12 hours after an ischaemic insult. Samples of troponin were taken during the experimental phase of the study and the recovery phase, with typically less than two hours duration between the samples. It could be postulated therefore, that our study protocol sampled too early and could miss the troponin peak. Speculatively, a more real-time marker of ischaemia is required to pick up any immediate myocardial injury. A potential candidate biomarker is cardiac myosin-binding protein C (C-myC), which peaks earlier and accumulates more rapidly than cardiac troponins (Baker et al., 2015).

In summary, to date, only our own research group and a study by Rana and colleagues have looked at the effect of hyperinsulinaemic hypoglycaemia on myocardial perfusion. This particular study has the
advantage of directly visualising the coronary artery, and additionally uses a biomarker of myocardial injury. Our own study benefits from having fewer confounding factors. In contrast to Rana’s study, we did not find any statistically significant changes during hypoglycaemia. We found numerically the lowest CFR values in people with T1DM during hypoglycaemia. It is unclear whether this is a potential signal to harm diminished by the underpowered study, or that other additional factors are required in order for hypoglycaemia to mediate cardiovascular harm. In contrast to our own study, Rana’s study recruited volunteers with microvascular complications, a significant confounding factor (Rana et al., 2011). As we have not reproduced the findings by Rana et al, our study does not resolve the controversy surrounding hypoglycaemia and cardiovascular disease. With this apparent neutral finding, further more robust studies are required to investigate this abnormality.

In clinical practice, minimising hypoglycaemia in people with high cardiovascular risk may be prudent until further research confirms or refutes this worrisome trend. Future broader perspectives include characterising the factors which actually induce harm during hypoglycaemia, and predicting more precisely sub-groups of people with diabetes who have high cardiovascular risk. This is discussed in the next section.
7.6 Broader Future Perspectives

The cardiovascular effects of the treatment of diabetes mellitus are complex and still include a multitude of unanswered research questions that have wide-ranging clinical implications. Diabetes mellitus itself is a heterogenous disease process, exhibiting multi-organ system ‘cross-talk’ between the gut, the circulatory system, the liver and pancreas, the brain, the visceral adipose tissue, and the body’s own humoral inflammatory and immune response. The pathophysiological perturbations in diabetes mellitus modulate the most evolutionarily-preserved biological pathways, such as the cell’s response to low energy levels and stress. It stands to reason therefore, that in attempting to influence these tightly regulated pathways, unexpected and unintended consequences can arise. Highly clinically relevant examples include the harm associated with insulin-induced hypoglycaemia, or an increase in cardiovascular mortality observed with the PPARγ-agonist rosiglitazone.

Given the extremely broad remit of this thesis, any meaningful insight necessitates examining some areas in detail whilst retaining a broader view of the disease process and its treatments. In this body of work, we have examined the cardio-metabolic effects of a CR mimetic. Some of the insights in CR have generalisable clinical implications, but cannot be divorced from a discussion of anti-diabetes agents with more potent cardiovascular benefit data, such as metformin, and the GLP-1RA and SGLT2 classes. Similarly, pathophysiological studies that investigate hypoglycaemia and myocardial ischaemia must be contextualised into glycaemic control professional guidelines, and inform the debate vis-a-vis tight glycaemic control. Analogous to the treatment of diabetes mellitus itself, the future perspectives straddle many disciplines and multi-faceted. We examine these themes in turn subsequently.
7.6.1 Optimising cardiovascular risk in people with diabetes mellitus- the role of diabesity

The Swedish Obese Subjects a prospective randomised-controlled trial which involved 4047 obese subjects. 2010 people in that subject were randomised to bariatric surgery, while 2037 were randomised to conventional therapy. This RCT was one of the first interventional study to show that weight loss was associated with decreased overall mortality, with the leading causes of death being myocardial infarction and cancer. After approximately 10 years of follow-up, 25 individuals in the control group died of myocardial infarction, versus 13 individuals in the bariatric surgery group (Sjöström et al., 2007).

In the UKPDS trial, there were no differences in macrovascular complications, except in the arm of the study containing obese subjects treated with metformin. In this arm, 342 subjects had approximately 11 years of treatment with metformin with an average glycated haemoglobin of 7.5% or 58 mmol/mol. Compared to the glyburide, glibenclamide and the insulin group, treatment with metformin was associated with a 42% reduction for any diabetes related death (UKPDS, 1998). This finding was a major driver for metformin being the first line drug of choice for treatment of T2DM. Metformin is recognised to be predominantly weight-reducing or weight neutral, whilst sulfonylureas and insulin both cause weight gain and hypoglycaemia. Thus, one can begin to speculate that both glycaemic control and a neutralising effect on weight are crucial components in improving cardiac mortality. Of course, a significant proportion of people do not tolerate metformin, and bariatric surgery is associated with a significant peri-operative risk and considerable post-operative considerations such as vitamin malabsorption.
Thus, advice on weight loss by diet and exercise should form a crucial part of the multi-pronged approach in tackling obesity and type 2 diabetes, but it is often overlooked in favour of pharmacological interventions. This is however due to several very important considerations. Chiefly, the fact that there is no clear guidance on which dietary treatment is optimum in people with type 2 diabetes, and there are gaps in our knowledge in the neurohormonal adaptative responses which mediate weight gain after initial weight loss. Secondly, that there are socio-economic contributory factors to obesity which require a public health paradigm more than clinic or small-group interventions.

7.6.2 Professional guidelines in the management of obesity, clues from caloric-restriction studies, and the DiRECT Trial

The Scottish Intercollegiate Guideline Network (SIGN) Guideline 115 for the management of obesity was created in 2010. The recommendations from this guidance were that of a 600-kilocalorie (kcal) deficit per day, with the choice of caloric deficit ‘tailored to the individual’. A summary of the evidence on the guidelines note that there were insufficient data of sustained weight loss over 12 months to recommend any particular kind of diet. However, the very-low-calorie-diet (VLCD) of less than 800 kcal per day do produce more sustained weight loss over 4 months (Tsai and Wadden, 2006). An RCT of 811 individuals over 2 years randomised participants to reduced calorie diets based on different emphasis of macronutrients. This trial showed that the 80% of participants in the trial who completed 2 year follow up lost 6 kilograms in weight at 12 months, and began to regain weight at the 12-month mark. The macronutrient composition did not influence the degree of weight loss (Sacks et al., 2009).
In summary, within the Scottish context our guidelines suggest aiming for a caloric deficit of 600 kcal deficit per day in a way that works individually, and to favour less processed high fibre foods. Additionally, an increase is in physical activity is recommended, and a reduction of sedentary behaviours (SIGN Network, 2010). The guideline acknowledges that there is insufficient evidence to recommend any weight maintenance programmes, and that any interventions should assess the participants’ ‘willingness to change’ using a ‘Stage of Change’ psychological screening model used in primary care (Verheijden et al., 2005). The latter is in the lowest level of recommendation category.

In clinical practice, physicians recommending weight management programmes often find engagement variable, and frequently programmes are over-subscribed. There is also often a limited appreciation of the scope of medical supervision required, and in Scotland the role of the diabetes physician is limited. This is perhaps surprising in the context of high prevalence of T2DM in obese individuals, and that relapse and remission of the same can occur during weight management interventions.

Over the past decade, there has been more research building on what is already known on bariatric surgery and VLCD diets, aiming to induce remission of T2DM, and to supervise the long-term sequelae over a period of years. Work by Taylor and colleagues showed that VLCD over a period of 8 weeks can induce remission of T2DM, thought to be secondary to reduced pancreatic and liver fatty deposition (Lim et al., 2011). The ‘Newcastle diet’ by Taylor et al contributed some granularity to the nuanced debate about diet. Although recognised to require medical supervision and probably difficult to adhere to, remission of diabetes is a strong motivating factor. Further work by this group characterised a larger cohort of participants in a clinical trial, and characterised ‘responders’ and ‘non-responders’, with responders having a higher fasting insulin values and shorter duration of diabetes (Steven et al., 2016). Even in the non-responders, weight fell by approximately 15 kilogrammes. The ‘responders’ group had re-establishment of the first phase insulin response. This is an example of the heterogeneity
of the of people with T2DM, and that different approaches may merit depending on fasting insulin and glucose values as well as a post-prandial response.

Some comparisons can be made with SIRT1 activation here. For example, a 30-day RCT of obese individuals with resveratrol found a reduction in intramuscular AMP-K and PGC-1α, while decreasing post-prandial lipolysis; i.e. similar to what we would expect metformin to do (Timmers et al., 2011). On the other hand, treating non-obese women with normal glucose tolerance did not induce a change in AMP-K and PGC-1α. As SIRT1 activation is NAD+ dependent, we could speculate that there is a rheostat effect, where available intracellular glucose for oxidative respiration (and hence NAD+) determines the beneficial downstream effect (Figure 18).
FIGURE 18: SUMMARY OF POSSIBLE RHEOSTAT EFFECT SIRT1 ACTIVATION AND INTERACTIONAL PATHWAYS

The centre of the schematic diagram shows NAD+ dependent SIRT1 Activation (green oval). NAD+ and the various factors that affect it are summarised, including glycolysis from ad-libitum diet, mitochondrial function and drugs such as metformin. In turn, SIRT1 activation modulates downstream effects, modelled as a ‘rheostat’. These downstream pathways effect cardiovascular biology, as described in the diagram.
The obesity milieu may represent a reset rheostat; however, activation of SIRT1 beyond the therapeutic window can create deleterious effects. As there is overlap in the pathways modulated with metformin, we might expect a problem with concurrent metformin use, and indeed this is observed in Study 1, and extrapolated from the study by Baksi and colleagues explored earlier. As the majority of people with established T2DM are likely to be on metformin, an interacting agent is of course problematic. Speculatively, there may be a role for SIRT1 activators in obese people not on metformin, but with a high fasting insulin. However, this approach might be fraught with difficulties in monitoring insulin and glucose responses over time, and may not be commercially viable on a large scale.

Nevertheless, the concept of SIRT1 activation looks at treating the underlying biology of T2DM, rather than simply treating progressive hyperglycaemia. As shown by Taylor and colleagues, non-pharmacological interventions in controlled settings can mimic the diabetes remission seen in people undergoing bariatric surgery. However, we also know that normalisation of blood glucose values via short term intensive insulin therapy can induce near normalisation of long-term glycaemic control, or indeed remission of diabetes (Kramer and Retnakaran, 2013). In Kramer’s meta-analysis, remission is predicted by lower fasting blood glucose and higher body mass index in study participants. Thus, we can appreciate that we can modulate the natural history of T2DM by different strategies, and a different approach may be required for different characteristics of the individuals with T2DM. What needs to be answered more clearly is whether such intensive treatment strategies are feasible on a large scale, and whether diabetes remission is durable. The Diabetes Remission Clinical Trial (DiRECT) aims to answer the aforementioned questions, and will be completed in 2019. In the first 12 months of the study almost half the participants in the study achieved remission of diabetes, and this was achieved within resources in primary care in Scotland (Lean et al., 2018). The results DiRECT are likely
to provide information that may change practice, but long-term follow up study on hard outcomes on this group is also crucial. In this aspect, Scotland is well-placed due to the vast wealth of data accorded by Scottish Care Information- Diabetes Collaboration (SCI-DC) national database. If the mortality effects of remission of diabetes in the SOS study is replicated, but without the significant risks and costs associated with surgery, then the resource implications are immense.

The main caveat of the very low CR approach is the regain in weight, thought to be due to neurohormonal adaptations in leptin, driving appetite in an ‘obesogenic environment’, and a decrease in energy expenditure and basal metabolic rate (Greenway, 2015). Dealing with these factors will pose a significant multifaceted challenge. Part of the solution may involve public health initiatives at a national level, such as the published Scottish government strategy ‘A Healthier Future – Action and Ambitions on Diet, Activity and Healthy Weight’, which has an ambition to halve obesity in Scottish children by 2030, aiming to change the diet milieu and promoting physical activity (Scottish Government, 2017). Another part to solving this challenge cannot ignore pharmacological interventions, especially in view of the mounting cardiovascular outcome trial data, which are very being widely reproduced, in the GLP-1RA and SGLT2 class.
7.6.3 Cardiovascular outcome data of GLP-1RA and SGLT2 drugs. Do NICE guidelines need to change, with consideration of a minimum HbA1c?

Partially in response to the EMPA-REG and LEADER trials, the SIGN guidelines for management of T2DM were updated in 2017 (SIGN 116, 2017). The guidance recommends clinicians consider SGLT2 inhibitors and GLP-1RA classes in view of the aforementioned cardiovascular outcome trials. Since 2017, further trials have shown consistent trends, as outlined in Chapter 1. The growing evidence have prompted some experts to call for a wider change in clinical practice, especially those driven by the National Institute for Clinical Excellence (NICE) guidance for the management of T2DM (Fisher, 2018).

We can envisage that GLP1-RA and SGLT2 classes being adjunctive treatments to dietary strategies described previously to maintain remission, and this is already being observed anecdotally in clinical practice. It is still unclear how the GLP-1RA and SGLT2 class promote cardiovascular benefit. In broad terms, these two drug classes improve glycaemic control without weight gain or hypoglycaemia (unless combined with sulfonylureas). Experts argue that GLP-1RA and SGLT2 class are likely to improve outcomes only partially due to improvement in glucose, as hyperglycaemia is a weak risk factor to CVD (Abdul-Ghani et al., 2017). Many theories have been put forward, including that GLP1-RA directly affects vascular endothelium and the myocardium, with beneficial effects beyond that seen with improvement in weight and lipids. The beneficial effects seen with GLP-1 agonists likely effect atherosclerosis, as the CV benefit take more than 3 years to manifest (Nauck et al., 2017). By contrast, SGLT2 inhibitors have salutary effects likely independent from atherosclerosis, as the benefits in CV outcomes manifest early. This has led experts to speculate on beneficial modulations of different pathways including decreased sympathetic nerve stimulation (Abdul-Ghani et al., 2016), to increased
metabolic utilisation of ketone bodies (Mudaliar, Alloju and Henry, 2016). Many of these putative molecular pathways are complex, dynamical and interactional. Synthesis of these data is crucial, but challenging due to complexity. More collaborative approaches between multi-disciplinary may be needed in the future, or more speculatively, using the computational ability of ‘big data’ systems may be the way forward.

Until such a time, the challenge of disentangling glycaemic control from other cardiovascular risk factors remain. Part of the answer lies in CVOTs in younger patients with T2DM. The Liraglutide in Young Adults with Type 2 Diabetes (LYDIA) trial is one such an example and is as yet unpublished (LYDIA, 2018). It is hoped that with these studies, more informative data on younger people with minimal cardiometabolic risk factors will be available. Another ‘piece of the puzzle’ is the oft-repeated perception that hyperglycaemia is a minor risk factor for CVD. However, treatment of hyperglycaemia has until recently been very intertwined with hypoglycaemia, especially when aiming for tight glycaemic control.

7.6.3 ‘Metabolic memory’ and tight glycaemic control

We have outlined clearly the potential deleterious effects of tight glycaemic control. The controversy pertaining to near normalisation of glucose control has an important connection with the concept of ‘metabolic memory’, and the long-term follow-up of the DCCT and UKPDS. Turnbull’s meta-analysis of the RCTs during the aftermath of that era is summarised in Figure 19 (Turnbull et al., 2009).
**FIGURE 19: META-ANALYSIS OF INTENSIVE GLYCAEMIC CONTROL RCTS**

(reproduced from Turnbull et al 2009 with permission): Effects of more- vs less-intensive glycaemic control on major cardiovascular events (cardiovascular death or non-fatal stroke or non-fatal myocardial infarction), stroke (fatal or non-fatal), myocardial infarction (fatal or non-fatal) and heart failure resulting in hospitalisation or death. The diamond incorporates the point estimate, represented by the vertical dashed line, and the 95% CI of the overall effect for each outcome. The HRs are given for more-intensive compared with less-intensive glucose control. $\Delta \text{HbA}_1c = \text{mean HbA}_1c$ of more-intensive group minus mean HbA$_1c$ of less-intensive group. UKPDS follow-up truncated at 5 years from the time of randomisation.
The authors of this meta-analyses conclude that intensive glucose control improves glycaemic control at the cost of an increase in severe hypoglycaemia. A meta-analysis from Ray et al included more studies than Turnbull’s group, such as ADOPT and the UGDP. Although recognised to introduce heterogeneity to the data, the authors conclude a more conservative and cautious conclusion toward tight glycaemic control (Preiss and Ray, 2011). Both these analyses recognise the need to evaluate the effect of hypoglycaemia on cardiovascular outcomes.

To add granularity to the debate, both meta-analyses did not include the Steno-2 mortality study, which was an RCT that enacted all ADA guideline targets in the intensive treatment group for a mean of 7.8 years and total follow-up for approximately 13 years. This study was notable for also monitoring detailed caloric intake and exercise. This study showed a benefit to all-cause mortality (HR 0.41, 95% CI 0.25-0.67, p<0.001). For our purposes it is worthwhile noting that the intensive conventional group had similar hypoglycaemic event rates, and both groups reduced caloric intake over time (Gaede et al., 2008). It is therefore tempting to speculate that the mortality outcomes of the DCCT/EDIC study may have shown more benefit, had participants with ‘double-diabetes’ been phenotyped, and an attempt made to reduce hyperinsulinaemia and hypoglycaemic risk to CV outcomes.

Both the Rana study and study 4 of our own group are worrisome, but not conclusive and require further larger scale reproducible data. On the one hand, complete normalisation of blood glucose may be desirable in the era of agents which can minimise hypoglycaemia altogether. On the other hand, hypoglycaemia is still a pervasive challenge in people with T1DM, a subset of which will have high cardiovascular risk. Older people with T2DM, those who have pre-existing CVD at the outset of diagnosis, and people with T2DM already on sulfonylureas and insulin still represent a large proportion of people treated by clinicians. In people with known CVD, a minimum glycated haemoglobin may be prudent to prompt discussions around hypoglycaemia and cardiovascular safety.
7.6.4 Screening for coronary artery disease

In the medium-term future, more accurate measures of cardiovascular risk have to be considered. This is to better guide clinicians when a tight glycaemic control strategy may be particularly hazardous, but also to guide potential referral to cardiology colleagues. Current clinical practice uses CV risk engines which tend to under-estimate overall risk in people with diabetes, especially in T1DM (Zgibor et al., 2006). Current NICE guidance for risk stratification of stable angina already advocate risk stratification with Computed Tomography Coronary Angiography (CT-CA). It is uncertain whether individuals with one risk factor for CVD like diabetes mellitus, or with silent ischaemic heart disease, may also benefit from this investigative method. The Factor-64 trial was an RCT examining 900 people with either T1DM or T2DM, randomised to HbA1c < 42 mmol/mol or HbA1c < 53 mmol/mol, with tight blood pressure and lipid control. The cohorts were routinely screening for CAD by CT-CA. This was in the era post ACCORD findings, and the HbA1c target were relaxed post study. The RCT concluded that there were no differences in outcomes and that these findings would not support the use of CT-CA (Muhlestein et al., 2014). The authors discuss that the lack of benefit may be related to optimised blood pressure and cholesterol in both cohorts already, again raising the notion that chronic hyperglycaemia is a ‘weaker’ risk factor than blood pressure and cholesterol. However, rates of hypoglycaemia or parameters of weight were not reported in the study, and it is possible that these two factors may have abolished any benefit. Indeed, recent CVOTs show it matters more how glycaemic control is achieved, and speculatively, a repeat study in the era of SGLT2 inhibitors and GLP-1RAs is highly relevant.
The recently published SCOT-HEART (Scottish Computer Tomography-HEART) trial demonstrates very encouraging results in people who have suspected angina in chest pain clinics in Scotland. When combined with standard care, CT-CA improves outcomes over 5 years, without the need for increasing coronary revascularisation (Newby et al., 2018). This investigative method is appealing due to direct anatomical correlation, and provides powerful prognostic information. Whilst there is insufficient data to promote widespread use in people with diabetes, CT-CA is a promising avenue for multi-disciplinary collaboration, especially if the subjects investigated are phenotyped better and novel glycaemic monitoring methods are incorporated.

7.6.5 Glycaemic Variability, ‘Flash’ and Continuous Glucose Monitoring Devices

Circumstantial evidence around the harm of hypoglycaemia is increasing. We have identified areas that need further clarity previously. It is not certain precisely what component of the hypoglycaemia syndrome which mediates harm: the low blood glucose per se, the hyper-insulinaemic milieu, or the counter-regulatory response. There are associations between glycaemic variability and poorer cardiovascular outcomes in people with T1DM and T2DM, independent of glycated haemoglobin. It is unclear whether the post-prandial excursion or hypoglycaemia which is more important. The data is also significantly limited by the retrospective nature of the studies (Nalysnyk and Krishnarajah, 2010).

Flash glucose monitoring devices like the Freestyle Libre potentially can improve markers of glycaemic variability. This class of device has now been adopted in clinical practice in people with T1DM locally. Further studies of efficacy are needed, but initial results are encouraging as more people can decrease
hypoglycaemia, whilst maintaining good glycaemic control and reducing glycaemic variability (Bolinder et al., 2016). In the era of rapid acting insulin analogues which approximate normal physiology, near normal glycaemic control is becoming closer to reality. There are however some subsets of individuals with T1DM (and some with long-standing T2DM) who can have highly variable blood glucose, and have impaired-awareness of hypoglycaemia. In the DCCT, severe hypoglycaemia is predicted by low c-peptide values (DCCT Research Group, 1997). As stated previously the relationship between intensive glycaemic control and cardiac autonomic neuropathy is less clear, but in the DCCT/EDIC cohort the signal appears to be of small benefit, perhaps reflective of the benefit of glucose control directly onto microvascular complications (Pop-Busui et al., 2017).

Future prospective RCTs using flash glucose monitoring examining standard care versus safe tight glucose control are now feasible. When combined with characterisation of C-peptide levels and baseline CT-CA, the important clinical questions we have highlighted can be clarified.

7.6.6 Conclusion

This thesis has explored the potential beneficial and deleterious cardiovascular effects of the treatments for diabetes mellitus. To better understand these effects, it has been necessary to review complex molecular pathways which underpin metabolism and cardiovascular physiology. ‘Classical’ risk factors such as insulin resistance, hypertension and dyslipidaemia interact in to produce endothelial dysfunction, and a pro-inflammatory and pro-thrombotic milieu in the vascular tree. Newer anti-diabetes agents like GLP-1RA and SGLT2 inhibitors may exert beneficial cardiovascular effects independent of glycaemic control. SIRT1 modulation is postulated to have many potential
beneficial effects to treat both diabetes mellitus and cardiovascular diseases; for example, via modulating AMPK, theoretically increasing mitochondrial biogenesis and inhibiting cardiac myocyte hypertrophy. If proven to be beneficial, SIRT1 activators would be a welcome addition to the range of available treatments. This programme of research has shown that SRT2104, a novel SIRT1 activator, was a well-tolerated agent. There is a signal toward benefit in terms of lipids and markers of arterial stiffness. In people with T2DM, SRT2104 may induce weight loss at a cost of short-term loss of glycaemic control. Although the use of SRT2104 as an anti-diabetes agent may be problematic, its effect of weight and lipids merit further assessment. Some fascinating clues have been generated by the data, including a rise in free fatty acids and possible decreased insulin, which mimics the early effects of fasting. This has allowed us to speculate in this thesis regarding important molecular pathways underpinning caloric restriction, lipid metabolism, weight, and cardiovascular disease. In turn, these insights inform the debate regarding caloric restriction and diabetes remission especially, and more generally broadens our understanding of the underlying biology of diabetes mellitus.

Whilst there are similarities in phenotypes between T1DM and T2DM in multiplying risk of CVD, one of the contrasts is the effect of intensive glycaemic control. Hypoglycaemia, which is associated with tight glucose control especially with insulin therapy, may exert important cardiovascular effects. In this research programme, experimentally-induced hypoglycaemia in people with and without type 1 diabetes did not cause direct myocardial injury, but appeared to be associated with a low coronary flow reserve. This finding is not reassuring, and may prompt clinicians to screen for problematic hypoglycaemia during treatment, and to proceed with caution in cohorts of people with diabetes and known cardiovascular disease. Looking forward, the recent trend of cardiovascular benefit in the GLP-1RA and SGLT2 inhibitor class have reinvigorated interest in the common molecular pathways underpinning diabetes and vascular disease. Furthermore, newer insulins with more physiological
action profiles combined with real-time glucose monitoring is making near-normal glucose control whilst minimising hypoglycaemia an achievable aim. Significant uncertainties remain, but new powerful tools in research means there is cause for optimism. Further multidisciplinary collaborative research is the key to improving outcomes in clinical care.
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Appendix: Published Papers
Cardiometabolic effects of a novel SIRT1 activator, SRT2104, in people with type 2 diabetes mellitus

Radzi M Noh,1 Sowmya Venkatasubramanian,2 Shruti Daga,3 Jeremy Langrish,2 Nicholas L Mills,4 Ninian N Lang,5 Ethan Hoffmann,6 Brian Waterhouse,6 David E Newby,7 Brian M Frier8

ABSTRACT

Background The cardiometabolic effects of SRT2104, a novel SIRT1 activator, were investigated in people with type 2 diabetes mellitus (T2DM).

Methods Fifteen adults with T2DM underwent a randomised, double-blind, placebo-controlled cross-over trial and received 28 days of oral SRT2104 (2.0 g/day) or placebo. Forearm vasodilatation (measured during intrabrachial bradykinin, acetylcholine and sodium nitroprusside infusions) as well as markers of glycaemic control, lipid profile, plasma fibrinolytic factors, and markers of platelet-monocyte activation, were measured at baseline and at the end of each treatment period.

Results Lipid profile and platelet-monocyte activation were similar in both treatment arms (p>0.05 for all). Forearm vasodilatation was similar on exposure to acetylcholine and sodium nitroprusside (p>0.05, respectively). Bradykinin-induced vasodilatation was less during treatment with SRT2104 versus placebo (7.753 vs 9.044, respectively, mean difference=−2.296, 95% CI=−2.296 to −0.285, p=0.012). Estimated net plasminogen activator inhibitor type 1 antigen release was reduced in the SRT2104 arm versus placebo (mean difference=−8.39 ng/100 mL tissue/ min, 95% CI=−27.54 to −23.05, p=0.038). There were no differences in other plasma fibrinolytic factors (p>0.05 for all). After 28 days, SRT2104 exposure was associated with weight reduction (−0.93 kg (95% CI=−1.72 to −0.15, p=0.0236), and a rise in glycated haemoglobin (5 mmol/ mol or 0.48% (0.26 to 0.70), p=0.004).

Conclusions In people with T2DM, SRT2104 had inconsistent, predominantly neutral effects on endothelial and fibrinolytic function, and no discernible effect on lipids or platelet function. In contrast, weight loss was induced along with deterioration in glycaemic control, suggestive of potentially important metabolic effects.

Clinical trial registration NCT01031108; Results.

KEY QUESTIONS

What is already known about this subject?

► Sirtuins are a group of proteins that regulate many important biological pathways. Specifically, SIRT1 mediates the beneficial effects of caloric restriction on endothelial function and on glucose regulation in animal models. The cardiometabolic effects of SIRT1 activation in humans are unknown; it is this research question that underpins this randomised clinical trial.

What does this study add?

► It appears that short-term SIRT1 activation in humans is well tolerated, and has predominantly neutral effects on markers of endothelial function and platelet-monocyte function. However, it is associated with weight reduction and a rise in glycated haemoglobin.

How might this impact on clinical practice?

► The reduction in weight suggests that SIRT1 activation indeed modulates important biological pathways related to energy metabolism. People with type 2 diabetes mellitus have a high prevalence of obesity, and this finding of weight loss merits closer investigation. The rise in glycated haemoglobin is not consistent with results from animal model studies. We speculate that the deterioration in glucose control may be a signal of inadequate reduction in insulin resistance in humans. Further studies are required to confirm this signal, which will impact on the feasibility of development of this novel class of drugs.

INTRODUCTION

The effect of caloric restriction on energy metabolism has attracted intense interest over the past decade because it prolongs the life span in yeast, nematodes and flies. Caloric restriction also lowers the incidence of age-related disorders such as diabetes and cancer in mammals.1-5 These beneficial effects may be mediated by sirtuins, (‘SIR-2-ins’ or silent mating-type information regulation 2). In yeast, the sirtuin gene regulates many cellular pathways including inflammation, apoptosis and mitochondrial biogenesis. The activity of sirtuins is dependent on nicotinamide adenine dinucleotide , thereby linking energy metabolism and caloric restriction to these important cellular pathways.5

The mammalian homologue of the sirtuins is SIRT1.7 In murine models, activation of
SIRT1 has been implicated in the modulation of several cellular substrates including peroxisome proliferator-activated γ cofactor 1-α, nuclear factor κB, foxhead box protein and uncoupling proteins. These pathways are implicated in skeletal muscle energy metabolism and adipocyte differentiation, and play a role in glucose regulation in skeletal muscle and pancreatic β cells, as well as modulating insulin sensitivity. Caloric restriction also improves endothelial function in rodent and human studies through weight loss, improvement of nitric oxide production and reduction in reactive- oxygen species. Inhibition of SIRT1 promotes atherogenesis and upregulation of SIRT1 increases nitric oxide in rodent models. As a caloric restriction mimetic, SIRT1 activation therefore has the potential to improve cardiovascular health and mitigate the adverse effects of atherosclerotic disease and its risk factors.

Type 2 diabetes mellitus (T2DM) is a highly prevalent chronic condition, estimated to affect 4.4% of the worldwide population by 2030. It is associated with an increasing economic burden and in the USA total costs are estimated to be $245 billion. The major cause of mortality in patients with T2DM is cardiovascular disease. One of the putative mechanisms of the link between cardiovascular disorders and T2DM is endothelial dysfunction. Thus, modulation of SIRT1 is a promising strategy for the treatment of both diabetes and its well documented association with cardiovascular disease.

To date, studies have primarily investigated the ex vivo effect of SIRT1 activation. We hypothesised that therapeutic SIRT1 activation would have beneficial effects on cardiometabolic health in people with diabetes. Specifically, we examined the effect of a novel SIRT1 activator, SRT2104, on vascular vasomotor and fibrinolytic function, platelet activation, lipid profile and markers of glycaemic control in people with T2DM.

METHODS

The study was approved by the research ethics committee, was given clinical trial authorisation by the Medicines and Healthcare products Regulatory Agency (MHRA), and carried out at the MHRA phase I accredited Wellcome Trust Clinical Research Facility at the Western General Hospital, UK. Written informed consent was obtained from each participant, and the study was carried out in accordance with the Declaration of Helsinki.

Study participants

Fifteen individuals with T2DM were recruited from outpatient clinics at the Royal Infirmary of Edinburgh. Inclusion criteria included age between 18 years and 70 years, glycated haemoglobin (HbA1c) <9.0% (75 mmol/mol) and resting blood pressure of <160/90 mm Hg. Exclusion criteria included current smokers, the use of ACE inhibition therapy (as the potentiation of bradykinin and effects on endothelial function would confound the vascular studies), the presence of major comorbidities, chronic illness, renal or liver impairment, history of gastrointestinal diseases or surgeries influencing drug absorption, history of alcoholism, history of neoplastic disease within the last 5 years, a positive urinary test for recreational drugs, pregnancy, and participation in other clinical trials or blood donation within the last 3 months. Eligibility of participants including absence of relevant medical history was confirmed through a standardised form completed by the subject’s primary care physician after obtaining informed consent. Tests for pregnancy (serum human chorionic gonadotropin (HCG) concentrations at screening and urinary HCG concentrations at study visits) were conducted on all female participants of childbearing potential.

Study design

This was a prospective, double-blind, randomised, placebo-controlled cross-over study. Subjects were randomised 1:1 to receive 2.0 g daily of oral SRT2104 or matched placebo (Sirtris Pharmaceuticals) for a 28-day period, followed by cross-over to the alternate study arm for another 28 days, giving a total dosing duration of 56 days. A safety visit was conducted on day 70, with a follow-up by telephone on day 86. Assessment of drug safety, tolerability and efficacy on vascular function was carried out at baseline, and during and at the end of each treatment period (figure 1). The overall study design included otherwise healthy smokers in addition to people with T2DM, and volunteers were stratified by these two categories. This manuscript focuses on the T2DM group, with observations in the otherwise healthy smokers group described previously.

Vascular studies

Vascular studies were undertaken before and at the end of each 28-day trial period. All studies were performed with the patient lying supine in a quiet temperature-controlled (22°C to 25°C) room. Participants were fasted for 10 hours, and avoided caffeine and alcohol for 24 hours before the study. Venous cannulae (17G) were inserted into large subcutaneous veins in the antecubital fossae of both arms to facilitate periodic venous sampling. Supine heart rate and blood pressure were monitored at intervals throughout the study using a semiautomated, non-invasive oscillometric sphygmomanometer (Omron 705 IT).

Forearm venous occlusion plethysmography

Forearm blood flow was measured in the infused and non-infused forearms using forearm venous occlusion plethysmography as described previously. Volunteers underwent brachial artery cannulation in the non-dominant forearm with a 27 standard wire gauge steel needle. After a 20 min baseline infusion with 0.9% saline, incremental intra-arterial doses of bradykinin (American Peptide) at 100 pmol/min, 300 pmol/min and 1000 pmol/min (an endothelium-dependent vasodilator that induces tissue plasminogen activator (t-PA) release), acetylcholine (Chem. Pharm Fabrik) at 5 μg/min, 10 μg/
min and 20 µg/min (an endothelium-dependent vasodilator that does not induce t-PA release) and sodium nitroprusside (Hospira) at 2 µg/min, 4 µg/min and 8 µg/min (an endothelium-independent vasodilator that does not induce t-PA release) were infused for 6 min at each dose, with a 30 min 0.9% saline washout infusion between drugs. The order of drugs was randomised between subjects but kept constant for each participant across the three visits.

**Blood sampling**

Paired venous blood samples were obtained from each forearm before and during the infusion of intra-arterial bradykinin. Samples were collected into acidified buffered citrate (Stabilyte; Trinity Biotech) and citrate (BD Vacutainer; BD UK) for determination of t-PA and plasminogen activator inhibitor type 1 (PAI-1) concentrations, respectively. Samples were placed on ice before centrifuging at 2000 g for 30 min at 4°C. Platelet-free plasma was decanted and stored at −80°C before further analysis. Venous blood samples were collected into EDTA at the beginning and end of the vascular study to determine haematocrit.

Plasma t-PA antigen and activity (t-PA Combi Actibind t-PA ELISA kit; Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed) concentrations were determined by ELISAs.

**Platelet and monocyte activation**

Flow-cytometric measurements of platelet–monocyte aggregation and platelet surface expression of P-selectin and monocyte CD11b expression (Mac-1/CD11b) were performed at baseline and at the end of each treatment period as described previously.23–26 Briefly, peripheral venous blood was drawn from a large antecubital vein and anticoagulated with the direct thrombin inhibitor D-phenylalanine-L-arginine chloromethyl ketone (Cambridge Biosciences) and immunolabelled within 5 min of phlebotomy for subsequent flow cytometric analysis. Directly conjugated monoclonal antibodies were obtained from DakoCytomation and Serotec. Samples were stained with the following conjugated monoclonal antibodies: phycoerythrin (PE)–conjugated CD14, PE-conjugated CD62p, PE-conjugated CD11b, fluorescein isothiocyanate (FITC)–conjugated 42a and FITC-conjugated CD14 and appropriate control isotypes. Once stained, samples were incubated for 20 min at room temperature before being fixed with Fluorescence-activated cell sorting (FACS) Lyse (Becton-Dickinson). All samples were analysed using a FACS Calibur flow cytometer using CellQuest Pro software (Becton-Dickinson). Venous blood was collected in citrate at baseline and after each dosing period to assess plasma-soluble CD40 ligand concentrations. Blood was centrifuged at 1500 g for 15 min at 4°C, and plasma was decanted and stored at −80°C for further analysis by ELISA (Bender Medsystems).

**Safety and pharmacokinetic analyses**

Venous blood samples were collected biweekly to measure haematological and biochemical parameters including full blood count, coagulation profile, liver and renal function, creatine phosphokinase, lactate dehydrogenase, lipid profile and free fatty acids. Analyses were conducted by the regional clinical haematology and biochemistry reference laboratories using an automated haematology analyser (XE2100, Sysmex Corporation and ACL TOP, Instrumentation Laboratory), an automated chemistry
Table 1  Summary of treatment-emergent adverse events. Multiple adverse events for each subject are counted once within each unique category

<table>
<thead>
<tr>
<th>System</th>
<th>Adverse event</th>
<th>Placebo (n=14)</th>
<th>SRT2104 (n=15)</th>
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<tbody>
<tr>
<td>Immune system</td>
<td>Any event</td>
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<td>Seasonal allergy</td>
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<td>0</td>
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<tr>
<td>Vascular</td>
<td>Any event</td>
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<td>2 (13%)</td>
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<td></td>
<td>Flushing</td>
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<td>2 (13%)</td>
</tr>
<tr>
<td>General</td>
<td>Any event</td>
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<td>3 (20%)</td>
</tr>
<tr>
<td></td>
<td>Fatigue</td>
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<td>1 (7%)</td>
</tr>
<tr>
<td></td>
<td>Flu-like illness</td>
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<td>1 (7%)</td>
</tr>
<tr>
<td></td>
<td>Asthenia</td>
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</tr>
<tr>
<td></td>
<td>Malaise</td>
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<tr>
<td>Infections</td>
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<td>1 (7%)</td>
</tr>
<tr>
<td></td>
<td>Nasopharyngitis</td>
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</tr>
<tr>
<td></td>
<td>Upper respiratory tract infection</td>
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</tr>
<tr>
<td>Renal and urinary disorders</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>Dysuria</td>
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</tr>
<tr>
<td>Any event</td>
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<td>11 (79%)</td>
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</tr>
<tr>
<td>Injury, poisoning and procedural complications</td>
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</tr>
<tr>
<td></td>
<td>Muscle strain</td>
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</tr>
<tr>
<td></td>
<td>Nail injury</td>
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</tr>
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<td>Diarrhoea</td>
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<td>4 (27%)</td>
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</tr>
<tr>
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<td>Dyspepsia</td>
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</tr>
<tr>
<td></td>
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<td>1 (7%)</td>
</tr>
<tr>
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<td>Constipation</td>
<td>1 (7%)</td>
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</tr>
<tr>
<td></td>
<td>Frequent bowel motions</td>
<td>0</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>Immune system</td>
<td>Any event</td>
<td>1 (7%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Seasonal allergy</td>
<td>1 (7%)</td>
<td>0</td>
</tr>
<tr>
<td>Vascular</td>
<td>Any event</td>
<td>0</td>
<td>2 (13%)</td>
</tr>
<tr>
<td></td>
<td>Flushing</td>
<td>0</td>
<td>2 (13%)</td>
</tr>
</tbody>
</table>

Continued
Safety
All of the participants tolerated the study medication. Commonly reported side effects occurring in two or more volunteers included headache (35%), diarrhoea (27%), nausea (13%) and hypoglycaemia (13%) (table 1). Apart from one subject describing diarrhoea as ‘severe’, the remaining reported adverse events were mild to moderate in intensity. All side effects resolved without any sequelae. There were no meaningful differences in the frequency of treatment-emergent adverse events between the active treatment and placebo groups. One individual underwent part of the first period of the study but was withdrawn because an adverse event criterion was met with the concentration of alanine aminotransferase being recorded at five times the upper limit of normal. After unblinding, this patient was found to have been taking placebo at the time of the event.

Pharmacokinetics of SRT2104
After 28 days of active treatment, the mean maximum plasma concentration (C\text{max}) of SRT2104 was 517±355 ng/mL and 716±359 ng/mL in the first and second treatment periods, respectively. The mean time at which the maximum plasma concentration was observed (T\text{max}) on day 28 of dosing was 2.6±1.2 hours and 2.9±1.5 hours in the first and second treatment periods, respectively. The mean area under the curve was 5300±3473 ng·h/mL in the first treatment period and 7312±3708 ng·h/mL in the second treatment period. The pharmacokinetics in this group was consistent with levels seen in previous studies.27

Cardiovascular effects of SRT2104
Blood pressure and heart rate remained unchanged throughout the study. No effects were observed on cardiac rhythm or the 12-lead ECG, and specifically the corrected and uncorrected QT intervals were unaffected. A dose-dependent increase in the infused forearm blood flow was observed with all three agonists (acetylcholine, bradykinin and sodium nitroprusside) in the presence of either SRT2104 or placebo (p<0.0001 for all three agonists; figure 3). There were no differences in response to acetylcholine (p=0.318) and sodium nitroprusside (p=0.083) in the presence of SRT2104 compared with placebo. There was a reduction in bradykinin-induced vasodilatation with SRT2104 (7.753 vs 9.044, SRT2104 vs placebo, mean difference=−1.291, (95% CI −2.296 to −0.285, p=0.012)) with a trend for a period-by-treatment effect (p=0.092).

Endogenous fibrinolysis and monocyte and platelet activation
Post hoc analysis showed that dose-dependent increments were recorded in bradykinin-induced net t-PA antigen and activity release (p<0.0001 for both) in the infused arm. Estimated net PAI antigen release was reduced with SRT2104 compared with placebo (mean difference=−38.89 ng/100 mL tissue/min, (95% CI −75.47 to −2.305, p=0.038)) with a non-significant period effect for the plasma PAI-1 antigen concentrations (p=0.138). There were no differences in net PAI-1 activity release, or t-PA antigen and activity release (p>0.05 respectively). SRT2104 had no effect on markers of in vivo platelet or monocyte activation (figure 4).

Metabolic effects
During SRT2104 administration, body weight decreased by 0.93 kg (95% CI −1.72 to −0.15, p=0.0236) with a treatment-by-period effect (p=0.080). HbA1c rose by 0.48% (95% CI 0.26% to 0.70%, p=0.004) after 28 days of SRT2104, as did plasma fructosamine, which rose by 33.41 µmol/L (95% CI 20.24 to 46.58 µmol/L, p<0.001). Post hoc analyses showed that the lipid profile did not change (table 2) although trends were noted towards lower concentrations of total cholesterol (−0.36 mmol/L, 95% CI −0.87 to 0.16 mmol/L, p=0.158) and triglycerides (−0.22 mmol/L, 95% CI −0.53 to 0.09 mmol/L, p=0.150; table 2) with SRT2104. Non-esterified fatty acids rose by 0.09 mmol/L (95% CI 0.04 to 0.15 mmol/L, p=0.003) with a treatment-by-period effect (p=0.0087).

Discussion
The cardiometabolic effects of SRT2104 have not been studied previously in people with T2DM. Here we present the data in patients with T2DM that are broadly consistent with our previously reported observations in otherwise healthy cigarette smokers.21 However, we did note some important contrasts especially in metabolic parameters. In otherwise healthy smokers, we have previously found no differences in forearm blood flow, platelet aggregation and fibrinolysis with SRT2104. Our findings in the current T2DM cohort were similar although we found modest reductions in bradykinin-induced vasodilatation and net PAI-1 antigen release with SRT2104. This finding is unexpected from previous animal models.15 17 We did observe some treatment-by-period interactions suggesting the potential for an inadequate

<table>
<thead>
<tr>
<th>System</th>
<th>Adverse event</th>
<th>Number of subjects with events (percentage)</th>
<th>Placebo (n=14)</th>
<th>SRT2104 (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>General</td>
<td>Any event</td>
<td>2 (14%)</td>
<td>3 (20%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fatigue</td>
<td>1 (7%)</td>
<td>1 (7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flu-like illness</td>
<td>0</td>
<td>1 (7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asthenia</td>
<td>1 (7%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malaise</td>
<td>0</td>
<td>1(7%)</td>
<td></td>
</tr>
<tr>
<td>Infections</td>
<td>Any event</td>
<td>3 (21%)</td>
<td>1 (7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nasopharyngitis</td>
<td>3 (21%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Upper respiratory tract infection</td>
<td>0</td>
<td>1 (7%)</td>
<td></td>
</tr>
<tr>
<td>Renal and urinary disorders</td>
<td>Any event</td>
<td>1 (7%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dysuria</td>
<td>1 (7%)</td>
<td>0</td>
<td></td>
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</tbody>
</table>
washout period. Moreover, our sample size was modest and this could represent a chance finding. An alternative explanation is that SRT2104 affects a hitherto undescribed pathway which works via bradykinin alone and not acetylcholine or sodium nitroprusside. This study cannot answer that question presently but generates a hypothesis for further examination in the future. Once again in contrast to the smokers’ cohort we found a statistically significant decrease in PAI-antigen release. There was no significant treatment-by-period interaction in this case (p>0.1) which would suggest a potential fibrinolytic benefit in the treatment arm, although this was not confirmed by other measures of t-PA and PAI-1 activity, which were unaltered. Taken in aggregate, the effect of SRT2104 on cardiovascular measures are predominantly neutral although we acknowledge some inconsistent changes in isolated measures of vasomotor and fibrinolytic function.

Obesity is a major factor in the development of T2DM through the promotion of insulin resistance. Weight reduction is difficult to achieve in many patients and remains a major focus of therapeutic and lifestyle intervention. In the present study a striking reduction in weight over a 28-day period was observed that was not observed with matched placebo. It is unknown whether weight reduction with SRT2104 in overweight patients with T2DM would be sustained and have long-term benefits. Whether it could be attributable to appetite suppression through SIRT1 activation or a consequence of enhanced metabolic effects is unclear, but this is a potentially important observation that warrants further investigation. A treatment-by-period interaction was observed with this result as well, so this result should be interpreted
The loss of weight was associated with an apparent short-term deterioration in measures of glycaemic control. The elevations of HbA1c and fructosamine were puzzling. These observations contradict the findings in models of mice and other higher mammals. It is possible that the acute administration of SRT2104 mimics the early changes of fasting states (decreased insulin secretion), and that the subsequent effects of decreased insulin sensitivity take longer to develop. This may explain the weight loss that was observed and to a certain extent the lack of benefit to glycaemic control. Supporting this theory, a trend towards decreased peak insulin secretion to a glucose challenge was observed in a separate study (2-hour postglucose challenge insulin concentrations 143 mmol/L vs 117 mmol/L, placebo vs SRT2104, p=0.046) where a similar degree of weight loss (approximately 1 kg in 28 days) was observed. It may be that in people with T2DM with established relative or absolute insulin deficiency, attenuation of insulin secretion, without a concomitant reduction in insulin resistance, is sufficient to permit a net rise in blood glucose.

Another potential putative mechanism linking SIRT1 modulation to an acute rise in insulin resistance is the inhibition of Peroxisome proliferator-activated receptor gamma (PPAR-γ) and the mobilisation of fat from white adipose tissue. This may be responsible for the modest rise in non-esterified fatty acids seen after SRT2104 administration that could contribute to the change in glycaemic control, leading to insulin resistance via intracellular competition with glucose metabolism. People with T2DM have complex changes to other important glucoregulatory hormones such as glucagon and cortisol. During acute starvation, important metabolic adaptations occur such as gluconeogenesis and an increase in glucocorticoids. These metabolic adaptations maintain the supply of blood glucose to the brain during periods of prolonged starvation, but result acutely in a constellation of effects similar to insulin resistance. However, in people with established T2DM, these protective glucoregulatory mechanisms described above may exacerbate hyperglycaemia in the short term.

Without further study, it is unclear whether long-term exposure to SRT2104 will ultimately be metabolically beneficial in patients with T2DM. The promotion of hyperglycaemia, which is undesirable, conflicts with the beneficial effects of weight loss, and it is unknown whether these...

**Table 2** Effect of SRT2104 on serum lipid concentrations

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=14)</th>
<th>SRT2104 (n=15)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cholesterol (mean (SD)), mmol/L</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4.09 (0.743)</td>
<td>4.24 (0.714)</td>
<td></td>
</tr>
<tr>
<td>Day 28/56</td>
<td>3.91 (0.843)</td>
<td>3.65 (0.710)</td>
<td>0.158</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>-0.18 (0.410)</td>
<td>-0.59 (0.766)</td>
<td></td>
</tr>
<tr>
<td>Mean difference (95% CI)</td>
<td>-0.36 (-0.87 to 0.16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HDL cholesterol, (mean (SD)), mmol/L</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.07 (0.212)</td>
<td>1.08 (0.207)</td>
<td></td>
</tr>
<tr>
<td>Day 28/56</td>
<td>1.09 (0.210)</td>
<td>1.07 (0.249)</td>
<td></td>
</tr>
<tr>
<td>Change from baseline</td>
<td>0.03 (0.080)</td>
<td>0.00 (0.167)</td>
<td>0.542</td>
</tr>
<tr>
<td>Mean difference (95% CI)</td>
<td>-0.03 (-0.13 to 0.07)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LDL cholesterol,(mean (SD)), mmol/L</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.21 (0.585)</td>
<td>2.29 (0.552)</td>
<td></td>
</tr>
<tr>
<td>Day 28/56</td>
<td>2.05 (0.692)</td>
<td>1.90 (0.656)</td>
<td></td>
</tr>
<tr>
<td>Change from baseline</td>
<td>-0.15 (0.467)</td>
<td>-0.39 (0.656)</td>
<td>0.259</td>
</tr>
<tr>
<td>Mean difference (95% CI)</td>
<td>-0.23 (-0.65 to 0.19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Triglycerides,(mean (SD)), mmol/L</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.81 (0.800)</td>
<td>1.93 (0.767)</td>
<td></td>
</tr>
<tr>
<td>Day 28/56</td>
<td>1.67 (0.497)</td>
<td>1.46 (0.445)</td>
<td></td>
</tr>
<tr>
<td>Change from baseline</td>
<td>-0.14 (0.568)</td>
<td>-0.46 (0.638)</td>
<td>0.150</td>
</tr>
<tr>
<td>Mean difference (95% CI)</td>
<td>-0.22 (-0.53 to 0.09)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Effects are sustained over time. The finding of weight loss certainly raises the question as to whether the drug might be beneficial in individuals with impaired fasting glucose who are treatment-naive, and whether an exposure to the drug beyond 28 days would improve glycaemia once the downstream effects of increased mitochondrial activity and decreased adiposity are further established.

The principal aim of the present study was to establish whether SRT2104 could improve a range of markers of cardiovascular health in patients with T2DM. Ultimately, we did not demonstrate any improvements in vasoconstrictor or fibrinolytic vascular function or measures of platelet and monocyte activation.

An important question was whether the dose of SRT2104 was sufficient to have an effect. As yet, the pharmacokinetics and pharmacodynamics of SRT2104 are not fully elucidated. However, an effect on metabolic measures was demonstrated with substantial exposure to SRT2104, achieving high plasma SRT2104 concentrations in the current study. Even if adequate SIRT1 activation is assumed, its downstream effects may vary in different tissues in different disease states. It may be that in advanced states of disease associated with ageing, such as T2DM, the beneficial effects are abolished by higher caloric consumption, or that the benefits require treatment for longer than 28 days to become apparent. It might therefore be anticipated that a more prolonged exposure is required before any meaningful effect is apparent.

In conclusion, SRT2104 appears to be well tolerated in patients with T2DM but has no demonstrable beneficial effects on a range of measures of cardiovascular health. It is possible that while short-term exposure to SRT2104 is effective in mediating weight loss, it appears to be associated with an inadequate effect on diminishing insulin resistance, thereby causing deterioration in glycaemic control. Further larger-scale studies are required to confirm or refute these preliminary findings.

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**Contributors** DEN is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. RMN and SV were the main researchers who recruited the volunteers and conducted the study. JL, SD and NLM had input in writing the study protocol. RMN prepared the main manuscript and all other authors reviewed and edited the contents.

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**Disclaimer** DEN has undertaken consultancy for GSK; SD is currently an employee of GlaxoSmithKline, UK, and owns stock; EH is an employee of Sirtris Pharmaceuticals, Massachusetts, and owns stock; BW is an employee of GlaxoSmithKline, Pennsylvania, and owns stock. RMN, SV, NLM, NN, and BMF declare no conflicts of interest.

**Competing interests** None declared.

**Ethics approval** Lothian Regional Ethics Committee.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Data sharing statement** All available data can be obtained by contacting the corresponding author.

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**REFERENCES**


Cardiovascular Effects of a Novel SIRT1 Activator, SRT2104, in Otherwise Healthy Cigarette Smokers

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Background—We examined the effect of the oral SIRT1 activator SRT2104 on cardiovascular function in otherwise healthy cigarette smokers.

Methods and Results—Twenty-four otherwise healthy cigarette smokers participated in a randomized double-blind, placebo-controlled crossover trial and received 28 days of oral SRT2104 (2.0 g/day) or matched placebo. Plasma SRT2104 concentrations, serum lipid profile, plasma fibrinolytic factors, and markers of platelet and monocyte activation were measured at baseline and at the end of each treatment period together with an assessment of forearm blood flow during intra-arterial bradykinin, acetylcholine, and sodium nitroprusside infusions. Three hours postdose, mean plasma SRT2104 concentration was 1328±748 ng/mL after 28 days of active treatment. Compared with placebo, serum lipid profile improved during SRT2104 administration, with reductions in serum total cholesterol (−11.6±20 versus 6±21 mg/dL), low-density lipoprotein cholesterol (−10±17 versus 3±21 mg/dL), and triglyceride (−39.8±77 versus 13.3±57 mg/dL) concentrations (P<0.05 for all). All vasodilators produced a dose-dependent increase in blood flow (P<0.0001) that was similar during each treatment period (P>0.05 for all). No significant differences in fibrinolytic or blood flow parameters were observed between placebo and SRT2014.

Conclusions—SRT2104 appears to be safe and well tolerated and associated with an improved lipid profile without demonstrable differences in vascular or platelet function in otherwise healthy cigarette smokers.


Key Words: cigarette smokers • endothelium • forearm plethysmography • platelet activation • SIRT1 • sirtuins • vascular

O riginally identified in yeast, sirtuins represent a class of highly conserved nicotinamide adenine dinucleotide (NAD)–dependent histone deacetylases that have 7 identified members in mammalian species.1,2 They have been implicated in the beneficial effects of calorie restriction on longevity in several species and are promising drug targets for a variety of diseases of aging.3 Sirtuin (silent mating type information regulation 2 homolog) 1 (SIRT1) is the best-known member of this class of proteins and is expressed broadly in multiple tissues and highly expressed in the vascular endothelium.4 SIRT1 inhibition is associated with vascular dysfunction and arterial thrombosis5 as well as alterations in fibrinolysis.6 Conversely, SIRT1 activation is associated with improved endothelial function,7 enhanced lipid metabolism,8 and inhibition of atherogenesis.9

Smoking tobacco remains one of the most important and consistent modifiable risk factors for coronary heart disease and is associated with an up to 7-fold increased risk of nonfatal myocardial infarction.10 It is associated with both accelerated atherosclerosis11 and a propensity for acute coronary thrombosis.12,13 This is mediated through a variety of mechanisms including alterations in vascular, endothelial, fibrinolytic, and platelet function.14–17 The precise cellular mechanism for these effects is as yet unknown, but cigarette smoke is associated with oxidative stress, endothelial nitric oxide synthase acetylation, and increased endothelial cell senescence that has been attributed to reduced SIRT1 levels.4

To date, there have been few clinical studies to assess the effect of SIRT1 activation in vivo in humans. Therefore, the...
aim of the present study was to examine the in vivo effects of a novel oral SIRT1 activator, SRT2104, on the lipid profile and vascular, endothelial, and platelet function in otherwise healthy cigarette smokers. We hypothesized that SIRT1 activation could improve the cardiovascular risk profile and reverse or improve the vascular and endothelial dysfunction associated with cigarette smoking.

Methods

The study was approved by the Research Ethics Committee, was given Clinical Trial Authorization by the Medicines and Healthcare products Regulatory Authority (MHRA), and carried out at the MHRA Phase 1 accredited Wellcome Trust Clinical Research Facility at the Royal Infirmary of Edinburgh, United Kingdom, between June 2010 and September 2011. Written informed consent was obtained from each volunteer, and the study was carried out in accordance with the Declaration of Helsinki.

Study Participants

Twenty-four otherwise healthy male and female volunteers aged between 18 and 70 years who smoked ≥10 cigarettes daily for at least 1 year were eligible for the study. Exclusion criteria included the presence of significant comorbidities, chronic illness, renal or liver impairment, history of gastrointestinal diseases or surgeries influencing drug absorption, history of alcoholism, history of neoplastic disease within the last 5 years, a positive urinary test for recreational drugs, pregnancy, and participation in other clinical trials or blood donation within the last 3 months. Eligibility of participants including absence of relevant medical history was confirmed through a standardized form completed by the registered general practitioners after informed consent. Tests for pregnancy (serum human chorionic gonadotrophin [HCG] concentrations at screening and urinary HCG concentrations at study visits) were conducted on all female participants of child-bearing potential.

Study Design

This was a prospective double-blind, randomized, placebo-controlled crossover study (1:1 SRT2104:placebo). Subjects were randomized to receive 2.0 g daily of oral SRT2104 or matched placebo (Sirtris Pharmaceuticals Inc) for a 28-day period, followed by crossover to the alternate study arm for another 28 days, giving a total dosing duration of 56 days. An end-of-study visit was conducted on day 70, with a phone call follow-up on day 86. Assessment of drug safety, tolerability, and efficacy on vascular function was carried out at baseline and during and at the end of each treatment period (Figure 1).

Vascular Studies

Vascular studies were undertaken before and at the end of each 28-day trial period. All studies were performed with the patient lying supine in a quiet temperature-controlled (22°C to 25°C) room. Participants were fasted and asked to refrain from smoking for 10 hours before the study and to avoid caffeine and alcohol for 24 hours before the study. Venous
cannulas (17G) were inserted into large subcutaneous veins in the antecubital fossae of both arms at the start of the study to facilitate periodic venous sampling. Supine heart rate and blood pressure were monitored at intervals throughout the study using a semiautomated noninvasive oscillometric sphygmomanometer (Omron 705 IT).

Forearm Venous Occlusion Plethysmography
Forearm blood flow was measured in the infused and noninfused forearms using forearm venous occlusion plethysmography as described previously. Subjects underwent brachial artery cannulation in the nondominant forearm with a 27 standard-wire-gauge steel needle. After a 20-minute baseline infusion with 0.9% saline, incremental intra-arterial doses of bradykinin (American Peptide Co) at 100, 300, and 1000 pmol/min (an endothelium-dependent vasodilator that evokes tissue plasminogen activator [t-PA] release); acetylcholine (Chem. Pharm Fabrik GmbH) at 5, 10, and 20 μg/min (an endothelium-dependent vasodilator that does not evoke t-PA release); and sodium nitroprusside (Hospira Inc) at 2, 4, and 8 μg/min (an endothelium-independent vasodilator that does not evoke t-PA release) were infused for 6 minutes at each dose, with a 30-minute 0.9% saline washout infusion between drugs. The order of drugs was randomized between subjects but kept constant for each subject across the 3 visits.

Blood Sampling
Paired venous blood samples were obtained from each forearm before and during the infusion of intra-arterial bradykinin. Samples were collected into acidified buffered citrate (Stabilyte; Trinity Biotech Plc) and citrate (BD Vacutainer; BD UK Ltd) for determination of t-PA and plasminogen-activator inhibitor type 1 (PAI-1) concentrations, respectively. Samples were placed on ice before centrifuging at 2000 × g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at −80°C before further analysis. Venous blood samples were collected into EDTA at the beginning and end of the vascular study to determine hematocrit.

Plasma t-PA antigen and activity (t-PA Combi Actibind t-PA ELISA kit; Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed) concentrations were determined by enzyme-linked immunosorbent assays (ELISAs).

Platelet and Monocyte Activation
Flow-cytometric measurements of platelet–monocyte aggregation (PMA) and platelet surface expression of P-selectin and monocyte CD11b expression (Mac-1/CD11b) were performed at baseline and at the end of each treatment period as described previously. Briefly, peripheral venous blood was drawn from a large antecubital vein and anticoagulated with the direct thrombin inhibitor d-phenylalnine-l-arginine chloromethyl ketone (75 μmol/L PPACK; Cambridge Biosciences) and immunolabeled within 5 minutes of phlebotomy for subsequent flow cytometric analysis. Directly conjugated monoclonal antibodies were obtained from DakoCytomation and Serotec. Samples were stained with the following conjugated monoclonal antibodies: phycoerythrin (PE)–conjugated CD14, PE-conjugated CD62p, PE-conjugated CD11b, fluorescein isothiocyanate (FITC)–conjugated 42a, and FITC-conjugated CD14 and appropriate control isotypes. Once stained, samples were incubated for 20 minutes at room temperature before being fixed with FACS-Lyse (Becton-Dickinson). All samples were analyzed using a FACS Calibur flow cytometer using CellQuestPro software (Becton-Dickinson).

Venous blood was collected in citrate at baseline and after each dosing period to assess plasma-soluble CD40 ligand (sCD40L) concentrations. Blood was centrifuged at 1500g for 15 minutes at 4°C, and plasma was decanted and stored at −80°C for further analysis by ELISA (Bender MedSystems).

Safety and Pharmacokinetic Analyses
Venous blood samples were collected biweekly to measure hematological and biochemical analytes including full blood count, coagulation profile, liver and renal function, creatine phosphokinase, lactate dehydrogenase, lipid profile°C and free fatty acids. Analyses were conducted by the regional clinical hematology and biochemistry reference laboratories using an automated hematology analyzer (XE2100, Sysmex Corporation and ACL TOP, Instrumentation Laboratory), an automated chemistry analyzer using colorimetric, kinetic and enzymatic ultraviolet and color assays (AU2700/AU640 analyzers, Beckman & Coulter), ion-selective electrodes (sodium, potassium, and chloride assays) and 2-point and multiple-point rate assays (Ortho Clinical Vitros 250 analyzer).

Venous blood samples were taken into prelabeled heparinized sodium tubes for pharmacokinetic assessment of plasma SRT2104 concentrations (Simbec Laboratories Limited). Serial blood samples were collected on days 1, 28, and 56 immediately before (0 minutes) and 15, 30, 60, 120, 180, 240, 480, 720 and 1440 minutes following study medication. Plasma was separated by centrifugation of whole blood at 1500g for 15 minutes, and decanted and stored at −80°C until analyzed.

Methodology of SRT2104 Analysis
Plasma concentrations of SRT2104 were measured using liquid chromatography with tandem mass spectrometry.
Data and Analysis Statistics

Plethysmographic data were analyzed as described previously. Estimated net release of t-PA and PAI-1 antigen and activity was defined as the product of forearm plasma flow (based on blood flow and hematocrit) and the difference in plasma antigen (or activity) concentrations between the 2 forearms. On the basis of previous power calculations, a sample size of 20 gives 80% power to detect a change in net t-PA antigen release of 27.0 ng/100 mL of tissue per minute, assuming a standard deviation of 40.0 and a 2-sided \( P < 0.05 \) (paired t test). To account for a 20% dropout rate, we recruited 24 subjects.

Fibrinolysis and forearm blood flow data were analyzed using a linear mixed-model repeated-measures analysis of covariance. Treatment differences were investigated in a model adjusting for period, treatment by period, vasodilator dose, treatment by vasodilator dose, and vasodilator dose by period using SAS for UNIX (version 9.1.3 or higher; SAS Institute). Values for these parameters are expressed as model adjusted (least square means) and 95% confidence intervals. Between-day reproducibility of forearm venous occlusion plethysmography data was assessed using the Bland–Altman method, and coefficient of reproducibility was determined for 95% confidence intervals using the Student t distribution. All other values are expressed as mean±SD.

Results

Study Participants

Volunteers had a mean age of 38±13 years (median, 37 years) and relatively equal sex distribution (58% male) and were normotensive without any significant coexisting medical conditions. Volunteers had a body mass index of 25±4 kg/m\(^2\) and a mean cigarette consumption of 17±6 cigarettes per day over 21±14 years. The mean urinary cotinine concentration at screening was 1352±950 ng/mL. All 24 volunteers completed all study visits. Before drug administration, 1 subject was withdrawn from the study because of problems with venous access and was replaced.

Pharmacokinetics, Tolerability, and Safety

Three hours postdose, mean plasma SRT2104 concentration was 1328±748 ng/mL after 28 days of active treatment (Figure 2). The median plasma SRT2104 concentration after 28 days of treatment was 366 ng/mL (IQR, 940 ng/mL). The median time at which the maximum plasma concentration was observed (\( T_{\text{max}} \)) on day 28 of dosing was 3.05 hours, which coincided well with study measurements performed on those days (2 to 4 hours postdose). The geometric mean area under the curve (AUC\(_{0-t}\)) was 6412 h·ng/mL. Consistent with previous observations, there was substantial intersubject variability in exposure during this study.

All subjects tolerated study medication well. Commonly reported side effects included headache (25%) and rhinitis, nasopharyngitis, and respiratory tract symptoms (17%) (Table 1). The reported adverse events were mild in intensity and resolved without any intervention or sequelae. There were no meaningful differences in the number of events between active treatment and placebo. There was only 1 reported serious adverse event in the study (SRT2104 arm): a traumatic facial bone fracture that was considered unrelated to SRT2104.

Blood pressure and heart rate remained unchanged throughout the study. There were no effects on cardiac rhythm or the 12-lead electrocardiogram, and specifically there were no effects on the corrected or uncorrected QT intervals. There were no clinically significant adverse effects involving any of the clinical hematological or biochemical analytes.

Lipid Profile

Treatment with SRT2104 had a favorable effect on the lipid profile. A statistically significant period effect was observed in the analysis of total and low-density lipoprotein (LDL) cholesterol concentrations. Baseline values were higher in subjects receiving placebo in the first period. Regardless of treatment arm, the level of change from baseline was greater in period 2 for total and LDL cholesterol and less in period 2 for triglycerides. Adjusted summaries combined over period are presented in Table 2. There was a reduction in total and LDL cholesterol as well as triglyceride concentrations. There

![Figure 2](http://ahajournals.org/doi/10.1161/JAHA.113.000042)
**Table 1. List of Adverse and Serious Adverse Events**

<table>
<thead>
<tr>
<th>System</th>
<th>Symptom (AE/SAE)</th>
<th>Placebo (n=24)</th>
<th>SRT2104 (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number of Events</td>
<td>Number of Events</td>
</tr>
<tr>
<td>Any event</td>
<td></td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Nervous system disorders</td>
<td>Headache</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Paresthesia</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Hypoesthesia</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Carpal tunnel syndrome</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Presyncope</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Burning sensation</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sciatica</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Respiratory, thoracic, and mediastinal disorders</td>
<td>Oropharyngeal pain</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Rhinorrhea</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Gastrointestinal disorders</td>
<td>Flatulence</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mouth ulceration</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Abdominal pain upper</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Hemorrhoids</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Reproductive system and breast disorders</td>
<td>Dysmenorrhea</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Musculoskeletal and connective tissue disorders</td>
<td>Back pain</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Musculoskeletal chest pain</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Musculoskeletal pain</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Myalgia</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Investigational</td>
<td>Blood bilirubin increased</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Creatinine phosphokinase increased</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>LDH increased</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AST increased</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>General disorders and administration site conditions</td>
<td>Influenza-like illness</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Fatigue</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Catheter site pain</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Catheter site rash</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Catheter site-related reaction</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Catheter site swelling</td>
<td>1</td>
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<tr>
<td></td>
<td>Edema peripheral</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>Pyrexia</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Swelling</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Infections and infestations</td>
<td>Nasopharyngitis</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rhinitis</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Upper respiratory tract infection</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Oral herpes</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Respiratory tract infection</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Injury, poisoning, and procedural complications</td>
<td>Contusion</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Excoriation</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Arthropod bite</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Facial bones fracture*</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Laceration</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
was no effect on high-density lipoprotein concentrations, and the 7% fall in total cholesterol was attributable to the 11% fall in LDL cholesterol concentrations.

Vasomotor Function
Noninfused forearm blood flow remained unchanged throughout all assessment periods, as were the predose measurements of blood flow in the infused arm between visits ($P>0.05$). There was a dose-dependent increase in the infused forearm blood flow with all 3 agonists (acetylcholine, bradykinin, and sodium nitroprusside) in the presence of either SRT2104 or placebo ($P<0.0001$ for all 3 agonists; Figure 3). There were no significant differences in response to either endothelium-dependent or -independent vasodilators in the presence of SRT2104 compared with placebo (bradykinin, $P=0.1169$; acetylcholine, $P=0.1683$; sodium nitroprusside, $P=0.9039$: placebo versus SRT2104). There were no differences in forearm vasodilatation between the baseline and placebo visits of the study for all 3 agonists ($P=0.5649$, $P=0.4009$, and $P=0.2908$ for bradykinin, acetylcholine, and sodium nitroprusside, respectively), confirming the good reproducibility of the measurements (Table 3).

Endogenous Fibrinolysis and Monocyte and Platelet Activation
There was a dose-dependent increase in bradykinin-evoked net t-PA antigen and activity release ($P<0.0001$ for both) in the infused arm that was unaffected by SRT2104 ($P=0.3691$ and $P=0.1377$, placebo versus SRT2104, for net t-PA antigen and activity, respectively; Table 4). Plasma plasminogen activator inhibitor-1 (PAI-1) activity decreased with time during all study visits ($P<0.05$), consistent with its circadian variation and t-PA release. Plasma PAI-1 antigen and activity concentrations were similar in both treatment arms ($P=0.8877$ and $P=0.6635$, placebo versus SRT2104, for plasma PAI antigen and activity, respectively).

SRT2104 had no effect on markers of in vivo platelet or monocyte activation (Figure 4).

Discussion
In this randomized, double-blind, placebo-controlled crossover trial of otherwise healthy cigarette smokers, we have demonstrated that oral SRT2104 is safe and well tolerated at a dose of 2.0 g daily. Importantly, we have shown that
Treatment with SRT2104 was associated with an 11% mean reduction in serum LDL cholesterol concentrations, but without demonstrable differences in vasomotor function, endothelial function, or platelet activation assessments compared with placebo. The favorable effects on lipid profile suggest that SIRT1 activation may have a beneficial role in

Figure 3. Effect of bradykinin (100, 300, 1000 pmol/min), acetylcholine (5, 10, 20 µg/min), and sodium nitroprusside (2, 4, 8 µg/min) on absolute forearm blood flow. Blue, placebo; red, SRT2104; closed circle, infused forearm blood flow; open circle, noninfused forearm blood flow. Data presented as mean±95% confidence interval.
patients at risk of developing or with established cardiovascular disease.

Elevated serum cholesterol is an established risk factor for atherosclerosis and coronary heart disease. In general, coronary heart disease risk is reduced by 2% to 3% for each 1% decrease in total cholesterol concentrations.23 We observed a 7% mean reduction in serum total cholesterol and an 11% mean reduction in LDL cholesterol concentrations without affecting serum high-density lipoprotein cholesterol concentrations. The mechanism of this lipid-lowering effect is not entirely clear but is consistent with observations associated with SIRT1 activation in animals. Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a naturally occurring polyphenolic compound that is believed to confer health benefits through SIRT1 activation.24 Resveratrol has been found to lower plasma triglycerides and cholesterol accumulation in guinea pigs25 and to suppress atherogenic lesion formation in apolipoprotein E-deficient mice.26 Indeed, SRT2104 also lowers triglyceride levels in preclinical murine models of dyslipidemia, diabetes, and obesity as well as improving insulin sensitivity and metabolic function in these animals.27 One mechanism whereby SIRT1 activators such as SRT2104 could improve lipid profiles may involve a positive regulatory effect on liver X receptor proteins (LXRs), nuclear receptors involved in cholesterol and lipid homeostasis. Nuclear receptor LXR is a substrate for SIRT1. Li and colleagues have shown that SIRT1 deacetylates and positively regulates this receptor, potentially altering cholesterol transport and metabolism.28 Although the exact mechanism of the improved lipid profiles seen with SIRT1 activation remains to be determined, our findings would suggest that SIRT1 activation could provide a therapeutic adjunct to current lipid-lowering strategies, leading to improvements in cardiovascular disease pathophysiology and thus clinical outcomes.

There are currently no published data directly examining the effects of SIRT1 activation on vasomotor function or endogenous fibrinolysis in vivo in humans. Despite the several beneficial effects of SIRT1 activation on endothelial function observed in preclinical in vitro studies,6,7,29–31 we were unable to demonstrate improvements in vascular, endothelial, or platelet function in these otherwise healthy smokers. Why was this?

Did we use appropriate and sufficiently sensitive techniques? Forearm venous occlusion plethysmography is a well-established technique that has been used extensively over the years to study human vascular physiology and has been considered a gold standard in the assessment of vascular function in health and disease.32 Using endothelium-dependent (bradykinin and acetylcholine) and -independent (sodium nitroprusside) vasodilators, we observed a dose-dependent increase in forearm arterial vasodilatation with all 3 agonists. Our results are comparable with those reported in previously published studies15,33–35 in otherwise healthy cigarette smokers including impaired t-PA release.15,36,37 Moreover, our data had low variance and were highly reproducible when we compared the baseline responses with those obtained during placebo administration. Similarly, flow cytometric analysis is considered a sensitive gold standard for measurement of in vivo platelet activation. We have previously shown that in patients with peripheral arterial disease, measurements of platelet–monocyte aggregates are reproducible and consistently reflect other markers of platelet and monocyte activation.38 In the present study, we again report comparable levels of platelet–monocyte aggregation19,39 that were reproducible between visits.

There is a body of published data that confirms a strong association between cigarette smoking, endothelial dysfunction, and impaired endogenous fibrinolysis.4,14,17,21,40 We were interested to see if this vascular and endothelial dysfunction could be improved or reversed by SIRT1 activation. There could be numerous explanations why we failed to achieve improvement with SRT2104 in these parameters in the current study. One possibility is that the SRT2104 exposure achieved in this study did not lead to adequate or consistent SIRT1 activation, which would be required to reverse the vascular and endothelial dysfunction in these smokers. Unfortunately, there is no current biomarker for SIRT1 activation or the ability to measure SIRT1 activation directly in humans. Therefore, we do not have a good understanding of the pharmacokinetic-pharmacodynamic relationship between SRT2104 drug exposure and SIRT1 activation. Although we were able to demonstrate improved lipid profiles, it is unclear whether the same exposure levels

### Table 3. Between-Day Repeatability of Forearm Blood Flow

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Mean of Differences in Forearm Blood Flow (mL/100 mL per minute)</th>
<th>Coefficient of Repeatability (mL/100 mL per minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradykinin, pmol/min</td>
<td>100</td>
<td>−0.3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>−0.2</td>
<td>7</td>
</tr>
<tr>
<td>Acetylcholine, µg/min</td>
<td>5</td>
<td>−0.1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>−0.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.3</td>
<td>7</td>
</tr>
<tr>
<td>Sodium nitroprusside, µg/min</td>
<td>2</td>
<td>0.2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.7</td>
<td>6</td>
</tr>
</tbody>
</table>

Between-day reproducibility (baseline vs placebo visit) in absolute forearm blood flow for bradykinin (100, 300, 1000 pmol/min), acetylcholine (5, 10, 20 µg/min), and sodium nitroprusside (2, 4, 8 µg/min).

DOI: 10.1161/JAHA.113.000042
Table 4. Effect of SRT2104 on Endogenous Fibrinolysis

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>SRT2104</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bradykinin Dose, pmol/min</td>
<td>Bradykinin Dose, pmol/min</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>100</td>
</tr>
<tr>
<td>Net release t-PA antigen, ng/100 mL tissue per minute</td>
<td>0.5 (−8.6 to 9.7)</td>
<td>4.7 (−4.4 to 13.9)</td>
</tr>
<tr>
<td>Net release t-PA activity, ng/100 mL tissue per minute</td>
<td>−0.0 (−4.7 to 4.6)</td>
<td>3.7 (−1.0 to 8.3)</td>
</tr>
<tr>
<td>Net PAI-1 antigen, ng/100 mL tissue per minute</td>
<td>−5.7 (−68.2 to 56.8)</td>
<td>—</td>
</tr>
<tr>
<td>Net PAI-1 activity, LS mean±95% c</td>
<td>−0.0 (−1.2 to 1.2)</td>
<td>—</td>
</tr>
</tbody>
</table>

Day 28/56

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>SRT2104</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>100</td>
</tr>
<tr>
<td>Net release t-PA antigen, ng/100 mL tissue per minute</td>
<td>−1.05 (−8.0 to 5.9)</td>
<td>3.9 (−3.1 to 10.8)</td>
</tr>
<tr>
<td>Net release t-PA activity, ng/100 mL tissue per minute</td>
<td>−0.1 (−3.2 to 3.0)</td>
<td>2.4 (−0.8 to 5.5)</td>
</tr>
<tr>
<td>Net PAI-1 antigen, ng/100 mL tissue per minute</td>
<td>−4.8 (−52.2 to 42.6)</td>
<td>—</td>
</tr>
<tr>
<td>Net PAI-1 activity, LS mean±95% c</td>
<td>−0.0 (−1.2 to 1.2)</td>
<td>—</td>
</tr>
</tbody>
</table>

Data presented as LS mean±95% confidence interval. t-PA, tissue plasminogen activator; PAI-1, plasminogen-activator inhibitor type 1.

∗P < 0.0001, for dose response to agonist.
†P > 0.05, SRT2104 vs placebo.
would also lead to improved vascular and endothelial function. There are at least 70 known substrates for SIRT1. SRT2104 may differentially deacetylate certain substrates in preference to others, depending on the precise interaction between SRT2104 and the substrates as well as the level and activity of the substrates in a particular disease state. It is also possible that certain abnormalities may be reversed more readily than others through SIRT1 activation. Although a 28-day exposure may be adequate for observing improvement in lipid profiles, longer treatment may be required to reverse some of the vascular and endothelial abnormalities. The small sample size of our study may also be a potential limitation. SRT2104 is the first selective SIRT1 activator to be studied in human clinical trials. As the biology of SIRT1 becomes more established and additional data are gathered from small exploratory trials such as this one, the optimal approach for developing SIRT1 activators and identifying disease states with the greatest therapeutic potential will become better defined.

In conclusion, we have demonstrated that the oral SIRT1 activator SRT2104 is safe and well tolerated in otherwise healthy cigarette smokers and provides positive effects on lipid profiles, but were unable to demonstrate beneficial effects on vascular, endothelial, or platelet function compared with placebo.

Acknowledgments
We thank the staff of the Wellcome Trust Clinical Research Facility in Edinburgh and Eric Thomson and Neil Johnston for their help with this study. We also thank Alison Hinds and Michelle Rostant-Belle from the Scottish Primary Care Research Network for their help with recruitment and the colleagues at Sirtris Pharmaceuticals Inc, Cambridge, Massachusetts, for their support throughout the study.

Sources of Funding
The study was funded and supported by Sirtris Pharmaceuticals Inc, Cambridge, Massachusetts. They also supplied the study drug SRT2104 and its matching placebo.

Disclosures
Drs Venkatasubramanian, Noh, Langrish, Joshi, Mills, Lang, and Newby report no disclosures; Dr Daga is currently an employee of GlaxoSmithKline, United Kingdom; Drs Hoffmann, Jacobson, and Vlasuk are employees of Sirtris Pharmaceuticals, Massachusetts, and own stock; Dr Waterhouse is an employee of GlaxoSmithKline, Pennysylvania, and owns stock.

References
SIRT1 Activators in Endothelial Dysfunction

Venkatasubramanian et al


Effects of the small molecule SIRT1 activator, SRT2104 on arterial stiffness in otherwise healthy cigarette smokers and subjects with type 2 diabetes mellitus

Sowmya Venkatasubramanian,1 Radzi M Noh,2 Shruti Daga,3 Jeremy P Langrish,1 Nicholas L Mills,1 Brian R Waterhouse,5 Ethan Hoffmann,4 Eric W Jacobson,4 Ninian N Lang,1 Brian M Frier,2 David E Newby1

ABSTRACT
Objective: Arterial stiffness increases with age, and is associated with adverse cardiovascular outcome including increased mortality. The effect of the oral small molecule SIRT1 activator, SRT2104, on arterial stiffness was examined in otherwise healthy cigarette smokers and participants with type 2 diabetes mellitus.

Methods: 24 otherwise healthy cigarette smokers and 15 people with stable type 2 diabetes were randomised in a double-blind placebo-controlled crossover trial and received 28 days of oral SRT2104 (2.0 g/day) or matched placebo. Blood pressure was measured using non-invasive oscillatory sphygmomanometry. Pulse wave analysis and velocity were measured using applanation tonometry at baseline and the end of each treatment period. Owing to the small sample size and similar trends for both groups, data for the two groups were pooled (post hoc analysis).

Results: Compared to placebo, treatment with SRT2104 was associated with a significant reduction in augmentation pressure (p=0.0273) and a trend towards improvement in the augmentation index and corrected augmentation index (p=0.05 for both). However, no changes were observed in pulse wave velocity and time to wave reflection (p>0.05). Systolic and diastolic blood pressures remained unchanged throughout the study. Treatment by cohort interaction was not significant for any of the pulse wave parameters, suggesting that the response to SRT2104 in otherwise healthy smokers and people with diabetes was consistent.

Conclusions: SRT2104 may improve measures of arterial stiffness in otherwise healthy cigarette smokers and in participants with type 2 diabetes. Definitive conclusions are not possible given the small sample size and exploratory nature of this analysis.

Trial registration number: NCT01031108.

KEY QUESTIONS
What is already known about this subject?
Among the seven known sirtuins, SIRT1 has been identified as the most critical modulator of vascular function. Animal and laboratory studies have amply demonstrated its prominent role in the regulation of vascular homeostasis and diseases. However, little is known about their direct vascular effects in man.

What does this study add?
The present study has provided evidence that suggests treatment with the oral SIRT1 activator, SRT2104, may lead to an improvement in measures of arterial compliance in otherwise healthy cigarette smokers and people with type 2 diabetes. The exact mechanism of this improved arterial compliance and the effects of prolonged treatment with SRT2104 on vascular health remain to be elucidated.

How might this impact on clinical practice?
Given that aortic stiffness and endothelial function are key factors in predicting cardiovascular outcomes, identification of novel pharmacological means of improving these predictive parameters is important and highly relevant in populations with known cardiovascular risk factors.

INTRODUCTION
The enzyme sirtuin (silent mating-type information regulation 2 homologue) 1 (SIRT1) belongs to the sirtuin family of nicotinamide adenine dinucleotide-dependent histone deacetylases and is highly expressed in the vascular endothelium. In addition to other characteristics, its activation is associated with improved endothelial function and inhibition of atherogenesis. Particular interest has been focused on the potential of therapeutic SIRT1 activators to act as anti-ageing agents. Arterial stiffness rises with age and is recognised to be an independent predictor of...
cardiovascular risk. In particular, elevations in pulse pressure and aortic stiffness are associated with increased risk of coronary events and overall mortality. Indeed, central aortic stiffness is associated with the presence of coronary atherosclerosis and ischaemic heart disease.

Cigarette smoking and diabetes mellitus are significant risk factors for the development of cardiovascular disease. A wealth of data has established a strong correlation between diabetes and cigarette smoke exposure with increased aortic stiffness, endothelial dysfunction and cardiovascular risk. New pharmacological strategies that improve arterial compliance would therefore be highly relevant to these groups at increased cardiovascular risk.

The aims of the present study were to assess the effect of the oral SIRT1 activator, SRT2104, on measures of arterial compliance in otherwise healthy cigarette smokers and patients with type 2 diabetes. It was hypothesised that SIRT1 activation in these ‘at risk’ groups could lead to an improvement in arterial compliance and therefore reduce their cardiovascular risk.

**METHODS**

The study was approved by the Berkshire Research Ethics Committee, received Clinical Trial Authorisation from the Medicines and Healthcare products Regulatory Agency (MHRA, UK), and was conducted at the MHRA phase I accredited Wellcome Trust Clinical Research Facility at the Royal Infirmary of Edinburgh, UK between June 2010 and September 2011 (EudraCT #: 2009-016765-28; Clinical trials identifier: NCT01031108). Written informed consent was obtained from each volunteer and the study was carried out in accordance with the declaration of Helsinki.

**Study participants**

Twenty-four otherwise healthy cigarette smokers and 15 participants with stable type 2 diabetes, aged between 18 and 70 years, were eligible for the study. Healthy cigarette smokers were required to have smoked ≥10 cigarettes daily for at least 1 year. Participants with type 2 diabetes were non-smokers and were selected on the basis of having a diagnosis of type 2 diabetes mellitus for at least 6 months prior to inclusion in the study, with no change in medications having been made for at least the preceding 3 months. Patients with type 2 diabetes mellitus on ACE inhibitors, antiplatelet or anticoagulant therapies were excluded from the study. Tests for pregnancy (serum human chorionic gonadotropin (HCG) concentrations at screening and urinary HCG concentrations at study visits) were conducted on all female participants of childbearing potential.

**Study design**

This was a prospective double-blind randomised placebo-controlled cross-over study. Participants were randomised to receive 2.0 g daily of oral SRT2104 or matched placebo (Sirtris, a GSK company, Massachusetts, USA) for a 28-day period, followed by cross-over to the alternate study arm for a further 28 days, giving a total dosing duration of 56 days. An end of study visit was conducted at day 70 with a telephone call follow-up on day 86. Measures of arterial stiffness were undertaken prior to and at the end of each 28-day trial period. Figure 1 outlines participant enrolment, intervention allocation, follow-up and data analysis for both groups.

All studies were performed in a quiet temperature controlled (22–25°C) room. Participants were fasted and asked to refrain from smoking for 10 h, and abstain from caffeine and alcohol for 24 h prior to assessment. Participants remained supine for at least 30 min before any recordings were started. Systolic and diastolic blood pressures were recorded using a non-invasive oscillatory sphygmomanometer (Omron705 IT, Omron Healthcare Europe, the Netherlands).

Pulse wave analysis of the radial artery was performed at the wrist using micromanometer application tonometry (Millar Instruments, Texas, USA) and the SphygmoCor system (AtCor Medical, Sydney, Australia) in accordance with the manufacturer’s recommendations. Briefly, pulse wave analysis derives an aortic pulse pressure waveform from the radial artery waveform via a mathematical transfer function. The arterial pressure waveform is a composite of the forward pressure wave created by ventricular contraction and a reflected wave generated by peripheral vascular resistance. The augmentation pressure is the pressure difference between the second and first systolic peaks. The augmentation index, augmentation pressure as a percentage of the pulse pressure, is a measure of systemic arterial stiffness and wave reflection. Corrected augmentation index represents the augmentation index corrected for heart rate. The time to wave reflection declines with increasing arterial stiffness, and provides a surrogate measure of aortic pulse wave velocity. At least three independent waveform analyses were obtained from each participant, with measurements only accepted on meeting SphygmoCor quality control criteria. Pulse wave velocity was calculated by measuring the time for the pulse wave to travel between the carotid and femoral arteries. The operator performing the analysis was kept constant for each participant throughout the study.
Blood sampling
Venous blood samples were collected at fortnightly intervals to measure haematological and biochemical analytes including full blood count, coagulation profile, liver and renal function, creatine kinase, lactate dehydrogenase and lipid profile. Analyses were conducted by the regional clinical haematology and biochemistry reference laboratories using an automated haematology analyser (XE2100, Sysmex Corporation, Japan) and ACL TOP, Instrumentation Laboratory), an automated chemistry analyser using colorimetric, kinetic and enzymatic ultraviolet and colour assays (AU2700/AU640 analysers, Beckman and Coulter), ion selective electrodes (sodium, potassium and chloride assays) and two point and multiple point rate assays (Ortho Clinical Vitros 250 analyser, USA).

Data analysis and statistics
Data were analysed, where appropriate, using repeated measure analysis of covariance on the change from baseline for all parameters. Initially, analyses were conducted separately on cohorts. As a result of the small sample size and similar trends for the two cohorts, these data were pooled post hoc. Treatment differences were investigated in a model adjusting for baseline, period, treatment by period and treatment by cohort using SAS for UNIX (V9.1.3 or higher) (SAS Institute, Cary, North Carolina, USA). Unless stated otherwise, values are expressed as mean±SD. Tests for treatment effect were two-sided with a significance level of 0.05.

RESULTS
Baseline characteristics
Participants in the study had a mean age of 45±15 years and were predominantly male (68%). Participants in the type 2 diabetes cohort were older (mean age 58±8 years) when compared with the participants in the otherwise healthy smokers group (mean age 38±13 years). All participants were normotensive with comparable systolic...
and diastolic blood pressures at baseline (table 1). No clinically significant changes in haematological or biochemical analytes occurred throughout the study. Biochemical measures of renal function (serum urea, creatinine and electrolytes) were within normal limits at baseline and remained unchanged with placebo and treatment with SRT2104 in both subgroups (table 2).

**Blood pressure**
Resting systolic and diastolic blood pressures remained unchanged throughout the study with no significant differences between treatment and placebo treatment periods.

**Pulse wave analysis and velocity**
In a combined analysis of otherwise healthy cigarette smokers and participants with type 2 diabetes, a reduction in the augmentation pressure was observed in participants receiving SRT2104 compared with placebo (mean change from baseline: SRT2104 $-1.60$ (5.304) vs placebo $-0.06$ (4.205); $p=0.0273$) and a trend towards improvement in the augmentation index (mean change from baseline in AIx: placebo $-0.64$ (8.361) vs SRT2104 $-3.47$ (9.728); $p=0.0813$) and the corrected augmentation index (mean change from baseline AIx75: placebo $-2.2$ (7.453) vs SRT2104 $-4.84$ (9.299); $p=0.0747$) (figure 2A). Pulse wave velocity and time to wave reflection remained unchanged between placebo and treatment arms ($p>0.05$ for both parameters; figure 2B). The effects of SRT2104 administration on measures of arterial compliance were consistent across the two cohorts. For example, in the SRT2104 arm, mean augmentation index at 75 bpm was reduced for healthy smokers and participants with type 2 diabetes ($-4.97$ vs $-4.63$, respectively). Measures of arterial compliance and stiffness for the individual cohorts have been presented in the online supplementary table S1. A statistical interaction between cohort and treatment was not observed ($p>0.05$ for all variables tested).

**Tolerability and safety**
Participants in both study groups (healthy cigarette smokers and patients with type 2 diabetes) tolerated the study medication well. There were no meaningful
differences in the number of adverse events between active treatment and placebo. All reported adverse events were mild in intensity and resolved without any intervention or sequelae (table 3). Headaches occurred with nearly equal frequency in the treatment (SRT2104) group in both cohorts. Participants with type 2 diabetes appeared to have more frequent gastrointestinal disturbances, such as diarrhoea and nausea in comparison with healthy smokers. Elevated liver enzymes (alanine transaminase) resulted in withdrawal of one participant in the placebo period (day 36) of the diabetes group. There was only one reported serious adverse event in the study (SRT2104 arm of healthy cigarette smokers) of traumatic facial bone fracture that was considered unrelated to SRT2104.

DISCUSSION
This randomised double-blinded cross-over study demonstrated for the first time that the oral SIRT1 activator, SRT2104, may improve arterial compliance in otherwise healthy cigarette smokers and in people with type 2 diabetes, without affecting resting measures of blood pressure.

The assessment of arterial stiffness is increasingly being used in clinical practice as an independent measure of cardiovascular risk, including those in high-risk groups.14 Ageing is associated with an increase in the stiffness of large elastic arteries induced by structural alterations in the vascular media such as an increase in collagen and a decrease in elastin content.15 This process of biological ageing is accelerated in the presence of conditions such as diabetes mellitus and hypertension. Semba et al16 and Hofmann et al17 have demonstrated an association between the presence of advanced glycation end products and increased arterial stiffness. Indeed, vascular change induced by cigarette smoke is considered to be a model of accelerated vascular ageing. The relationship between tobacco exposure,9–10 diabetes7,8,18 and increased arterial stiffness is well established.

Calorie restriction can attenuate age-related arterial stiffness in animal models through reduced oxidative stress and altered endothelial nitric oxide bioavailability.15 Indeed, calorie restriction can extend lifespan in lower organisms and mammals, and improves several metabolic and inflammatory parameters.21–26 SIRT1 has been implicated as an important mediator of lifespan

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline characteristics of participants who were otherwise healthy cigarette smokers or who had type 2 diabetes mellitus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Otherwise healthy cigarette smokers (n=24)</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>38±13</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14 (58)</td>
</tr>
<tr>
<td>Female</td>
<td>10 (42)</td>
</tr>
<tr>
<td>Baseline blood pressure (mm Hg)</td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>129±6</td>
</tr>
<tr>
<td>Diastolic</td>
<td>77±2</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>68±1</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25±4</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
</tr>
<tr>
<td>Number of cigarettes/day</td>
<td>17±6</td>
</tr>
<tr>
<td>Number of pack years</td>
<td>16</td>
</tr>
<tr>
<td>Urinary cotinine concentration (ng/mL)</td>
<td>1352±950</td>
</tr>
<tr>
<td>Glycaemic profile</td>
<td></td>
</tr>
<tr>
<td>Baseline blood glucose (mg/dL)</td>
<td>85±0</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>–</td>
</tr>
<tr>
<td>Concomitant medications</td>
<td></td>
</tr>
<tr>
<td>Antiplatelet agents</td>
<td>–</td>
</tr>
<tr>
<td>Antihypertensive agents</td>
<td>–</td>
</tr>
<tr>
<td>ARB</td>
<td>–</td>
</tr>
<tr>
<td>Diuretics</td>
<td>–</td>
</tr>
<tr>
<td>Lipid lowering agents</td>
<td>–</td>
</tr>
<tr>
<td>Hypolipaemic agents</td>
<td>–</td>
</tr>
<tr>
<td>Biguanide</td>
<td>–</td>
</tr>
<tr>
<td>Sulfonamide</td>
<td>–</td>
</tr>
<tr>
<td>Thiazolidine</td>
<td>–</td>
</tr>
<tr>
<td>Insulin</td>
<td>–</td>
</tr>
<tr>
<td>Others</td>
<td>–</td>
</tr>
</tbody>
</table>

Values expressed as mean±SD. ARB, angiotensin receptor blocker; HbA1c, haemoglobin A1c.
extension mediated by calorie restriction. The current hypothesis, therefore, was that activation of SIRT1 may inhibit this process of vascular ageing and be associated with improvements in arterial stiffness.

No studies have examined the direct effect of SIRT1 activation on measures of arterial compliance. Botden et al. were unable to demonstrate an improvement in augmentation index or central or peripheral blood flow. Venkatasubramanian S, Noh RM, Daga S, et al. Open Heart 2016;3:e000402. doi:10.1136/openhrt-2016-000402

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Changes in biochemical measures of renal function in otherwise healthy cigarette smokers and participants with type 2 diabetes mellitus administered placebo and SRT2104</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otherwise healthy cigarette smokers (n=24)</td>
<td>Participants with type 2 diabetes (n=15)</td>
</tr>
<tr>
<td>Treatment period</td>
<td>Treatment period</td>
</tr>
<tr>
<td>Placebo (n=13)</td>
<td>SRT2104 (n=11)</td>
</tr>
<tr>
<td>Day 1</td>
<td>Day 28</td>
</tr>
<tr>
<td>Serum urea (mg/dL)</td>
<td>17±3</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Electrolytes</td>
<td></td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>140±2</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>4.0±0.4</td>
</tr>
<tr>
<td>Chloride (mmol/L)</td>
<td>109±3</td>
</tr>
</tbody>
</table>

Values expressed as mean±SD.

Figure 2 Effect of treatment with SRT2104 on measures of arterial compliance in otherwise healthy cigarette smokers and participants with type 2 diabetes mellitus–change from baseline. (A) Pulse wave analysis—augmentation index, corrected augmentation index, augmentation pressure and time to wave reflection. (B) pulse wave velocity. Solid column: placebo; checked column: SRT2104. (C) baseline parameters of measures of arterial compliance-combined data.
pressure following treatment with red wine polyphenols.
In the present study, a 28-day period of treatment with
the oral SIRT1 activator SRT2104 was associated with a
reduction in augmentation pressure and trends towards
improvement in augmentation index and corrected aug-
mentation index. Augmentation pressure and index are
measures of arterial compliance and wave re
fl
exion

from small to medium sized arteries. As such, they can
be influenced by endothelial function and a number of
other dynamic and functional factors, such as heart rate
and peripheral circulatory tone. Preclinical studies
have demonstrated improved vascular function with
SIRT1 activation, and this may explain our obser-
vations of improvement in dynamic measures of arterial
stiffness following short-term administration of SRT2104.
Pulse wave velocity is a more direct measure of arterial
stiffness that is determined by the structural and physical
composition of the arterial wall. Changes in pulse wave
velocity are therefore more gradual and less dependent
on the function of small to medium sized arteries. In


Aortic and vascular disease

Table 3 Summary of treatment emergent adverse events occurring in two or more participants in OHS and participants with
T2DM

<table>
<thead>
<tr>
<th>System organ class</th>
<th>Adverse event</th>
<th>Number of events</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OHS</td>
<td>SRT2104</td>
<td>T2DM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo (n=24)</td>
<td>SRT2104</td>
<td>Placebo (n=14)</td>
</tr>
<tr>
<td>Any event</td>
<td>Any event</td>
<td>18</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>Nervous system disorders</td>
<td>Headache</td>
<td>6 (25%)</td>
<td>11 (46%)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td></td>
<td>Paraesthesia</td>
<td>4</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Hypoesthesia</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Presyncope</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Respiratory, thoracic and mediastinal disorders</td>
<td>Any event</td>
<td>1 (4%)</td>
<td>3 (13%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Oropharyngeal pain</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Rhinorrhea</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Gastrointestinal disorders</td>
<td>Any event</td>
<td>3 (13%)</td>
<td>1 (4%)</td>
<td>4 (29%)</td>
</tr>
<tr>
<td></td>
<td>Diarrhoea</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Nausea</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Abdominal pain upper</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dyspepsia</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Reproductive system and breast disorders</td>
<td>Any event</td>
<td>3 (13%)</td>
<td>1 (4%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Dysmenorrhrea</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Musculoskeletal and connective tissue disorders</td>
<td>Any event</td>
<td>4 (17%)</td>
<td>1 (4%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Back pain</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Any event</td>
<td>2 (8%)</td>
<td>1 (4%)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td></td>
<td>Blood bilirubin increased</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Alanine amino transferase increased</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Abnormal liver function test</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>General disorders and administration site conditions</td>
<td>Any event</td>
<td>4 (17%)</td>
<td>4 (17%)</td>
<td>2 (14%)</td>
</tr>
<tr>
<td></td>
<td>Influenza like illness</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Fatigue</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Infections and infestations</td>
<td>Any event</td>
<td>3 (13%)</td>
<td>5 (21%)</td>
<td>3 (21%)</td>
</tr>
<tr>
<td></td>
<td>Nasopharyngitis</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Rhinitis</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Upper respiratory tract infection</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Injury, poisoning and procedural complications</td>
<td>Any event</td>
<td>4 (17%)</td>
<td>2 (8%)</td>
<td>3 (21%)</td>
</tr>
<tr>
<td></td>
<td>Contusion</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Excoriation</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Skin and subcutaneous tissue</td>
<td>Any event</td>
<td>1 (4%)</td>
<td>0</td>
<td>1 (7%)</td>
</tr>
<tr>
<td></td>
<td>Pruritus</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Metabolism and nutrition disorders</td>
<td>Any event</td>
<td>0</td>
<td>1 (4%)</td>
<td>2 (14%)</td>
</tr>
<tr>
<td></td>
<td>Hypoglycaemia</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Vascular disorders</td>
<td>Any event</td>
<td>0</td>
<td>1 (4%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Flushing</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

OHS, otherwise healthy cigarette smokers; T2DM, type 2 diabetes mellitus.
the present study, a change in pulse wave velocity was not observed with SRT2104 administration. This is perhaps not surprising given the short-time period of exposure to SRT2104 (28 days) and the brief period of observation. An improvement in pulse wave velocity might be anticipated with a longer period of treatment with SRT2104, to allow more favourable structural changes in the larger arterial tree.

STUDY LIMITATIONS
Some limitations of this trial should be considered. Although favourable trends in parameters of arterial compliance were observed, some did not achieve statistical significance. This may partly be attributed to the trial being designed specifically to examine the acute effects of treatment with SRT2104. A longer period of treatment may be required for benefits to emerge on variables such as pulse wave velocity that involve structural changes in the arterial wall. Moreover, the sample sizes of the two groups examined were small. Two disparate populations were studied in this trial, in whom the mechanisms of vascular dysfunction may be very different. However, the direction of beneficial effects on treatment with SRT2104 was similar between the two groups, providing reassurance of a consistency of effect and allowing the post hoc presentation of the results pooled across the two groups.

Conclusion
The present study has provided evidence that suggests treatment with the oral SIRT1 activator, SRT2104, may lead to an improvement in measures of arterial compliance in otherwise healthy cigarette smokers and people with type 2 diabetes. The exact mechanism of this improved arterial compliance and the effects of prolonged treatment with SRT2104 on vascular health remain to be elucidated. Given that aortic stiffness and endothelial function are key factors in predicting cardiovascular outcomes, identification of novel pharmacological means of improving these predictive parameters is important and highly relevant in populations with known cardiovascular risk factors.

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Contributors
SD, JPL, NLM, BRW, BMF and DEN were involved in planning of the trial, data analysis and review and revision of manuscript. SV, RMN, NNL and DEN were involved in the conduct of the trial, data analysis and preparation and review of the manuscript. BW, EH, EWJ and DEN were involved in planning of the trial, data analysis and statistics and review of the manuscript.

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Competing interests
SV, RMN, JPL, NLM, NNL, BMF and DEN—no disclosures; SD is currently an employee of GlaxoSmithKline and owns GSK stock; UK, EH and EWJ are employees of Sirtris Pharmaceuticals, Massachusetts, USA and own stock; BRW is an employee of GlaxoSmithKline, Pennsylvania, USA and owns stock.

Patient consent
Obtained.

Ethics approval
Berkshire Research Ethics Committee.

Provenance and peer review
Not commissioned; externally peer reviewed.

Data sharing statement
No additional data are available.

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REFERENCES